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Optimization of a Vaginal Suppository Formulation to Deliver SHetA2 as a Novel Treatment for Cervical Dysplasia

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Abstract

Cervical dysplasia induced by the human papilloma virus (HPV) unpredictably progresses to cervical cancer. Therapeutic options are invasive and affect the patient's quality of life. SHetA2 has demonstrated therapeutic efficacy against human and murine HPV- induced tumors, but its oral bioavailability is <1%. An optimized vaginal suppository formulation can deliver SHetA2 in sufficient doses to prevent cervical dysplasia. The quality by design (QbD) approach was employed to optimize the suppository formulation consisting of cocoa butter as base with 5% Kolliphor and 40% SHetA2. The suppository had a content uniformity of 105.44±0.42%, melted in <8 minutes and had a complete release of SHetA2 in water. Administration of the suppository to mice achieved cervix concentrations that were significantly higher than the SHetA2 therapeutic concentration, with the maximum concentration ($C_{\text{max-cervix}} = 336.78 \mu\text{g/g}$) being more than 100-fold the therapeutic SHetA2 concentration. Furthermore, the levels of cyclin D1 protein decreased 9-fold indicating a correlation of drug concentrations with the pharmacodynamic endpoint. These proof-of-concept studies suggest that the SHetA2 optimized vaginal suppository formulation may have a potential use in the prevention of cervical dysplasia, but detailed efficacy studies are required to confirm this assumption.

Keywords

Mucosal drug delivery; Formulation; Solid dosage form; Dissolution; Absorption; Vaginal suppository; Quality by Design; Cervical dysplasia; Cancer; SHetA2

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Supplementary material

Dissolution studies of a model drug from suppository formulations having the fastest disintegration time in DoE2

INTRODUCTION

Cervical cancer is the second most common form of gynecologic cancer worldwide affecting about 528,000 women and caused approximately 266,000 deaths in 2012¹. Infection of the cervix with the human papilloma virus (HPV) is widespread in women under 25 years of age². Epidemiological and experimental data suggest a strong association between cervical HPV infection and cervical cancer³. High risk HPV infection causes premalignant lesions in the cervical epithelium, which are known as cervical dysplasia. If untreated, cervical dysplasia can progress to cervical cancer through a series of cellular mechanisms. In the United States, there are 330,000 new cases of high grade dysplasia⁴ compared to only 12,000 cases of cervical cancer every year^{2, 3}. Therefore, treatment of cervical dysplasia may be a practical way to prevent cervical cancer and has the potential of benefiting more patients.

Current treatment for high grade cervical dysplasia involves cryogenic destruction of tissue or surgical removal of tissue⁵. While this treatment is effective, many women are unnecessarily treated because many cases will resolve on their own, but there is no way to know which dysplasia patients will progress to cancer. Moreover, because these therapeutic measures are invasive, they can cause infertility and poor quality of life for the patient⁵. The use of a chemopreventive drug instead of these intrusive measures could reduce cost, discomfort and potential loss of fertility.

SHetA2 (NSC721689) is a novel, non-toxic, flexible heteroarotinoid compound that has demonstrated therapeutic and preventive efficacy in human and murine HPV-induced tumors by causing G1 cell cycle arrest by suppressing cyclin D1 levels and ultimately inducing apoptosis⁶. In association with cyclin-dependent kinase 4 or 6, cyclin D1 forms a complex that phosphorylates retinoblastoma protein, which in turn can act as tumor suppressor^{7, 8}. Previous studies demonstrated that SHetA2 induced G1 arrest by suppressing cyclin D1 levels *in vivo*⁹. Given that SHetA2 can counteract the influence of HPV on cell cycle progression by suppression of cyclin D1 cellular levels, a reduction in cyclin D1 levels can be considered as pharmacodynamic endpoint in preclinical studies. However, the potential efficacy of SHetA2 is limited by its low oral bioavailability caused by its poor aqueous solubility¹⁰. Kabirov et al.¹¹ reported that the oral bioavailability of SHetA2 is null and that this bioavailability increases to approximate 10% when Kolliphor, a self-emulsifying drug delivery system is added to the suspension. Kolliphor was selected to enhance bioavailability because of its lack of toxicity at lower concentrations on epithelial cells¹² and its enhancement of tumor inhibitory effect observed with other chemotherapeutic agents¹³.

