

Original article

Topical SMIP-7.7, a toll-like receptor 7 agonist, protects against genital herpes simplex virus type-2 disease in the guinea pig model of genital herpes

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Background: Development of more effective therapies for genital herpes simplex virus type-2 (HSV-2) infections remains a priority. The toll-like receptors (TLR) are attractive targets for the immunomodulation of primary and recurrent genital herpes infection. The guinea pig model of genital HSV-2 disease was therefore used to evaluate the efficacy of a new TLR-7 agonist, SMIP-7.7.

Methods: The effects of SMIP-7.7 at concentrations between 0.90% and 0.09% were compared to the vehicle control or Aldara® (3M Health Care Limited, Northridge, CA, USA) as treatment for genital HSV-2 infections. Following intravaginal inoculation of Hartley guinea pigs with 10⁶ pfu HSV-2 (MS strain), animals were treated intravaginally beginning at 36 h post-infection. Animals were evaluated for acute disease, acute virus replication, recurrent disease and shedding, as well as infection of the dorsal root ganglia.

Results: Treatment with SMIP-7.7 significantly decreased mean total lesion scores during primary infection (all doses, $P < 0.01$ compared with vehicle control, and similar to Aldara®). Vaginal virus titres were reduced in treated animals compared with vehicle control ($P < 0.001$ for each treatment versus vehicle control on day 4). Treatment with SMIP-7.7 also significantly decreased the number of recurrent lesion days, the number of days with recurrent virus shedding and the infection of the dorsal root ganglia compared to the vehicle control, and was similar to Aldara®. As opposed to Aldara®, SMIP-7.7 did not induce fever or weight loss during treatment.

Conclusions: SMIP-7.7 improves the outcome of primary and recurrent HSV-2 disease comparable to Aldara® but without some of the side effects associated with Aldara®.

Introduction

Immune response modifiers have the potential to be effective anti-infective and anti-cancer drugs and may be useful as vaccine adjuvants. Toll-like receptor (TLR) agonists seem to be particularly suitable in these regards [1]. Previous reports have documented the potent effects of imiquimod and resiquimod, TLR-7 and -8 agonists [2], as treatments for acute and recurrent genital herpes infections in guinea pigs [3–5] and as an adjuvant [6,7]. Using this model, we and others were able to show that early therapy can prevent acute disease and genital lesions, reduce or eliminate viral replication from the genital tract, reduce latency and reduce the subsequent number of recurrences [3–5]. When evaluated as therapy for genital herpes in guinea pigs

with established infections, therapy not only reduced recurrent disease during the period of administration but for weeks after therapy was discontinued [8,9]. Furthermore, a relationship between augmentation of certain cell-mediated immune responses and the efficacy of these drugs was shown [8]. Administration of higher doses, although more potent, were associated with fever and weight loss [2].

When initially evaluated in humans with recurrent genital herpes, resiquimod appeared to have the same effects we noted using the guinea pig model [10]. However, subsequent trials were not as effective except for a reduction in recurrent virus shedding [11,12]. Furthermore, when Aldara® (3M Health Care Limited,

Northridge, CA, USA) was evaluated as therapy for herpes labialis some subjects developed more severe lesions than the control group [13]. Imiquimod 5% cream (Aldara®) is presently approved for the treatment of genital warts as well as actinic keratosis and basal cell carcinomas [14].

Therefore, although this class of drugs has great promise it appears that modifications are needed to improve safety and enhance efficacy. New TLR-7 agonists were therefore developed and the data from initial evaluations are presented in this manuscript

Materials and methods

Animals

Female Hartley guinea pigs (250–350 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA, USA) and housed under AAALAC-approved conditions at Cincinnati Children's Hospital Medical Center (Cincinnati, OH, USA).

Drug

SMIP-7.7 (Novartis Vaccines and Diagnostics, Inc., Cambridge, MA, USA) is a benzonaphthyridine derivative. Aldara® (5% imiquimod cream) was obtained from the Cincinnati Children's Hospital pharmacy.

