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Mathematical modeling of herpes simplex virus-2 suppression with pritelivir predicts trial outcomes

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Abstract

Pharmacokinetic and pharmacodynamic models estimate the potency of antiviral agents but do not capture viral and immunologic factors that drive the natural dynamics of infection. We designed a mathematical model that synthesizes pharmacokinetics, pharmacodynamics and viral pathogenesis concepts to simulate the activity of pritelivir, a DNA helicase-primase inhibitor that targets herpes simplex virus. Our simulations recapitulate detailed viral kinetic shedding features in five dosage arms of a phase 2 clinical trial. We identify that *in vitro* estimates of EC₅₀ are lower than *in vivo*

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Author contributions

JTS, LC, AW and AB conceived the study. JTS, DAS, AM, MRM, SAVW and CMR performed the modeling and statistical analyses. JTS, AM, LC, AW, JO, HRS, SS, BT, HZ, MRM, SAVW, CMR and AB wrote the manuscript.

Competing interests

Dr. Magaret reports receiving consulting fees from Immune Design and AiCuris. Drs. Rubino, Vanwart, and Melhem report receiving research funding from AiCuris. Dr. Corey reports being on the scientific advisory board for and holds stock (<1% of company) in Immune Design Corp, and is a coinventor listed on several patents involving potential HSV vaccine development. Dr. Wald reports receiving consulting fees from Aicuris, Amgen, and grant support through her institution from Agenus, Genentech, Genocoea, Gilead, and Vical. Drs. Ossig, Ruebsamen-Schaeff, Stoelben, Timmler, Zimmermann, and Birkmann are employees of AiCuris; Drs. Ruebsamen-Schaeff, Timmler, Zimmermann, and Birkmann report holding stock options in AiCuris. Dr. Birkmann reports contributing to patents related to galenics and the synthesis of drug substances through AiCuris. Drs. Rubino, Van Wart, and Melhem report that AiCuris has provided research funding to the Institute for Clinical Pharmacodynamics. No other potential conflict of interest relevant to this article was reported.

values for the drug. Nevertheless, pritelivir potently decreases shedding at appropriate doses based on its mode of action and long half-life. While pritelivir directly inhibits replication in epithelial cells, our model indicates that pritelivir also indirectly limits downstream viral spread from neurons to genital keratinocytes, within genital ulcers, and from ulcer to new mucosal sites of infection. We validate our model based on its ability to predict outcomes in a subsequent trial with a higher dose. The model can therefore be employed to optimize dose selection in clinical practice.

Introduction

Dose selection for clinical trials of antiviral agents lacks precision. While pharmacokinetic (PK) models reproduce drug absorption, distribution and elimination, and pharmacodynamic (PD) models capture concentration dependent viral inhibition(1), complex viral immune interactions must be considered for optimization of dosing regimens(1–7). For instance, currently licensed antiviral agents for herpes simplex virus-2 (HSV-2), only partially suppress genital tract shedding(8). Using mathematical models, we identified that breakthrough shedding occurs due to the short half-life (3–4 hours) of these agents(9, 10), and predicted rapid viral replication during narrow time intervals when drug concentrations are sub-therapeutic.(10, 11)

Here we use data from a phase 2 trial of pritelivir, an HSV DNA helicase-primase inhibitor with serum half-life of ~80 hours(12),(13), to identify that increasing doses of pritelivir limit shedding frequency by decreasing shedding episode frequency, duration, and viral load. We superimpose dose-specific PK and PD effects of pritelivir onto a spatial stochastic mathematical model of HSV-2 replication and immunological response, and reproduce detailed dynamic features of shedding in all 4-dosage arms. Our model estimates a higher *in vivo* plasma drug concentration requirement for 50% viral inhibition (EC_{50}) than *in vitro* assays. Pritelivir persists above this EC_{50} when given at 75 mg daily, and limits HSV-2 spread during all stages of pathogenesis including ganglionic reactivation, cell-to-cell spread within ulcers, and secondary seeding of new sites of infection. The model is validated mechanistically based on analyses correlating high drug levels with low shedding in ensuing days, and clinically based on its ability to predict outcomes in a subsequent trial with 100 mg daily dosing. Our results demonstrate that highly informed mechanistic models allow rational dose selection in clinical trials of antiviral compounds.

Results

Pritelivir exhibits a dose response on HSV-2 shedding

We analyzed results from a randomized, double-blind phase 2 study, in which 150 participants were distributed among 5 doses (5 mg, 25 mg and 75 mg daily; 400 mg weekly; placebo)(12). To rapidly achieve steady-state drug concentrations, participants received appropriate loading doses (**Methods**). To assess viral shedding, each person performed daily self-genital swabs for 28 days. Swabs from days 2–29 were analyzed for quantitative HSV DNA using PCR.

There was a pronounced dose response of quantitative shedding rate and quantity to pritelivir. Quantitative shedding frequency on 5 mg approximated that of placebo, while there was a sequential decrease in shedding from 5 mg daily to 25 mg daily, from 25 mg daily to 400 mg weekly, and from 400 mg weekly to 75 mg daily (Table S1). Participants on 25 mg shed above 10^4 HSV DNA copies less frequently than placebo. Shedding in the 75 mg arm was rare and rarely exceeded 10^4 HSV DNA copies. Participants on 400 mg weekly had a low shedding rate but some breakthrough shedding at $>10^6$ HSV DNA copies (Fig 1a). Quantitative shedding rate in the placebo group was equivalent to patterns observed in a historical 531 patient cohort with $>14,000$ samples (Fig 1b)(10, 11). The dose response on shedding rate was evident on most days of the protocol and dissipated when therapy was stopped after day 30 of the protocol (Fig S1).