Vaginal suppositories are commonly employed to treat urogenital infections and other local diseases^{14–17} due to the ease of drug administration. Direct delivery of SHetA2 at the site of dysplasia would overcome its limited oral bioavailability and circumvent the need for systemic absorption. Previous studies demonstrated that topical application of SHetA2 did not irritate mice skin¹⁸, therefore, we hypothesized that a vaginal suppository would be able to deliver an effective dose of SHetA2 that in turn, may be capable to treat and prevent cervical dysplasia.

The present study developed and optimized a suppository formulation to deliver SHetA2 by the vaginal route to achieve therapeutic drug concentrations at the cervix. Preliminary *in vivo* studies were performed as proof-of-concept to evaluate the drug tissue concentrations and cyclin D1 protein levels that administration of the optimized SHetA2 suppository would be able to achieve.

MATERIALS AND METHODS

Materials

SHetA2 was synthesized by Cayman Chemical company, Inc. under a contract from the Rapid Access to Preventive Intervention Development (RAPID) National Cancer Institute (NCI) program. Cocoa butter was purchased from Nature's Oils (Streetsboro, OH). Polyethylene glycols (PEG) (MW 400, 3350), acetonitrile (HPLC grade 99.5%), sodium chloride, lactic acid, acetic acid, potassium hydroxide (KOH), bovine serum albumin (BSA), glucose were purchased from Sigma Aldrich (St Louis, MO). Glycerol USP and mineral oil USP were purchased from VWR International (Radnor, PA). Kolliphor was obtained from BASF (Germany). Captiva[®] filtration plates were purchased from Agilent Technologies Inc. Anti-cyclin D1 antibody was purchased from Cell Signaling Technology (Boston, MA), and anti- β -actin peroxidase/FITC conjugated secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA) for Western Blot experiments. Mammalian Protein Extraction Reagent (m-PER), Pierce BCA protein assay kits were purchased from Thermo Fisher Scientific (Grand Island, NY). Protease inhibitor cocktail and phosphatase inhibitor cocktail were purchased from Roche (New York, NY). Polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence (ECL) reagent were purchased from Bio-Rad (Hercules, CA).

HPLC determination of SHetA2 in solutions

Determination of SHetA2 in solutions was performed using a Waters Alliance HPLC System with a V_{ydac} 201 TP C₁₈ 5 μ (250 mm $\times$ 2.1 mm) column equipped with a guard column (V_{ydac} 201 TP, Grace), and a UV detector set at 341 nm. The mobile phase consisted of acetonitrile:water (80:20, v/v). The flow rate was 0.3 mL/min with a retention time of 3.65 minutes.

Determination of the partition coefficient (Log P) and pKa of SHetA2

Given that SHetA2 is a new drug, the Log P was estimated using the interactive Log P calculator on Molinspiration[®] website (<http://www.molinspiration.com/>). Log P was also determined experimentally by the shake flask method¹⁹ using three different octanol:water volume ratios: 1:2, 1:1 and 1:4.

The pKa of SHetA2 was determined using the potentiometric titration method described by Benet et al.²⁰ using potassium hydroxide 0.1N. Ethanol was used as co-solvent, as SHetA2 is poorly soluble in water. The spKa values (defined as the pKa obtained from semi aqueous titration) were determined from each titration curve at half equivalence point. The average spKa+ Log [water%] was plotted against 1/D (D= dielectric constant of the semi aqueous solvent)²¹.

Human size suppositories

Formulation and optimization of SHetA2 suppository formulation—Quality by design (QbD) methodology was employed to optimize the formulation of SHetA2 into vaginal suppositories by first considering the material attributes (Figure 1). The desired characteristics for the SHetA2 vaginal suppository formulation were: i) ease of insertion; ii) fast drug release of from the base; and, iii) meet quality control specifications outlined by the United States Pharmacopoeia (USP) ²². Thus, the critical quality attributes (CQA) in the optimum formulation were defined as to: - i) be solid at room temperature and maintain physical integrity during administration; ii) disintegrate/melt within 5–10 minutes; iii) have content uniformity (85%–115%) and weight variation within 7.8%.