Experimental design

For experiment 1, 60 Hartley guinea pigs (300–350 g) were divided into five groups ($n=12$): group 1, vehicle (PEG-400/EtOH); group 2, 0.90% SMIP-7.7; group 3, 0.30% SMIP-7.7; group 4, 0.09% SMIP-7.7; and group 5, Aldara®. Animals were challenged intravaginally with 1×10^6 pfu of HSV-2 (MS strain) by rupturing the vaginal closure membrane with a moistened calcium alginate tipped swab (Calgiswab number 3; Thermo Fisher Scientific, Waltham, MA, USA) and instilling 0.1 ml of a virus suspension into the vaginal vault [3,4]. Intravaginal treatment with 0.1 ml of compound delivered via a positive placement pipette was initiated at 36 h after inoculation and continued once daily until day 6. Rectal temperatures were measured at 18 h after challenge and again on day 2, 11 h after treatment started, and then 5 h after treatment on days 3–9 with a final temperature measured on day 12. Weights were obtained on days 3, 5, 7, 9 and 12, measured 5 h after each treatment. Vaginal swabs were obtained at days 2, 4, 6 and 8 days post-infection to measure acute vaginal virus replication and inoculated onto rabbit kidney cells grown in Basal Medium Eagle (Life Technologies, Grand Island, NY, USA) and 10% fetal bovine serum (Hyclone; Thermo Fisher Scientific, Waltham, MA, USA) as described previously [15].

Guinea pigs were evaluated daily and primary genital skin disease quantified using a lesion score-scale ranging

from 0 representing no disease to 4 representing severe vesiculoulcerative skin disease of the perineum [15]. Following recovery from primary infection, animals in all groups, except group 3, were examined daily from 21–63 days post-infection for evidence of spontaneous recurrent herpetic lesions [15]. The number of lesion days (days on which a recurrent lesion was observed on the perineum) was recorded. Vaginal swabs were obtained every Monday, Wednesday and Friday during this time to evaluate for recurrent virus shedding. Swabs were stored frozen (-80°C) until they were processed for PCR analysis to determine the frequency of viral shedding into the genital tract. At the end of the study, the guinea pigs were sacrificed, and the dorsal root ganglia (DRG) were harvested aseptically. DRGs were stored frozen (-80°C) until DNA was extracted for PCR evaluation of latent virus.