HSV-2 shedding consists of highly heterogeneous episodes. We applied statistical tools(10, 11), to describe episode kinetic features (Fig S2) and compared these on and off pritelivir. The dose response on each episode kinetic feature is summarized in Table S1. The dose-response on shedding rate occurred due to a decreased episode rate (Fig 2a) and duration (Fig 2b) as dose increased, though not all differences were statistically significant (Table S1). Median expansion slope from initiation to episode peak was equivalent among all groups (Fig 2c). Median clearance slope from episode peak to termination was higher in 25 mg, 75 mg and 400 mg weekly than placebo and 5 mg daily (Fig 2d). Episodes on 75 mg daily had lower first positive (Fig 2e) and peak positive (Fig 2f) HSV DNA copy number, highlighting pritelivir's direct effect on replication. Last positive HSV DNA copy number per episode was equivalent across groups with slightly lower values in the 75 mg arm (Fig 2g), highlighting that other factors contribute to late viral clearance whether or not pritelivir is given. Episode re-expansion (a 0.5 log increase in viral load following a period of 0.5 log clearance) is a common cause of prolonged episodes(10). We observed non-significantly lower re-expansion rates as dose increased (Fig 2h), which explains why viral clearance rates were slower on placebo (Fig 2d). More episodes were associated with genital lesions in the placebo than treatment arms (Fig 2i) but this trend did not achieve statistical significance.

A mathematical model of pritelivir on HSV-2 shedding

Our model represents a synthesis of three sets of equations: viral-host pathogenesis in the absence of treatment(14); impact of the host on plasma drug concentration (PK); and pritelivir concentration-dependent inhibition of HSV-2 replication (PD)(10). Our pathogenesis model (Fig 3)(14) and its parameters are explicated in the **Methods** and Supplement (parameters in Table S2). The model captures spatial behavior of virus, infected cells and the cell-mediated immune response within 200 genital tract microenvironments(10). Equations describe HSV-2 replication and spread, and CD8+ T-cell elimination of infected cells within ulcers (Fig 3a). Cell-free virus from one ulcer can seed adjacent regions, allowing multiple concurrent foci of HSV production and clearance (Fig 3b)(10, 15).

We used data from several Phase 1 and 2 studies to develop a population PK model (**Methods**, Supplement and Fig S3) that characterizes pritelivir absorption and time-course

in plasma. Pritelivir clearance and steady-state volume of distribution varied by weight and gender. Therefore, weight and gender were randomly assigned using normal and uniform distributions. Parameters that varied amongst doses included peak antiviral concentration ($C_{\max}$), time to peak concentration ($T_{\max}$), rate of absorption, and plasma clearance and elimination half-life (Table S3).

The PD model (**Methods** and Supplement) includes three parameters: percent of drug unbound to protein (2.8%), *in vivo* EC_{50} and the Hill coefficient (m). *In vivo* EC_{50} is the unbound plasma drug concentration at which viral replication is inhibited 50% in infected persons. m is the slope of the dose response curve and captures the range over which therapeutic effect increases, up to peak inhibition achieved at $C_{\max}$. If $m > 1$, then a molecule binds its target cooperatively, achieves intensification of antiviral effect across small increases in concentrations, and more potently limits viral replication at $C_{\max}$ (1).

Our previous models suggest that HSV-2 targeting small molecular agents exert activity within ganglia where reactivation initiates via gene products synthesis in the nucleus of latently infected neurons(10). We therefore synthesized our PK/PD and viral pathogenesis models by assuming that pritelivir inhibits HSV-2 replication in genital keratinocytes and neuronal cell bodies within dorsal root ganglia: the latter effect manifests in our model as a downstream decrease in HSV-2 release from neurons that innervate the genital tract.

***in vivo* pritelivir EC_{50} estimates exceed *in vitro* measures**

Published *in vitro* EC_{50} values indicate variability in viral sensitivity to pritelivir amongst isolates (mean=11.7 ng/mL, range=3.6–26.1)(16). We hypothesized that *in vivo* EC_{50} might differ from *in vitro* estimates because HSV-2 is better adapted to the cellular machinery of its human host than cells in culture. The percentage of unbound drug in genital secretions may also differ from plasma. *In vitro* estimates of m are also variable (mean=1.4, standard deviation=0.6)(16).

To identify *in vivo* EC_{50} and m , we repeatedly simulated trials with 30 patients per arm who swabbed for 30 days each at all 4 doses. Each simulation assumed varying EC_{50} and m values. The output associated with each parameter set was graded for fit to each feature of viral kinetic data using weighted residual sum of squares (Figs 1 and 2). *In vivo* EC_{50} values between 50 and 70 ng/mL provided best fit (Fig 4a). Thus, our *in vivo* EC_{50} estimate is 2–15 fold higher than *in vitro* measures. There was similar fit for m values from 1–3 (Fig 4a), demonstrating that shedding kinetics are less sensitive to m than *in vivo* EC_{50} .

The *in vivo* EC_{50} estimate is higher than trough and peak pritelivir concentrations for 5 and 25 mg daily dosing (Fig 4b,c). Unbound plasma pritelivir levels exceed the *in vivo* EC_{50} for the entire dosing interval at 75 mg (Fig 4d) and 40% of the time at 400 mg weekly (Fig 4e). By inputting dynamic pritelivir levels from the PK equations into the PD equation inclusive of the *in vivo* EC_{50} , we estimated percent inhibition of HSV-2 replication for each dose. At 75 mg, inhibition of replication was sustained but incomplete (80%). At 25 mg, inhibition was ~30%. At 400 mg weekly, there was minimal inhibition at the end of the dosing interval (Fig 4f).