Suppositories were manufactured using the fusion molding method ²³ and USP stainless steel suppository molds. The formulation ingredients consisted of a hydrophilic base (PEG mixture) or a lipophilic base (cocoa butter), drug and Kolliphor. These ingredients were entered at different proportions into the Design of Experiments (DoE) software (Design-Expert, version 8.0.1, Stat-ease®) to evaluate statistically the effects of the formulation components on the characteristics of the product ²⁴. The final formulation composition was determined using two sequential DoEs as follows.

The first DoE (DoE1, 16 formulations) was an “optimum mixture” design to identify the proportions of PEGs and Kolliphor that would yield a fully formed suppository that would remain solid and stable at room temperature. Combinations of PEG 400 (10–40%), PEG 3350 (40–60%) and Kolliphor (5–30%) were entered as factors and the responses evaluated were: (A) Is the suppository fully formed? and (B) Does the suppository remain solid at room temperature?

In the second DoE (DoE2), the type of base, the proportion of drug and the proportion of Kolliphor were optimized. DoE2 was a 2³ factorial design, with each of the three factors having two levels: (1) Percentage of drug (20% and 40% w/v), (2) Types of base, (cocoa butter as the lipophilic base and the best combination of PEG 400 and PEG 3350 that was identified in DoE1 as the hydrophilic base); (3) Percentage of Kolliphor (5% and 30%). To save SHetA2, suppositories in DoE2 were prepared using rifampicin as model drug because, like SHetA2, it has low solubility in water and a similar Log P=3.71^{25, 26}. The responses evaluated in DoE2 were: Is the suppository fully formed? (B) Does the suppository remain solid at room temperature? and (C) What is the disintegration time/softening time for PEG suppositories and cocoa butter suppositories, respectively. The dissolution profile was also determined for the three formulations that had the shortest disintegration/softening time.

Quality control of human size suppositories—For PEG suppositories, disintegration tests were performed as indicated by Saleem et al ²⁷ using a USP disintegration tester (QC-21 Disintegration Test System, Hanssen Research, Utah) and water at 37 °C.

For cocoa butter based suppositories, the softening time was determined by “Method B” specified in the guidelines of the World Health Organization ²⁸, with a custom made apparatus as follows. A 25 cm tube containing 10 ml of water was immersed in a water bath at 37°C. The glass tube was fixed vertically and immersed to a depth of at least 7 cm below

the surface but without touching the bottom of the water-bath. For the test, a suppository was introduced tip first, into the tube followed by the rod with the free gliding plastic cover into the glass tube until the metal needle touches the flat end of the suppository. The cover was placed on the tube (beginning of time measurement) and the time elapsed until the rod sank down to the bottom of the glass tube was recorded as softening time and response for DoE2.

The dissolution profile of the model drug rifampicin was determined for three formulations exhibiting the shortest disintegration time in DoE2 using the USP rotating basket apparatus at 75 rpm with water at 37 °C as the dissolution media (Vanderkamp 600 Six-Spindle Dissolution Tester, Vankel Corp., NC). Samples were withdrawn at different time points and the rifampicin concentration determined by UV spectroscopy at 339 nm.

Mouse-sized suppositories

Preparation of mouse-sized SHetA2 suppositories—Suppository molds in mouse sizes are not commercially available, therefore, we designed and custom-made a mold based on the average dimensions of the vaginal cavity in FVB female mice of 8–10 weeks of age. For this purpose, the diameter and depth of the vaginal cavity of 5 mice were measured with calipers and determined to be 0.4 cm diameter and 0.5 cm depth. Mouse-size suppository molds were custom-made using 0.2 ml PCR tubes trimmed at the desired length while ensuring that the conical shape was maintained to facilitate insertion.