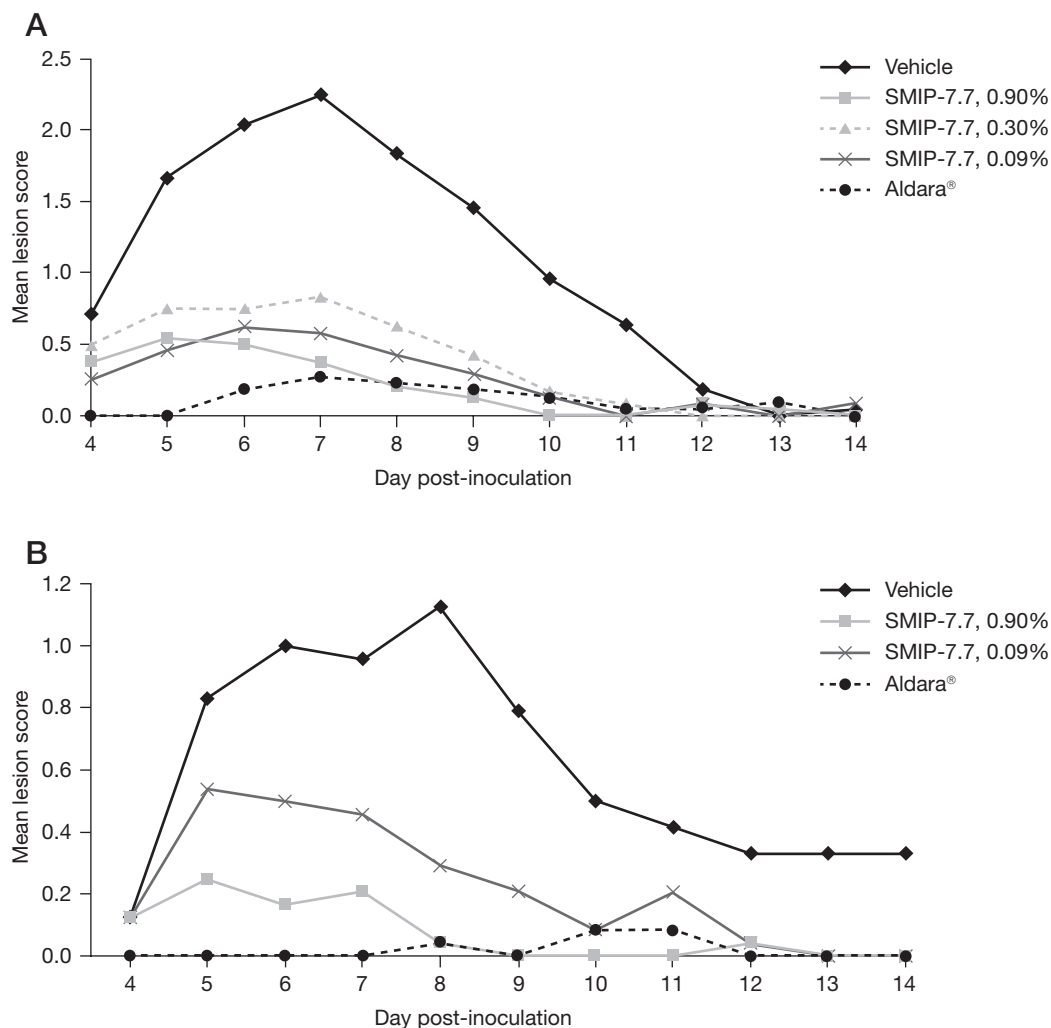
For experiment 2, 48 Hartley guinea pigs (300–350 g) were divided into four groups ($n=12$): group 1, vehicle (PEG-400/EtOH); group 2, 0.90% SMIP-7.7; group 3, 0.090% SMIP-7.7; and group 4, Aldara®. Animals were challenged intravaginally, and treated and evaluated as described above except that weights were obtained on days 0, 3, 7, 10 and recurrent virus shedding was not assessed.

In order to evaluate the effects of therapy on weight loss and temperature without the effects of HSV infection a separate experiment was performed. A total of 18 uninfected animals were divided equally and received 5 days of daily intravaginal therapy, administered as described above, with 0.90% or 0.09% SMIP-7.7 or Aldara®. Temperatures were obtained 5 h after each treatment and animals weighed every other day.

Quantitative PCR

To determine whether treatment with SMIP-7.7 affected the establishment of latent infection, the DRG were harvested at the end of the study (days 64–73) and analysed for viral DNA levels. Viral DNA was detected using primers specific for the HSV-2 gG gene (kindly provided by Debbie Long, Genocoe Biosciences, Inc., Cambridge, MA, USA), yielding a 71 base-pair DNA product. The primers were obtained from Sigma-Aldrich (St Louis, MO, USA) and the sequences were: forward 5'-CGG/AGA/CAT/TCG/AGT/ACC/AGA/TC-3', reverse 5'-GCC/CAC/CTC/TAC/CCA/CAA/CA-3' and probe FAM-ACC/CAC/GTG/CAG/CTC/GCC/G-tamRA. Bio-Rad iCycler using Tam/Fam fluorescent dye was used. Each PCR reaction contained 50–100 ng of sample DNA, 50 μM of each primer, and 10 μl of Taqman Gene Expression Master Mix (ABI, Life Technologies, Grand Island, NY USA). Total volume of each sample was 20 μl . A standard curve was generated with 10-fold serial dilutions of purified HSV-2 DNA (ATCC, Manassas, VA, USA) containing 10^5 to 10^0 HSV-2 copies in 50 ng of