Model simulations recapitulate viral kinetic data from clinical trials

To validate our optimized parameter sets, we simulated 100 trials, each with 30 subjects in five treatment arms. For each participant, PK, PD and pathogenesis parameters were randomly selected from known distributions (Tables S2 and S3). We normalized EC_{50} around 60 ng/mL and m to *in vitro* ranges(16).

Given the model's stochastic structure, each trial generated unique results. Mean simulated shedding rate was 14.7% (range: 11.2–19.2%) versus 16.6% in the trial for placebo; 13.5% (range: 9.4–23.8%) versus 18.2% in the trial for 5 mg; 7.9% (range: 3.3 – 11.1%) versus 9.3% in the trial for 25 mg; 2.5% (range: 0.7–6.2%) versus 2.1% in the trial for 75 mg; and 4.6% (range: 1.8–10.5%) versus 5.3% in the trial for 400 mg weekly. Simulated values fell mostly within 95% confidence interval ranges of quantitative shedding rate in the trial (Fig 5a–e). Mean shedding rate at each quantity was similar in simulated (Fig 5f) and actual trials (Fig 1a). Model simulations reproduced episode rate (Fig S4) and episode characteristics in the five arms including: first (Fig S5), peak (Fig S6), and last (Fig S7) HSV DNA copy per episode. The model overestimated proportion of one-day episodes (Fig S8).

Pritelivir dose is the sole correlate of shedding rate

We examined shedding rate in 1500 simulated participants to ascertain which model parameters correlate with clinical outcomes. In keeping with the trial(12), we noted heterogeneous shedding rates amongst simulated participants (Fig 6a). Median participant shedding rates differed by dose (placebo: 7.4%, 5 mg: 6.4%, 25 mg: 3.3%, 75 mg: 0.8%, and 400 mg weekly: 1.7%). Shedding rate variability and the percentage of high shedders (rate >20%) decreased with increasing dose: 25.3% for placebo, 25.0% for 5 mg, 12.7% for 25 mg, 5.3% for 400 mg weekly and 0.3% for 75 mg (Fig 6a).

Within each cohort, no PK parameter (C_{max} , C_{min} , drug half-life, gender or weight), PD parameter (*in vivo* EC_{50} or m) or pathogenesis parameter (rate of HSV-2 release from neurons, replication, or clearance; CD8+ T-cell expansion or decay rate) predicted individual shedding rate: all parameters had $R^2 < 0.05$ indicating poor correlation. Only *in vivo* EC_{50} at 25 mg had a slight positive correlation with shedding rate ($R^2 = 0.07$). When all 900 simulated patients on daily regimens were analyzed together, only parameters specific to dose (C_{max} ($R^2 = 0.13$) and C_{min} ($R^2 = 0.13$)) had slight inverse correlation with lower shedding rate.

The lack of correlation between model parameters and shedding rate likely in part reflects the short 30-day sampling period for each patient. In all study arms, more than 25% of simulated study participants had no shedding based on the stochastic nature of our model (Fig 6a). A high correlation between dose specific PK parameters (C_{max} and C_{min}) would be expected if sampling continued for a year or longer in each simulated patient.

Pritelivir indirectly lowers cell-to-cell spread of HSV-2

Though pritelivir directly inhibits replication, model output demonstrates that by limiting intracellular viral load, pritelivir indirectly prevents downstream viral spread to new cells.

First, pritelivir results in less HSV-2 spread from neurons into genital tract epithelial cells, and lowers episode rate (Fig 6b, Fig S4).

Second, upon episode initiation, pritelivir limits cell-to-cell spread within an ulcer. Increased drug concentration at the time of reactivation lowers the effective reproductive number or R , (mathematically defined in the Supplementary text) which is the average number of cells infected by the first infected keratinocyte in a region. As a result, the first and peak detected viral load in an episode are generally lower (Fig 2e, 2f, S5 & S6). Given that drug levels often suppress R to less than one, brief blips of $<10^4$ HSV DNA copies were more common on 75 mg (49% of episodes) relative to other doses (42% for 400 mg weekly, 28% for 25 mg, and 26% for 5 mg). At each simulated dose, blips represent infection of a median of 2–3 cells: episodes on pritelivir 5 and 25 mg were more likely to involve spread of HSV-2 to thousands of cells, than episodes on 75 mg and 400 mg weekly (Fig 6c).

Finally, pritelivir inhibits secondary seeding of new regions of infection via cell free HSV-2 from a single ulcer. New ulcer formation in spatially discrete mucosal regions allows concurrent replication in multiple loci, and explains frequent viral rebound during prolonged episodes off treatment(10, 17). During simulated therapy with DNA nucleoside analogues (half-life=3–4 hours), we noted that rebound occurs when drug concentrations decay below the *in vivo* EC_{50} for half of the dosing interval(9, 10). With daily dosing, pritelivir concentrations reach a relative steady-state due to its longer half-life (Fig 4b–d): accordingly, the percent of episodes associated with secondary seeding decreased at higher doses (placebo: 25.7%, 5 mg: 26.0%, 25 mg: 19.1%, 75 mg: 9.7%, 400 mg weekly: 13.2%) (Fig 6d). This occurred because the threshold viral load within an ulcer required for seeding adjacent regions ($\sim 10^5$ HSV DNA copies) was less frequently surpassed at high doses. Due to infrequent secondary seeding, observed and simulated episode duration was usually less than one day and rarely exceeded 5 days on 75 mg (Figs 2b, 6e & S8). The decrease in viral rebound probability also explains the higher peak to termination slope (Fig 2d) at higher doses.