Previous studies delivering SHetA2 by the oral route used a dose of 30 mg/kg body weight⁹, consequently, we adopted the same dose for this study for comparison. Considering an average weight of 21 g for female mice at eight weeks of age²⁹ and to achieve the 30 mg/kg dose, the amount of drug in each suppository was 0.63 mg of SHetA2. The optimum formulation identified at the end of DoE2 was adapted to achieve this dose using the equations and plots generated from the analysis of the parameters in DoE2.

Quality control of mouse-sized suppositories—In addition to the quality control tests performed on the human-sized suppositories, the mouse-sized suppositories were evaluated for content uniformity and weight variation as specified in the USP²². Determination of the dissolution rate of SHetA2 was evaluated in both, deionized water and simulated vaginal fluid at 37 °C as described above using the USP rotating basket apparatus. The simulated vaginal fluid (SVF) was prepared as described by Marques et al.³⁰. Samples were withdrawn at different time points and the SHetA2 content analyzed by HPLC.

Preliminary *in vivo* disposition studies

Two sets of *in vivo* studies were performed as proof of concept to evaluate the optimized suppository formulation. The first study estimated the residence time of the suppositories in the mouse vagina and the second study determined the extent of drug absorption after suppository administration. Friend Leukemia Virus B (FVB) female mice (8 weeks of age; Charles River, USA) were used in this study. Mice were housed at 22°C on a 12 h light/dark cycle and allowed access to water and food *ad libitum*. All animal experiments were approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee.

To evaluate the residence time of each suppository formulation as a function of the suppository base, FVB female mice (n=3) were anesthetized with isoflurane to facilitate suppository insertion and kept under sedation for 3 minutes to prevent suppository leakage. The time from suppository insertion to either suppository coming out of the vaginal cavity or when no suppository or fluid leaked out of the vaginal cavity, was recorded as residence time. The suppository formulation having the longest residence time without evidence of leakage was selected for the absorption study.

To determine the extent of SHetA2 absorption, two suppositories containing a 30 mg/kg dose were administered vaginally to each mouse to ensure that enough SHetA2 could be detected. Mice were anesthetized as described above and one suppository was first inserted in the vaginal cavity. The mouse was allowed to recover for 30 minutes and then it was anesthetized again to administer a second suppository. The mouse remained anesthetized for three additional minutes to prevent drug leakage. Mice (n=3) were euthanized at 0.25, 0.5, 2 and 4 hours after administration of the second suppository and their cervix tissues were collected, weighed, snap frozen in liquid nitrogen and stored at -80 °C until drug analysis.

Determination of SHetA2 concentrations in cervix tissue—For SHetA2 extraction, cervix tissues were weighed and homogenized in an acetonitrile:saline mixture (80:20, v/v). Samples of 2 ml/g of tissue were centrifuged at 14000 rpm for 10 minutes. Supernatants were separated and loaded onto a Captiva filtration plate. Filtrates were collected and injected directly into the HPLC using the same conditions described above. SHetA2 concentrations were determined from a standard curve that was prepared by spiking SHetA2 solution into untreated cervix tissue homogenates.

Determination of Cyclin D1 levels in cervix and uterus

Gynecologic tissues were collected from mice treated with suppositories containing SHetA2 or placebo (suppositories containing base and Kolliphor but no drug) for 30 mins. From each gynecologic tissue sample, the two uterine horns, each with part of the cervix attached were separated. Then each uterine horn was horizontally dissected and homogenized in ice-cold m-PER protein extraction solution containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail. The homogenate was incubated on ice for 1 hour with occasional shaking and centrifuged at $13,000 \times g$ for 15 minutes at 4°C.

Protein concentrations in supernatants were determined using the BCA assay as per manufacturer's instructions. Equal amounts of protein were separated by gel electrophoresis and then transferred to an Immuno-Blot™ PVDF membrane. The membrane was blocked with 5% skim milk and then incubated with primary cyclin D1 antibody at 1:1000 dilution overnight at 4 °C. The membrane was then washed and incubated with a secondary peroxidase-conjugated antibody for 1 h. Antibody binding was detected using enhanced chemi-luminescence detection system (Bio-Rad). After developing, the membrane was stripped and re-probed using an antibody against β -actin at 1:2000 dilution to confirm equal loading. Each experiment was repeated three times to assess for consistency of results. Densitometric quantitation of the bands in the most representative Western blots was

performed using a Bio-Rad ChemiDoc Touch Imaging System. Relative cyclin D1 levels were calculated by dividing the cyclin D1 band intensity by the β -actin band intensity.