Figure 1. Effect of treatment on the severity of acute disease



Animals were inoculated intravaginally with herpes simplex virus type-2 and topical treatment started 36 h after challenge. Clinical disease was evaluated daily. Animals first developed disease on day 4 and were followed until day 14. The severity of disease was scored on a scale of 1–4. (A) First experiment of 12 animals per group. (B) Second experiment of 12 animals per group.

uninfected guinea pig DNA. The amplification program used included a pre-incubation step at 50°C for 2 min and at 95°C for 10 min, followed by 50 cycles consisting of a denaturation step at 95°C for 15 s, annealing at 60°C for 1 min and elongation at 72°C for 10 s. The limit of detection of the assay was between 10⁰ to 10¹ copies.

Statistics

For comparison of means, data were analysed by ANOVA followed by a Student's *t*-test comparison. The primary comparison were those of the treated groups to vehicle alone. Statistics were not adjusted for the multiple comparisons. Incidence data were compared by

Fisher's exact test. All comparisons are two-tailed. Data are presented as means and standard deviation.

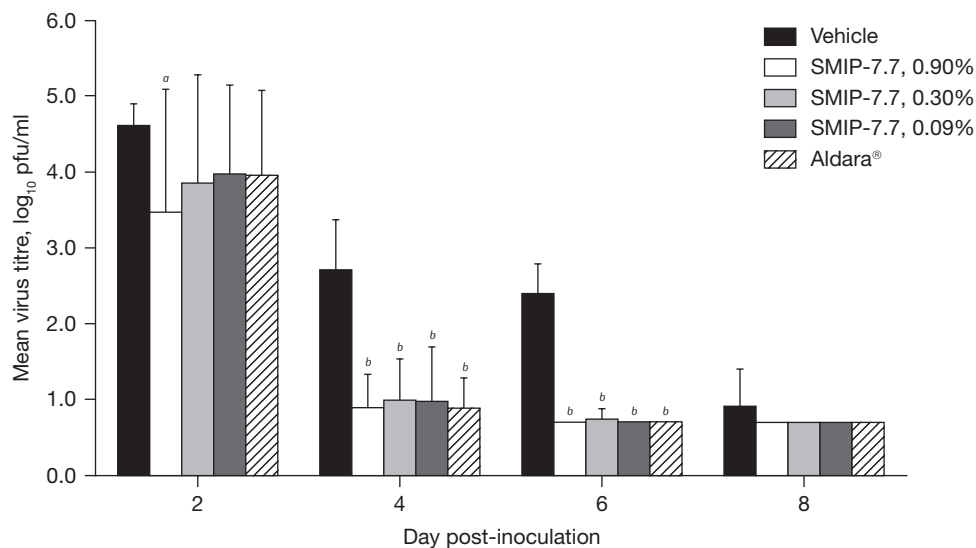
Results

Acute disease

In experiment 1, treatment with any dose of SMIP-7.7 beginning 36 h after virus inoculation significantly reduced the severity of the acute disease compared to vehicle control (Figure 1A). The most effective SMIP-7.7 group (0.90%) reduced the mean lesion score by 80% ($P < 0.001$; Table 1). This reduction was similar to but not as great as Aldara®. In experiment 2, SMIP-7.7 also significantly reduced the severity of