The effect of drug concentration on spatial features of HSV-2 pathogenesis is again demonstrated by considering viral growth potential using the effective reproductive number (R). Off therapy, our model recapitulates spatial heterogeneity in immune cell density and predicts that this leads to certain genital tract micro-regions with high viral growth potential ($R>10$) due to low density of immune cells(14). If HSV-2 reactivates in regions with high immune cell density ($R<1$), then virus is contained within hours before spreading beyond a few cells. A surge in immune cells occurs in response to high copy number viral reactivation leading to a period where $R<1$. This protection wanes over months, possibly due to death of local immune cells. Breakthrough shedding is always possible, as $R>1$ in a majority of genital tract regions.

This pattern is demonstrated with pritelivir 5 mg, as this dose has little impact on shedding (Movie 1). With 25 mg (Movie 2) and 75 mg (Movie 3) daily, a greater percentage of genital tract regions exist at $R<1$ due to concurrent pharmacologic and immunologic pressure. On 400 mg weekly, viral growth potential in all regions increases as drug levels

decline. HSV loads $>10^6$ DNA copies occasionally occur. However, each weekly spike in pritelivir levels coincides with rapid HSV-2 clearance (**Movie 4**).

The model recapitulates the observation that the final observed viral load distribution during episodes varies less according to dose (Figs 2g & S7). The explanation is that free viral lifespan exceeds the lifespan of infected cells under immune pressure in the model and is independent of drug dosing. The lower viral loads observed in 75 mg daily dosing likely reflect the generally lower peak viral loads in these simulated patients.

High pritelivir concentrations predict limited concurrent and subsequent shedding

To reinforce model predictions, we re-examined trial data and demonstrated that high unbound pritelivir concentrations correlated with low shedding levels concurrently (Fig 7a) and in the ensuing 48 hours on drug (Fig 7b). High copy shedding occurred exclusively with unbound drug levels below the *in vivo* EC₅₀.

The model accurately predicts decreased shedding rate at a higher dose

While 75 mg daily achieved an 85% reduction in shedding rate versus placebo and was the most effective dose in the trial, we observed decreased shedding rates at low and high viral quantities with increasing simulated dose and dosing frequency (Fig 8a). Up to 96% reduction versus the placebo shedding rate was reached at 150 mg per day, and average daily cumulative dose regardless of dosing frequency was highly predictive of simulated and observed shedding rate (Fig 8b).

We performed 10 simulations of a 90 participant trial at 100 mg daily and obtained a median shedding rate of 1.7% (range: 0.6% – 2.7%). In a subsequent trial of 91 participants who performed swabs every 6 hours in which pritelivir 100 mg daily was compared to valacyclovir 500 mg daily, the shedding rate on pritelivir was 2.4% (173 of 7276 swabs positive for HSV DNA) (18), thereby confirming the predictions of our model.

Discussion

We demonstrate that *in silico* clinical trials are predictive tools if based on well-characterized host-pathogen dynamics, and drug characteristics. Pritelivir directly inhibits HSV-2 replication in our simulations, but also limits downstream spread from neuron to genital mucosa, cell-to-cell within ulcers, and ulcer-to-new skin sites, an effect that is likely amplified by pritelivir's ability to protect both infected and uninfected cells from infection. Typical PK/PD models neglect these features of infection and cannot reliably simulate the entirety of the biologic system. Our techniques may be used for other viral pathogens with the goal of leveraging phase 2 clinical trial results for optimal dose selection in late-stage licensure trials.

Our model provides evidence that a long half-life is a critical property of drugs that target rapidly replicating viruses such as HSV-2. We previously developed the hypothesis that the short half-life of acyclovir and famciclovir is the explanation for breakthrough shedding on these agents(9, 10). Here we demonstrate that a drug with longer half-life can achieve potent

suppression of viral replication. The veracity of this prediction is strengthened by the model's ability to recapitulate detailed viral kinetics in all study arms.

We also demonstrate that *in vitro* estimates of EC₅₀ may not reflect conditions during human infection and develop the concept of an *in vivo* EC₅₀, which captures antiviral activity as a function of unbound plasma drug levels. Our *in vivo* EC₅₀ estimate is substantially higher than *in vitro* measures, suggesting higher protein binding to drug in genital secretions, degradation of active drug in mucosa, or a higher threshold for HSV-2 inhibition within infected cells *in vivo*.

A high Hill coefficient predicts efficacy for HIV antiretroviral agents(1). Though *in vitro* estimates suggest variability in this value depending on the HSV-2 isolate, we cannot estimate the Hill coefficient of pritelivir as all reasonable values allow model fit to the data. It appears that the Hill coefficient has little impact on shedding rate in this study because even in the 75 mg daily arm, unbound drug values only exceed the *in vivo* EC₅₀ two-fold. The impact of a large Hill coefficient is amplified when drug concentrations exceed the EC₅₀ 5–10 fold(1).

One strength of our approach is fitting to extremely detailed viral kinetic measures from participants in four therapeutic arms while only allowing two parameters in the model to vary. Parameters that describe viral replication and T-cell response in a population of 531 study participants were used to simulate shedding off therapy(10). Pritelivir PK values were imputed directly from experimental data. *In silico* trial results were not sensitive to variability in any of these parameters. Only one parameter drove model fit (*in vivo* EC₅₀). Therefore, the model was only minimally trained to reproduce the clinical trial. The highly predictive simulated data is an emergent mathematical property of our multi-scale model, which is enhanced further by its prediction of shedding rate in a trial of 100 mg daily dosing.

A few potential limitations are worth noting. Participants in the placebo arm and four treatment arms of the actual study were not matched. Some difference between arms may be due to different baseline HSV-2 shedding rates, rather than differential drug effects. While there is heterogeneity amongst patients in regards to HSV-2 shedding frequency(19–21), our model generates substantial variability in shedding based on its stochastic output structure. Yet, variability in viral, PK and PD parameters did not impact shedding rate.