Data analysis

The times measured for disintegration/ melting time of each suppository formulation in DoE2 were analyzed by the Design-Expert software as follows: First, the absolute values of the effect of each analyzed variable were displayed in a half normal probability plot and after the effects with the largest magnitude (above the “t” value) were selected, the chosen model effects (variables) were analyzed with ANOVA based on F-test. The effect is considered significant if (prob>F) is <0.05. The equation quantifying the effect of each variable is built with the effects considered significant for each response.

Preliminary pharmacokinetic parameters for SHetA2 ($C_{\text{max-cervix}}$ = maximum concentration in cervix, $T_{\text{max-cervix}}$ = time to achieve maximum concentration in cervix, $t_{1/2\text{-cervix}}$ = half-life in cervix, Vz_F = apparent volume of distribution/F, Cl_F = Clearance/F) were determined by non-compartmental analysis using Phoenix WinNonlin® software.

RESULTS

Partition coefficient (Log P) pKa and of SHetA2

The Log P value estimated by the Molinspiration property engine was 4.48, whereas the Log P value determined by the shake flask method was 3.84 ± 0.34 . The pKa determined for SHetA2 was 13.01 ± 0.04 .

Human size suppositories

Table 1 shows the composition of the 16 formulations in DoE1 and the responses measured. All formulations produced suppositories that were solid (fully formed and not brittle) and retained their shape/integrity (not distorted or melting) at room temperature, indicating that the presence of Kolliphor did not affect the integrity of the suppositories. Thus, the formulation in experiment #16 was selected because it has the PEG combination that is closest to that of commercially available progesterone vaginal suppositories³¹.

The selected PEG combination was then entered into DoE2 together with cocoa butter (figure 1) to evaluate the effect of the base on the physical integrity of the suppository at room temperature and the rate of drug release. Table 2 lists the formulations prepared for DoE2 and the corresponding measured responses. Among these formulations, only six were well formed and remained solid at room temperature. Overall, cocoa butter suppositories melted faster than PEG suppositories disintegrated, regardless of the percentage of Kolliphor in the formulation. However, the larger percentage of Kolliphor appeared to be detrimental in the formation of cocoa butter suppositories, and appeared to have a minimal effect on the disintegration time of PEG suppositories (37 versus 31 minutes for suppositories containing 20% drug, and 95 versus 65 minutes for suppositories containing 40% drug). In contrast, an increase in the percentage of drug in the formulation appeared to double the disintegration time of PEG suppositories (from 37 to 95 minutes for suppositories containing 5% Kolliphor and from 31 to 65 minutes for suppositories containing 30% Kolliphor), whereas it appeared

to have a minimal effect on the melting time of cocoa butter suppositories containing 5% Kolliphor (6 minutes versus 7.4 minutes).

To understand the impact of materials on CQAs (Figure 1), the influence of formulation ingredients on the disintegration/melting time of suppositories was evaluated graphically and mathematically using the DoE software. The cube plot mapping the influence of each formulation ingredient (Figure 2) confirmed that the most influential factor for the disintegration/melting time of a suppository was the base, with cocoa butter suppositories melting faster than those made with the PEG combination. Likewise, the equation showing the contribution of each formulation factor (Equation 1) indicates that the type of base contributes much more to the disintegration/ melting time (26.8) than either the percentage of drug (11.3) or the interaction between the drug and base (11.7):

$$\text{Disintegration/Melting time} = 30.20 + 11.30 (\text{Drug}\%) + 26.8(\text{Base}) + 11.70(\text{Drug} * \text{Base})$$

[Equation 1]

The mathematical analysis also revealed the effect of an interaction between the drug and the base. This interaction is important because it had opposite effects on the disintegration/ melting time depending on the base, with the magnitude being bigger when PEG was used as the base. For example, when the drug percentage was increased from 20% to 40% in cocoa butter suppositories, melting time decreased from 3.7 minutes to 3 minutes (figure 2). In contrast, a similar increase in the percentage of drug in PEG suppositories resulted in an increase in disintegration time from 34 minutes to 80 minutes.