Table 1. Effect of SMIP-7.7 on acute genital herpes simplex virus type-2 disease

Group	Disease, <i>n</i>	No disease, <i>n</i>	Mean lesion score (sd)	<i>P</i> -value versus vehicle	<i>P</i> -value versus Aldara
Experiment 1					
Vehicle	12	0	11.7 (4.0)	-	<0.001
SMIP-7.7 0.90%	9	3	2.3 (2.5)	<0.001	0.246
SMIP-7.7 0.30%	10	2	4.1 (3.5)	0.004	0.019
SMIP-7.7 0.09%	11	1	2.9 (3.5)	<0.001	0.151
Aldara®	5	6	1.2 (1.7)	<0.001	-
Experiment 2					
Vehicle	12	0	6.8 (3.1)	-	<0.001
SMIP-7.7 0.90%	6	6	0.8 (1.3)	0.001	0.159
SMIP-7.7 0.09%	8	4	2.4 (2.9)	0.008	0.015
Aldara®	2	10	0.2 (0.6)	<0.001	-

Figure 2. Effect of treatment on acute vaginal virus replication

Vaginal swabs were obtained on days 2, 4, 6 and 8 after virus inoculation and the amount of infectious virus quantitated by plaque assay. All error bars are standard deviations. Treatments significantly reduced shedding compared to vehicle with effects seen as early as 24 h after initiation of SMIP-7.7 therapy. ^a*P*=0.03 versus vehicle. ^b*P*<0.001 versus vehicle.

acute disease compared to vehicle control ($P<0.001$ for both the 0.90% and 0.09%) but not as much as Aldara® (Figure 1B and Table 1).

As for acute virus replication, in experiment 1 all treated groups had significant ($P<0.001$ versus vehicle) reduction in virus titres beginning on day 4 (approximately 48 h after therapy was begun) and this continued through day 6 ($P<0.001$ for all treated groups versus vehicle; Figure 2). Virus was detected in only 2–3 of the 12 animals in any of the SMIP-7.7 treated groups on day 4 compared to all of the vehicle control animals. Similarly, on day 6, virus was detected in only 1–2 of any of the SMIP-7.7 group compared to all of

the vehicle control animals. There was no significant difference between any of the SMIP-7.7-treated groups and Aldara®. In the second experiment, dramatic reductions in virus shedding ($P<0.001$) were again seen on day 4 in all treated groups and again these reductions persisted through day 6 ($P<0.001$; data not shown).

Recurrent disease, latency and recurrent virus shedding
Recurrent disease was seen in the majority of animals in all groups (Table 2). In the first experiment, the number of days with recurrent lesions was reduced in all treated groups. This was significant for the SMIP-7.7 0.09% group ($P=0.034$) and the Aldara®-treated

Table 2. Effect of SMIP-7.7 on recurrent genital herpes simplex virus type-2 disease

Group	With recurrence, <i>n</i>	Without recurrence, <i>n</i>	Mean lesion days (sd)	<i>P</i> -value versus vehicle	<i>P</i> -value versus Aldara®
Experiment 1					
Vehicle	8	1	7.3 (6.3)	–	0.022
SMIP-7.7 0.90%	11	1	5.0 (4.1)	0.316	0.058
SMIP-7.7 0.09%	9	3	2.6 (3.1)	0.034	0.686
Aldara	6	5	2.1 (2.7)	0.022	–
Experiment 2					
Vehicle	12	0	13.1 (6.9)	–	<0.001
SMIP-7.7 0.90%	10	2	4.5 (4.9)	<0.001	0.201
SMIP-7.7 0.09%	11	1	7.2 (6.1)	0.019	0.018
Aldara	6	6	2.3 (3.2)	<0.001	–

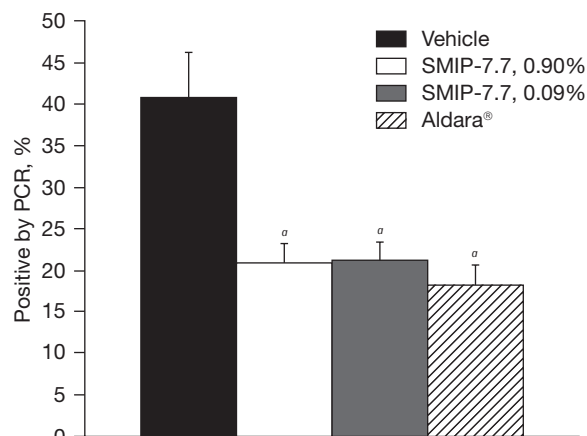
group ($P=0.022$). In the second experiment, reductions in recurrent lesion days reached significance for all the treated groups but were reduced most by Aldara® therapy.