While our model is effective for predicting outcomes of a clinical trial, it is unable to predict outcomes in treated individuals(22). Our results suggest that in the majority of patients, treatment with pritelivir 75 mg daily or higher will potently suppress shedding. Treatment with 150mg is predicted to decrease shedding by 96%. Real and simulated shedding was a rare event for participants on 75 mg daily. Yet if even one patient in this arm had high shedding rates, due to medical non-compliance, or high basal shedding rate, then this might skew study outcomes and decrease the model's predictive accuracy.

Finally, the model generally overestimates the proportion of short, one-day episodes, particularly in low dose simulations. One reason is that short episodes are associated with low viral loads, which approach the statistical cut off for positive (23): the briefest episodes may therefore be missed with our daily sampling protocol. Moreover, our statistical

assessment of episode duration is biased by the uncertain duration of observed episodes that begin prior to, or terminate after the sampling period. Our interval censoring approach may slightly overestimate the proportion of long episodes.

Phase 3 randomized controlled clinical trials are the gold standard for proving therapeutic efficacy but are expensive and labor intensive. Accurate dose selection is crucial. We present evidence that mathematical models can extrapolate the results from phase 2 trials to optimize dose selection for licensure studies. These models have the added benefit of identifying generic features of therapeutic agents, such as long half-life, that are of particular importance in containing viral infections.

Materials and Methods

Study Design

The purpose of this study was to develop a mathematical model of HSV-2 pathogenesis during pritelivir therapy that captures the fundamental features of viral replication and spread, the cytolytic immune response against infected cells, and PK/PD of pritelivir. We first developed the model and tested its ability to reproduce the most relevant dynamic features of HSV-2 shedding in each of five treatment arms in a Phase 2 clinical trial. Upon successful model fitting, we analyzed emergent properties of numerical simulations to identify the mechanistic underpinning of dose dependent viral suppression according to increasing pritelivir dosing. Finally, we simulated previously untested doses to allow for dose optimization in future trials.

Clinical trial data

We selected data for mathematical model fitting from a randomized, double-blind Phase 2 trial with 4 treatment arms and one placebo arm (n=30). To rapidly achieve representative drug concentrations, participants in the 5 mg arm (n=33) received an initial loading dose of 20 mg, participants in the 25 mg arm (n=32) received an initial loading dose of 100 mg and participants in the 75 mg arm (n=29) received an initial loading dose of 300 mg. No loading dose was administered in the 400 mg weekly arm (n=31). Participants took daily swabs of genital secretions for quantitative measures of HSV DNA for a total of 28 days and noted the result of daily examination for the presence of genital lesions. All study subjects signed informed consent prior to enrollment and the protocols were approved by the University of Washington Institutional Review Board as well as by the other included clinical trial sites (<https://clinicaltrials.gov/ct2/show/NCT01047540>).

The validation clinical trial was a crossover trial in which 91 participants received 28 days of valacyclovir 500 mg po daily and 28 days of pritelivir 100 mg po daily. The order of drug was selected randomly, and there was a 28 day washout period between each drug. Participants performed swabs 4 times per day during the dosing period.

Viral shedding outcomes

Shedding rate was defined as number of swabs with HSV DNA detected by PCR out of the total number of swabs collected. Lesion rate was number of days with reported lesions out of

total number of days with reports. Shedding episodes were defined by a series of consecutive swabs containing HSV DNA ≥ 150 copies/mL(23), and not including more than one consecutive time point with a negative or missed swab. To calculate relevant population-level estimates of annualized episode rate, we enumerated the number of shedding episodes initiated during the observation period, divided this number by the total number of daily swabs performed and multiplied by 365. Episodes of certain duration were those that started and ended with two negative swabs. We described each shedding episode according to its duration, peak copy number, expansion rate and decline rate (Fig S2), and arranged all outcomes in frequency histograms to define ranges for these outcomes.

As swabs were taken every 24 hours, episodes may have initiated within 0–24 hours of the first positive swab of the episode, and terminated within 0–24 hours of the last positive swab. We assumed that the midpoint of this interval (12 hours) would provide an unbiased estimate of length. To account for the fact that duration of some episodes could not be observed when shedding was present on the first or last swab of the session, we constructed an additional definition of episode that allowed the duration to be uncertain. The time to completion of an episode was then a survival outcome which could terminate as a censored episode; and we performed interval censoring to estimate duration of these(24). This technique assumes that within each study arm, censored episodes last at least that long (non-informative censoring).

For measures of episode peak copy number, expansion and decay, we only included episodes of known duration since we were not otherwise certain to capture either the start or termination. We defined episode peak as the maximum copy number obtained over the episode. We calculated the rate of viral increase from initiation to the estimated peak of each episode by computing the slope of a linear regression line over the copy numbers up to and including estimated maximum copy; for this calculation, we set the time of the most proximal negative swab at 0.5 days prior to first positive. We calculated the rate of decrease from peak to termination of each episode in a similar fashion by setting the time of termination to 0.5 days post the last positive. We identified proportion of episodes with and without re-expansion (defined as a rise in HSV copy number by 0.5 logs after a prior decline within the episode of at least 0.5 logs): of note, this measure may underestimate the true frequency of viral re-expansion as studies with every 2 hour sampling identified that viral rebound is a nearly universal feature of episodes lasting more than 3 days(10, 15).

Rate outcomes were compared by arm between persons using generalized estimating equations models with a log link, to estimate risk ratios for shedding rates, lesion rates, and episode rates by study arm. Continuous outcomes like maximum copy per episode were compared using generalized estimating models with an identity link.