To predict drug release from the suppositories, the rate of dissolution was evaluated, using rifampicin as a model drug, for the three formulations with the shortest disintegration/melting time in DoE2 (table 2: formulations 1, 2 and 7). The dissolution rate of rifampicin correlated well with the disintegration time (figure in supporting information), with cocoa butter suppositories containing 40% drug releasing more than 20% of drug in 15 minutes, whereas PEG suppositories released less than 5% of the drug in the same time period. Therefore, the formulation having cocoa butter as base, 40% drug and 5% Kolliphor was selected to prepare mouse size suppositories for *in vivo* studies.

Mouse-sized suppositories

The statistical analysis, the cube plot generated by DoE2 (Figure 2) and Equation 1 were used in conjunction to adapt the human size formulation to mouse size suppositories with the desired CQAs. The resulting suppositories were yellow due to the natural color of SHetA2, conical in shape to facilitate vaginal insertion, with a 5 mm length and 3 mm diameter of the base (figure 3). As expected, the suppositories were fully formed, remained solid at room temperature and melted in 3.86 ± 0.64 minutes. The content uniformity of SHetA2 in the suppositories was $105.44 \pm 0.42\%$ with a 3.34% individual weight variation, thus meeting USP specifications.

For comparison in animal studies, mouse-sized SHetA2 suppositories were also prepared using the PEG combination as base with 40% drug and 5% Kolliphor. These PEG suppositories had a $107.54 \pm 0.07\%$ of SHetA2 content uniformity and a 8.90% individual weight variation, thus also meeting USP specifications.

The dissolution profile of SHetA2 from the cocoa butter mouse-sized suppositories in water was similar to that of rifampicin in the human size suppositories (fast and 84% cumulative percent release within 25 minutes, figure 4). However, dissolution of SHetA2 from the same formulation but in SVF, was slower and irregular, achieving only 35% percent of drug released after 5 minutes (figure 4), indicating that the release and dissolution of SHetA2 from cocoa butter suppositories is pH-dependent.

Preliminary *in vivo* disposition studies

The residence time of suppositories in the vaginal cavity of mice was evaluated to estimate the time that mice should be sedated to prevent leakage of the melted suppository. This was important to ensure that the entire drug dose would remain in the vaginal cavity and available for absorption so that preliminary pharmacokinetic parameters could be determined accurately. Cocoa butter suppositories appeared to melt 4.33 minutes after vaginal insertion with no evidence of leaking for 10 minutes after the mouse was awake and moving around. This melting time *in vivo* correlated well with the melting time *in vitro* predicted by the softening test. PEG suppositories appeared to disintegrate after 5.5 minutes of vaginal insertion. However, slight leaking of the suppository was observed 15 minutes after suppository insertion.

Analysis of SHetA2 concentrations in the cervixes of these mice correlated well with the observed melting/disintegration times and suppository residence times, as the SHetA2 concentration in the cervix of mice receiving the cocoa butter suppository was 2-fold larger ($14.09 \mu\text{g/g}$) than that of mice receiving the PEG suppositories ($6.98 \mu\text{g/g}$). Therefore, SHetA2 suppositories having cocoa butter as the base were employed in the preliminary PK study.

Administration of the optimized SHetA2 suppositories to mice achieved cervix concentration above the therapeutic levels ($4.0 \pm 0.4 \mu\text{M}$)⁶ at all evaluated time points (figure 5). Notably, at the time of maximum SHetA2 absorption in the cervix ($T_{\text{max-cervix}}=0.5 \text{ h}$), the maximum SHetA2 concentration ($C_{\text{max-cervix}}$) was 106-fold higher than the therapeutic concentration. Preliminary PK analysis of the cervix concentration versus time data determined the half-life ($t_{1/2\text{-cervix}}$) of the drug in the cervix to be 3.08 hours, with clearance (CL/F) of 0.00152 mL/h and an apparent volume of distribution (Vz/F) of 0.0067 mL .