The ability of treatment to reduce the number of days that virus is shed after the acute disease is perhaps the most important factor for controlling spread of the virus. Treatment of the acute infection with both SMIP-7.7 and Aldara® significantly ($P<0.02$) reduced the subsequent number of days that virus was shed after recovery from the acute disease (Figure 3). Reductions were similar across all treatment groups.

In order to measure the burden of latent virus, the DRG from each animal was evaluated for HSV-2 by quantitative PCR. In both experiments the burden of latent virus was significantly reduced in all treated groups (Figure 4). Both the amount of virus ($P\leq 0.002$ for each treatment) and the number of animals with detectable virus ($P<0.03$ for each treatment except the low dose SMIP-7.7 group) were reduced compared to the vehicle group.

Weight loss and temperature

We used temperature and weight changes as a gross evaluation of toxicity. Weight loss was observed during Aldara® treatment of HSV-2-infected animals but not during treatment with SMIP-7.7 (data not shown). Weight rapidly returned to normal by day 9–10 except for the vehicle group, which presumably lost weight due to their HSV-2 genital infection. Aldara®-treated animals also had significant increases in temperature on each day of treatment ($P<0.001$), whereas SMIP-7.7-treated animals were similar to the vehicle control (data not shown). In order to evaluate the effects of treatment without the compounding effects of HSV infection, a separate experiment was performed using uninfected guinea pigs. As seen in Figure 5, topical treatment with Aldara® increased the temperature of animals on each day of therapy, whereas weight decreased during therapy. By contrast, SMIP-7.7 treatment did not induce increases in temperature or weight loss at either concentration evaluated.

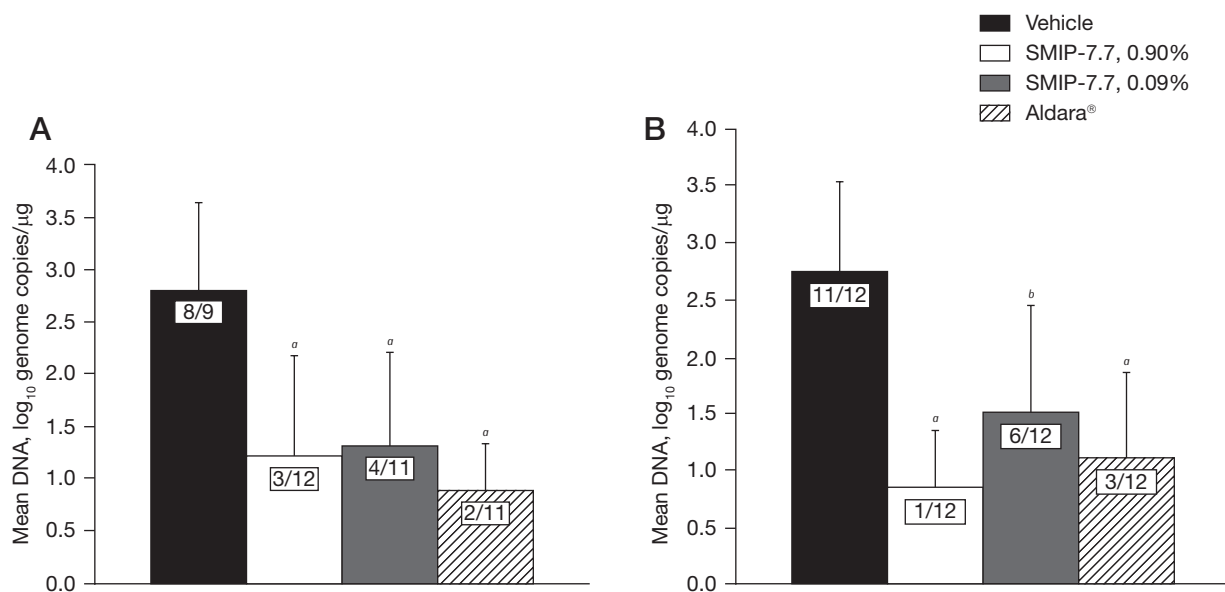
Figure 3. Effect of treatment on subsequent recurrent virus shedding