Mathematical model simulations

All simulations were performed using C++. The model was solved stochastically due to the random nature of shedding episode initiation and viral clearance, and to account for frequent presence of low numbers of infected cells: at each time step, integer values for equation terms were drawn randomly from binomial distributions. Model variables were updated at a narrow time interval (0.001 days). Graphical output was made with Prism and Microsoft Excel.

The *in silico* simulation model in this paper consists of 200 hexagonal micro-regions of infection in the genital mucosa. Previous versions consisted of 300 regions. However, we obtain similarly accurate model output and incur less computational cost with fewer regions (10, 15). Regions are linked by the ability of: 1) neurons to randomly release virus into any of the 200 regions and initiate shedding episodes by infecting a single keratinocyte, and 2) cell-free virus from one region to seed an adjacent hexagonal region leading to multiple concurrent foci of infection. The 200 micro-regions are arrayed in a two-dimensional matrix such that cell-free HSV from a region can only infect a maximum of six other regions (Fig 3b). In the past, simulation results were invariant to allowing anywhere between 4 and 12 potentially infected adjacent regions.

Below is the full set of model equations off of treatment. The HSV-2 replication cycle and immunologic response to infected cells can occur concurrently in multiple hexagonal regions (Fig 3b):

$$\begin{aligned}
 \Delta S(i=1 \dots 200) &= [\lambda - (\beta_i * S_i * V_i) - (\beta_i * S_i * V_{neu}) - (\beta_e * S_i * V_e)] \Delta t \\
 \Delta I(i=1 \dots 200) &= [(\beta_i * S_i * V_i) + (\beta_i * S_i * V_{neu}) + (\beta_e * S_i * V_{eadj}) - (a * I) - (f * I * E)] \Delta t \\
 \Delta E(i=1 \dots 200) &= [(F(I) * \theta * E) - (\delta * E)] \Delta t \\
 \Delta V_{neu}(i=1 \dots 200) &= [\phi - (c * V_{neu}) - (\beta_i * S_i * V_{neu})] \Delta t \\
 \Delta V_i(i=1 \dots 200) &= [(p * I) - (a * V_i) - (\beta_i * S_i * V_i)] \Delta t \\
 \Delta V_e(i=1 \dots 200) &= [(a * V_i) - (c * V_e)] \Delta t \\
 \lambda &= d(S - S_0) \\
 F(I) &= I / (I + r) \\
 V_{eadj} &= V_e \text{ from 6 adjacent regions} \\
 V_{etot} &= V_{e1} + V_{e2} + \dots + V_{e200} \\
 V_{itot} &= V_{i1} + V_{i2} + \dots + V_{i200}
 \end{aligned}$$

HSV-2 (V_{neu}) is randomly released from neuronal endings into the dermal-epidermal junction at a rate (ϕ). Neuronal HSV-2 is cleared from the genital tract at rate (c) during which time it can infect local epithelial cells (S). If an infection occurs, then one of the 200 genital tract regions is randomly selected as the site of infection take off. Viral infectivity (β_i) determines how effectively cells are infected by HSV. If an epithelial cell becomes infected (I), then it dies via direct lysis at rate (a) or via CD8+ lymphocyte (E) mediated lysis at a rate ($E * f$). p is the rate of viruses produced by an infected cell per day. Re-growth of susceptible keratinocytes in one region occurs according to a growth rate, λ or $d * (S_0 - S)$ with growth limited by S_0 , the carrying capacity of the system. The formation of a genital lesion is accompanied by accumulation of CD8+ lymphocytes at peak rate (θ), followed by slow decay of these cells at rate (δ). $\theta/2$ is the rate when infected cells are equal in number to parameter r , which represents how many epithelial cells need to be infected prior to half-maximal CD8+ expansion. Cell-associated HSV-2 (V_i) is differentiated from cell-free HSV (V_e) in the model: cell-associated particles can pass from cell-to-cell within one ulcer micro-environment as soon as they form within a cell and convert to cell-free virus (V_e) after infected cells rupture. V_e can initiate formation of new plaques within adjacent regions by local seeding in our model, and is assigned an infectivity parameter (β_e). Parameter (ε) is included to account for delay in viral production within secondary plaques after seeding. We use V_e as the variable to assess congruence with our empirical shedding data in treated trial

participants as cell-free virus is detected on our genital swabbing protocols. Of note, the CD8+ variable in the model is intended as a measure of full cytolytic potential in a micro-region which is likely due to concurrent effects of multiple cell types including NK cells, CD4+ T-cells and CD8+ T-cells, and is not intended as a literal interpretation of local CD8+ T-cell count.

PK model

A population PK model was previously developed to characterize the time-course of pritelivir in plasma following single and multiple-dose administration using data from five Phase 1 trials conducted in a total of 162 healthy volunteers and one Phase 2 clinical trial conducted in 113 HSV-2 infected patients. Given the complex absorption profiles observed for pritelivir, a three-compartment model with linear elimination along with both a time-dependent and saturable absorption process (Fig S3 and Table S3) was required to provide adequate fit to the drug concentration data. The oral pritelivir dose was first introduced into a depot compartment, which conceptually represents the stomach from which the rate of drug release into the intestine was characterized using a Michaelis-Menten function. We defined this function using a maximum absorption rate ($V_{\max}$ in mg/hr) and the dose achieving 50% of $V_{\max}$ (K_m in mg). The $V_{\max}$ parameter was allowed to increase over time after a dose (TAD) from its starting value [$V_{\max(0)}$] using a Hill function in which the maximum fractional increase in $V_{\max}$ relative to $V_{\max(0)}$ [$TV_{\max}$] and the time at which 50% of $TV_{\max}$ was achieved (T_{50} in hr) were estimated. The sigmoidicity factor (γ) was fixed at a value of 10 due to the apparent steepness of the relationship and the instability of the model when this parameter was fit.