Cyclin D1 levels in uterine tissue including cervix homogenate

For analysis of the cyclin D1 as a pharmacodynamic endpoint, Western blots were performed on protein extracts from the cervix-uterine tissue specimens collected from two mice 30 minutes after administration of the second suppository. Figure 6A shows that lower levels of cyclin D1 were observed in the tissues of two mice treated with SHetA2 compared to the two mice treated with placebo. Quantitation of the bands indicated that average cyclin

D1 protein levels were reduced on average 9 fold in the treated mice compared to the placebo-treated mice (figure 6B).

DISCUSSION

The decision about how to treat patients with cervical dysplasia is complicated because in most cases it is impossible to predict which dysplastic lesions will progress to cervical cancer³. The primary region where cervical dysplasia begins to appear is the “transformation zone” or “squamo-columnar junction”⁵, where the pre-cancerous dysplastic cells are fostered over long periods of time. To avoid the possibility of progression from dysplasia to cancer, physicians often prefer to employ procedures such as cryotherapy, cold knife conization or local ablation therapy to destroy the dysplastic cells or to remove the transformation zone. These invasive procedures have negative effects on the reproductive life of patients, from having pre-term birth to total loss of fertility^{5, 32}. Therefore, alternative, non-invasive and relatively less expensive approaches to treat this disease are greatly needed.

In the last two decades, several topical preparations have been evaluated in clinical trials as local treatments for cervical dysplasia using mainly two compounds: tretinoin³³ and imiquimod³⁴. In phase I-III trials, tretinoin was delivered to patients with mild to severe dysplasia using an inert collagen sponge and a cervical cap. Vaginal side effects occurred at every dose studied and included mild inflammation, increased vaginal discharge and itching. Thus, this treatment was not developed further³⁵. The topical use of imiquimod has been evaluated in clinical trials, employing different formulations in patients with high grade cervical intraepithelial neoplasia^{36–39}. The extent of efficacy in these trials as well as the observed side effects appeared to be dependent on the formulation, the dose and the number of doses^{36–39}. Pachman et al³⁸ employed a cream to deliver 2.5 mg of imiquimod twice a week and concluded that this treatment had no effect on cervical dysplasia but caused significant unwanted side effects. In another trial, Grimm *et al*³⁴ employed suppositories to deliver 6.25 mg of imiquimod (1–3 doses per week) for 16 weeks and concluded that this treatment induced disease regression in 73% of patients. However, most patients experienced side effects and some withdrew from the study while others continued on a reduced dose. These side effects were due to imiquimoid rather than the formulation, as similar or worse side effects were reported when imiquimod was used to treat other diseases^{36, 39}. In contrast to these compounds, formal toxicological studies indicated that SHetA2 does not elicit adverse effects when given orally to rats and dogs at doses that were more than 30 times larger than the effective dose¹¹. Other studies reported no side effects or irritation after repeated administration of SHetA2 at a 2-log concentration dosage range¹⁰, thus indicating the safety of SHetA2 for topical administration.

Besides the safety of the therapeutic compound, the formulation employed to deliver the drug is important. Vaginal creams and gels are difficult to dose^{15, 40} and leakage of medication leads to non-uniform dose delivered. In contrast, vaginal suppositories are a convenient way to deliver a fixed dose of drug without administration error and result in better treatment outcomes, as shown for bacterial vaginosis treated with clindamycin suppositories versus cream⁴¹. Therefore, we hypothesized that vaginal delivery of SHetA2

in an optimized suppository formulation would be able to achieve therapeutic drug concentrations at the cervix for longer times.

We applied the QbD principles to develop and optimize a suppository formulation to deliver SHetA2. The first step was to select the type of base that would enhance the release and absorption of SHetA2 in the vaginal cavity. To enhance the release of a drug, it is generally recommended that the drug has little to no solubility in the suppository base⁴². As SHetA2 is an investigational drug, it was necessary to determine its permeability and pH dependent solubility.