Vaginal swab samples were obtained intermittently during the 21–63 days after vaginal virus inoculation. The percentage of days with virus detected is shown. Each treatment significantly reduced the number of days with recurrent virus shedding compared to vehicle but there were no significant differences comparing treatments. ^a $P<0.02$ for each treatment compared to vehicle.

Discussion

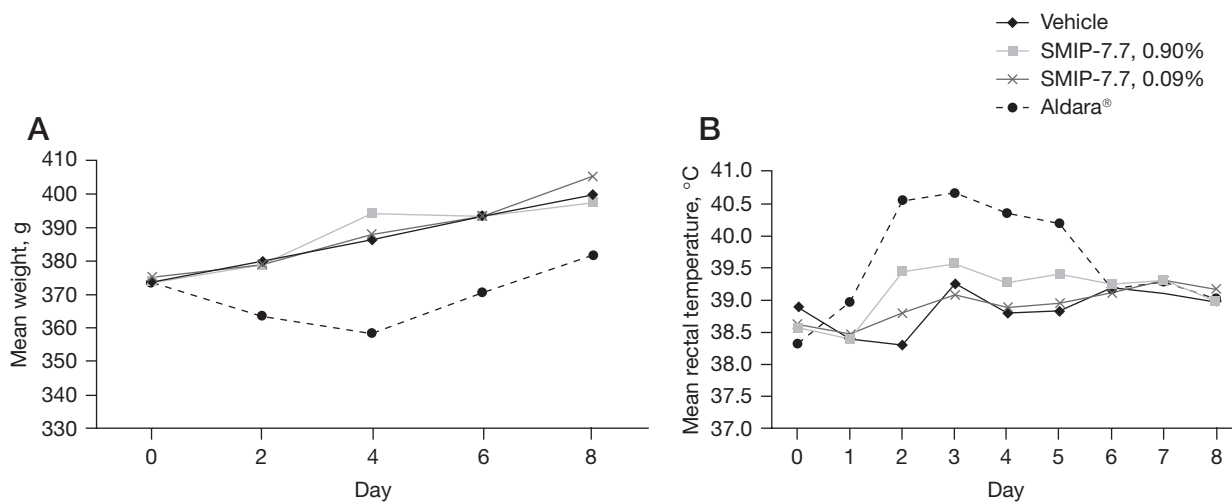
Treatments for acute and recurrent genital herpes are available but remain suboptimal. Treatment with acyclovir and its analogues reduced the severity of primary disease and virus replication when started early after infection, but there is an ongoing effort to develop more potent drugs. Furthermore, resistant viruses can develop easily and are a problem in immunocompromised patients [16]. Recent reports suggest that treatment with immune response modifiers can be useful in this situation [17,18]. Previous experience with the TLR-7 and -8 agonists, imiquimod (Aldara®) and the more potent analogue resiquimod, using the guinea pig model of genital herpes, showed the drugs were quite effective in limiting virus replication as well as acute

Figure 4. Effect of treatment on detection of latent herpes-simplex virus type-2 in the dorsal root ganglia



Drug-treated and vehicle recipients were sacrificed ≥ 65 days after herpes simplex virus type-2 infection, the dorsal root ganglia (DRG) removed and latent virus present in the DRG assessed by PCR. The n animals with virus detected/total n and the mean genome copy number are shown. All error bars are standard deviations. (A) First experiment. (B) Second experiment. ^a $P < 0.001$ comparing the quantity of latent virus in each treatment group to the vehicle. ^b $P = 0.002$ comparing the quantity of latent virus in the treatment group to the vehicle. The difference between the proportion of animals with detectable virus was also significantly different ($P < 0.03$ for each treatment group compared to vehicle except for the lower dose SMIP-7.7 group).