Pritelivir was allowed to leave the intestinal compartment to enter the central (plasma) compartment using a first-order rate constant (k_a). Once drug reached the central compartment, the distribution and elimination was modeled using a conventional linear three-compartment disposition model parameterized using the apparent total clearance (CL/F in L/hr), central volume of distribution (V_c/F in liters), volume of distribution for the second peripheral compartment (V_{p2}/F in L), steady-state volume of distribution (V_{ss}/F in L), the distribution clearance between the central and the first peripheral compartment (CL_{d1}/F in L/hr), and the distribution clearance between the central and the second peripheral compartment (CL_{d2}/F in L/hr).

$$\frac{dA_{\text{stom}}}{dt} = -\frac{V_{\max}(t) \cdot A_{\text{stom}}}{K_m + A_{\text{stom}}}, \quad IC = \text{Dose} \quad (1)$$

$$\text{Where: } V_{\max}(t) = V_{\max(0)} \cdot \left(1 + \frac{TV_{\max} \cdot TAD^\gamma}{T_{50}^\gamma + TAD^\gamma}\right)$$

$$\frac{dA_{\text{int}}}{dt} = \frac{V_{\max}(t) \cdot A_{\text{stom}}}{K_m + A_{\text{stom}}} - k_a \cdot A_{\text{int}}, \quad IC = 0 \quad (2)$$

$$\frac{dA_c}{dt} = k_a \cdot A_{int} + \frac{CLd1}{V_{p1}} \cdot A_{p1} + \frac{CLd2}{V_{p2}} \cdot A_{p2} - \left(\frac{CLd1}{V_c} + \frac{CLd2}{V_c} + \frac{CL}{V_c} \right) \cdot A_c, \quad IC=0 \quad (3)$$

$$\frac{dA_{p1}}{dt} = \frac{CLd1}{V_c} \cdot A_c - \frac{CLd1}{V_{p1}} \cdot A_{p1} \quad IC=0 \quad (4)$$

$$\frac{dA_{p2}}{dt} = \frac{CLd2}{V_c} \cdot A_c - \frac{CLd2}{V_{p2}} \cdot A_{p2} \quad IC=0 \quad (5)$$

Inter-individual variability was assumed for CL/F, V_c/F, K_m, k_a, T₅₀, V_{p2}/F and V_{ss}/F by drawing from normal distributions with %SEM listed in Table S3. The relationship between body weight and CL/F and V_{ss}/F were described using power functions while a proportional decrease in V_{ss}/F of 20% was identified for females (SEXF=1). Food was also shown to increase the relative bioavailability (F_{rel}) by 23% but significantly slowed k_a. We assumed that oral pritelivir was administered during or after food intake and also simulated trials in the fasting state: these simulations achieved equivalent results.

The model described above was developed using non-linear mixed effects models as implemented in the population PK software NONMEM (NONMEM 7 [computer program]. Version 7.1.2. Ellicott City, MD: ICON Development Solutions, 2010). For the purposes of this publication, the model was re-programmed in C++ in order to allow for integration with the viral kinetic model.

PD model

We included a PD equation to describe the instantaneous effect of drug on viral replication. Pritelivir is a helicase-primase inhibitor that inhibits viral replication. The 50% effective concentration (*in vivo* EC₅₀) is defined as the unbound plasma drug concentration at which HSV replication rate is decreased by 50%. PK and PD models are linked by (CONC(t)*0.028)/EC₅₀, where EC₅₀ is invariant, total plasma drug concentration (CONC) fluctuates according to the PK equations, and 0.028 is fraction of pritelivir unbound. We assumed that the rate of HSV DNA production (p_d) within infected skin cells was $p/[1+((CONC(t)*0.028)/EC_{50})^m]$, where p is the number of HSV DNA copies per cell per day without drug and m is the Hill coefficient. We assumed that the rate of HSV DNA release from neurons (φ_p) was $\phi/[1+((CONC(t)*0.028)/EC_{50})^m]$, where φ is the number of HSV DNA copies released from all neural ganglia per day. The Hill coefficient (m) is included to capture the fact that pritelivir may bind to its helicase-primase complex target in a cooperative fashion leading to a steeper dose response curve and potentially more potent drug activity at high drug to EC₅₀ ratios.

Mathematical model fitting

Our fitting techniques and parameter selection are described in the Supplement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Increased pritelivir dose results in decreased HSV-2 shedding, particularly at high viral copy numbers

(A) Frequency histograms of HSV-2 shedding in cohorts of untreated and pritelivir treated participants. (B) Placebo group shedding (n=30) closely approximates shedding in larger a historical control of untreated patients (n=531).

Figure 2. Higher pritelivir dose results in decreased HSV-2 shedding episode rate, as well as decreased shedding episode duration, increased clearance rate, decreased early viral load and decreased peak viral load

Frequency histograms of HSV-2 shedding in cohorts of untreated and pritelivir treated participants. **(A)** Annualized episode rate; **(B)** Episode duration; **(C)** Median initiation to peak expansion and **(D)** Median peak to termination clearance slopes; **(E)** first, **(F)** peak, and **(G)** last HSV DNA copy number per episode; and **(H)** episode re-expansion rate, and **(I)** percent of episodes associated with lesions.