Figure 5. Effect of treatment on weight gain and temperature in uninfected guinea pigs



Temperatures were assessed 5 h after daily (for 5 days) topical therapy and weights were obtained every other day. (A) Mean weight of the animals during and after the treatment. (B) Mean rectal temperatures of the animals. Weight loss and increased temperatures are seen only in the Aldara®-treated animals.

and subsequent recurrent disease. However, at higher doses, the drugs caused fever and weight loss [3] suggesting that there was dose limiting toxicity. It is possible that this contributed to the disappointing results in clinical trials [11,12]. Therefore, new TLR-7 agonists were developed and evaluated using the guinea pig model and compared with Aldara®. SMIP-7.7, the first of the new agonists, significantly reduced acute disease and decreased virus replication during the acute infection in a somewhat dose-dependent manner. The effects were similar to, although somewhat less, than Aldara®.

In addition to affecting acute disease, the goal of an anti-HSV treatment is to decrease the virus load in the DRG and/or subsequent recurrent disease and recurrent virus shedding. Recent data has emphasized the frequency and role of asymptomatic virus shedding in the spread of disease [19–21]. Therefore, for the first time, the effects of treatment of an acute HSV infection with an immune modifier on recurrent virus shedding and latent infection of the DRG were evaluated. Treatment with both SMIP-7.7 and Aldara® at 36 h after virus inoculation significantly decreased the viral load in the DRG as well as recurrent disease and recurrent virus shedding.

It is difficult to compare these results to previous experiments using other therapies because each laboratory uses different challenge viruses with different protocols that differ in time of initiation and duration of therapy. However, in order to put these results into some context, it is useful to compare our results to those using a potent antiviral, helicase-primase inhibitor, Bay 57-1293, even though the compounds have obviously different mechanisms of action. Bay 57-1293 is estimated to be many times more potent *in vitro* and *in vivo* compared to available nucleoside analogues that are used for the treatment of genital herpes [22–24]. In a study by Baumeister *et al.* [23], using a similar guinea pig model, the authors reported that Bay57-1293 reduced acute disease and virus replication when started 6 h after virus inoculation to a similar extent compared to the experiments reported here with SMIP-7.7 started 36 h after inoculation Both reduced virus replication by 3–4 logs and reduced mean lesion scores to <1, on any day. In this same report [23], valacyclovir only marginally reduced the severity of the acute disease and had no effect on virus replication. Similarly, Bay 57-1293 reduced subsequent recurrences and infection of the DRG, whereas valacyclovir did not. These observations are similar to previous reports of acyclovir in this model [25,26]. Recurrent vaginal virus shedding was not evaluated in the study reported by Baumeister *et al.* [23]. Thus, it appears that topical SMIP-7.7 has similar activity to, perhaps, the most potent systemic antiviral evaluated in the guinea pig model.

Lastly, the safety of SMIP-7.7 was compared to Aldara®. Aldara® treatment induced fever and weight loss when evaluated in infected as well as uninfected animals, whereas SMIP-7.7 did not, even at the highest concentration evaluated. Therefore it may be possible to increase the concentration of SMIP-7.7 and further improve its effect on genital HSV infections.

In summary, SMIP-7.7 was safe and reduced the severity of acute and recurrent HSV disease and virus replication in the guinea pig model of genital herpes. Further evaluation of this drug or similar analogues is warranted.

Acknowledgements

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Disclosure statement

YL, PM, CL, BPN, ATM, TY-HW and MPC are members of the Genomics Institute of the Novartis Research Foundation, San Diego, CA, USA, and NMV is a member of Novartis Vaccines and Diagnostics, Inc. All other authors declare no competing interests.

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