(A) Schematic for HSV-2 infection within a single genital tract microenvironment. Equations capture seeding of epithelial cells by neuronal HSV-2, replication of HSV-2 within epithelial cells, viral spread to other epithelial cells, cytolytic CD8+ T-cell response to infected cells, transition of cell-associated HSV-2 to cell-free HSV-2 following lysis of infected cells, and elimination of free virus and infected cells. Parameters are HSV DNA release rate from neurons (Φ), HSV DNA clearance rate (c), CD8+ T-cell expansion rate (Θ), CD8+ T-cell decay rate (δ), number of cells at which Θ becomes half maximal (r), cell-associated HSV infectivity (β_i), cell-free HSV infectivity (β_e); infected cell death rate (a), viral replication rate (p), and production lag within newly infected region (ϵ). (B) Micro-regions are linked virally because cell-free HSV-2 can seed surrounding regions.

Figure 4. High doses of daily pritelivir result in sustained, incomplete inhibition of HSV-2 replication *in vivo* throughout the dosing cycle

(A) Optimal fit to the trial data is achieved at *in vivo* EC₅₀ values between 50 and 70 ng/mL, with nearly equivalent model fit (low residual sum of squares) at all values for the Hill coefficient (m). Thin lines are polynomial fits through the data each with $R^2 > 0.9$. (B–E) Simulated unbound pritelivir levels relative to the *in vivo* EC₅₀ (black line, 60 ng/mL). Ten simulations were performed per dose with randomly selected gender, weight and fasting status (thin colored lines): (B) 5 mg daily, (C) 25 mg daily, (D) 75 mg daily, and (E) 400 mg weekly. (F) Estimated inhibition of HSV-2 replication over time for different doses assuming *in vivo* EC₅₀=60 and m=2.

Figure 5. *In silico* clinical trial simulations recapitulate quantitative shedding rates at all doses. Representative simulated model trials (n=30 patients) of HSV-2 shedding (thin colored lines) in reference to empirical shedding data (median marked with black dot and 95% CI with black horizontal bars). Ten of the one hundred simulated trials are displayed. (A) Placebo, (B) 5 mg daily, (C) 25 mg daily, (D) 400 mg weekly, and (E) 75 mg daily. A majority of simulated values fall within 95% confidence intervals. (F) Mean shedding quantity across 10 simulated trials for each dose cohort. The frequency histogram generally recapitulates the empirical trial data (Figure 1a).

Figure 6. Mechanism of decreased shedding rate at higher simulated doses

(A) High inter-subject variability in shedding rate decreases at higher simulated doses.

Each point represents a simulated participant (300 per arm). This analysis assumed continuous rather than daily sampling for a more precise measure of shedding rate. Bars represent median and interquartile range. (Placebo versus 5 mg, NS; 5 mg versus 25 mg, $p < 0.0001$; 25 mg versus 400 mg weekly, $p < 0.0001$; 400 mg weekly versus 75 mg, $p < 0.0001$)

(B) Simulated episode rate decreases as a function of dose. Each point represents a simulated trial (10 trials with 30 participants per arm) and lines represent mean values. (Placebo versus 5 mg, NS; 5 mg versus 25 mg, $p = 0.001$; 25 mg versus 400 mg

weekly, $p=0.004$; 400 mg weekly versus 75 mg, $p=0.0002$). **(C–E)** Each point represents a simulated shedding episode (placebo = 749, 5 mg = 753, 25 mg = 574, 400 mg weekly = 413, 75 mg = 267). **(C) Number of infected cells within the first infected ulcer decreases with dose.** (Placebo versus 5 mg, NS; 5 mg versus 25 mg, $p=0.001$; 25 mg versus 400 mg weekly, $p=0.004$; 400 mg weekly versus 75 mg, $p=0.0002$). **(D) Number of infected genital regions per episode decreases with dose.** (Placebo versus 5 mg, NS; 5 mg versus 25 mg, NS; 25 mg versus 400 mg weekly, $p=0.01$; 400 mg weekly versus 75 mg, NS). **(E) Episode duration decreases at higher doses.** (Placebo versus 5 mg, NS; 5 mg versus 25 mg, NS; 25 mg versus 400 mg weekly, $p<0.0001$; 400 mg weekly versus 75 mg, NS).

Figure 7. Clinical trial measures confirm limited viral shedding when drug levels exceed the *in vivo* EC₅₀

Unbound pritelivir concentrations versus (A) concurrent HSV viral load and (B) peak HSV viral load within the ensuing 48 hours on drug. The blue vertical line represents the estimated *in vivo* EC₅₀ (60 ng/mL).

Figure 8. Pritelivir is predicted to inhibit HSV-2 shedding in a dose dependent fashion even when shedding is rare

(A) Mean shedding rates from 10 simulated trials with 30 participants each. Mean shedding rate in 10 simulated trials was 2.2% (1.2 – 3.2%) for 75 mg daily versus 1.7% (range: 0.6 – 2.7%) for 100 mg daily ($p=0.01$). Mean shedding rate was 0.6% (range: 0.2 – 1.2%) for 150 mg daily ($p<0.01$ versus 100 mg daily) and 0.5% (range: 0.1 – 1.4%) for 75 mg twice daily ($p<0.01$ versus 100 mg daily). (B) **Simulated and observed reductions in shedding rates relative to placebo as a function of average daily dose.** Simulated data is shown with open circles for 5 mg (red), 25 mg (light green), 57.1 mg (orange, averaged from 400 mg weekly) 75 mg (light blue), 100 mg (royal blue), and 150 mg daily (dark blue for 150 mg daily, and green for 75 mg twice daily); and 400 mg weekly. Actual data is shown with closed circles for 5 mg (red), 25 mg (light green), 57.1 mg (orange, averaged from 400 mg weekly) and 75

mg (light blue). A logarithmic model is fit to the data and predictions and demonstrates that daily dose is predictive of reduction in shedding rate relative to placebo ($R^2=0.98$, reduction =