The pKa of 13.01 ± 0.04 indicated that SHetA2 is a weak acid, and that at acidic pH, such as that in the vaginal fluid⁴³, most of the drug will be in the unionized state and thus, it would likely be absorbed in the vaginal cavity. Likewise, the Log P of 3.84 indicated that SHetA2 would be retained more in tissues than in the systemic circulation. Thus, initially we hypothesized that SHetA2 would be soluble in cocoa butter and that the release would be faster from the PEG mixture.

DoE2 indicated that suppositories having cocoa butter as base melted at least 5 times faster than the PEG mixture suppositories disintegrated. This was expected as cocoa butter has a melting point of 35°C, whereas the PEG 400:3350 has a melting point of 55 °C, which is why instead of melting, it slowly dissolves and releases the drug^{23, 26}. The difference in melting point of the bases may have also influenced the drug release as the dissolution rate of rifampicin in water correlated well with the melting/disintegration time. These results are in agreement with those of Bergren et al., in which the release of meteneprost potassium was faster when formulated in suppositories with bases having lower melting temperatures compared to suppositories having bases with higher melting temperature²⁶.

The mouse-sized suppositories had similar melting/disintegration times and release profile of SHetA2 in water as that of rifampicin from the human-sized suppositories. However, contrasting with the complete release of SHetA2 from suppositories in water, the release of SHetA2 in simulated vaginal fluid was slow and incomplete. This was surprising because Kolliphor was supposed to enhance SHetA2 solubility, as reported for other surfactants that enhance drug solubility in vaginal products^{44, 45}. The pH of biological fluids has been reported to affect the ionization status of a drug and its dissolution rate⁴⁶. SHetA2 is a weak acid and it is likely that at the reported pH value of the vaginal fluid⁴³ (4.5–5) it may be unionized at this pH and thus is less soluble and would dissolve less, resulting in its incomplete release from the suppository. It is also plausible that the dissolution of SHetA2 may have been limited by the presence of the compounds used to prepare the simulated vaginal fluid, including sodium chloride, potassium hydroxide, calcium hydroxide bovine serum albumin, lactic acid, acetic acid, glycerol, urea and glucose³⁰.

The residence times of the two suppository formulations in the vaginal cavities of mice were evaluated because it is known that residence time of the drug is a key determinant for drug absorption. Thus, the. Cocoa butter suppositories stayed longer than PEG suppositories in the vaginal cavity of mice The longer residence time is likely the reason for the 2-fold increase in SHetA2 cervix concentration after administration of cocoa butter suppositories

compared to PEG suppositories (14.09 $\mu\text{g/g}$ and 6.98 $\mu\text{g/g}$ respectively). Thus, cocoa butter suppositories were employed in the preliminary PK study.

FVB mice were employed in this study because they are the wild type species for the K14-HPV16 mice, which will be employed in future efficacy studies, as the cervix of these mice shows progression from intraepithelial neoplasia to invasive cancer similar to humans⁴⁷. The $T_{\text{max-cervix}}=0.5$ h, indicated that the drug is absorbed rapidly into tissues. This rapid absorption may have occurred because the drug released was mostly in the unionized state due to the acidic environment, thus favoring its absorption. Once absorbed, SHetA2 remained in the cervix for several hours and it was cleared very slowly as indicated by its estimated half-life ($t_{1/2\text{-cervix}} = 3.08$ h) and clearance ($\text{CL/F} = 0.00152$ mL/h). This correlates with the $\text{Log P} = 3.84$ for SHetA2, as it is reported that compounds having a $\text{Log P} > 3$ tend to partition more into the tissues and less into systemic circulation⁴⁸.

Only a few studies have determined concentrations in gynecological tissues after vaginal administration of drugs. Shanmugam et al⁴⁹ determined the concentration of sildenafil in gynecological tissues after vaginal administration of a suppository to rats. Sildenafil concentrations in gynecological tissues were almost 20-fold higher after vaginal administration of the drug compared to oral administration of the same dose. In another study, Mizutani *et al*⁵⁰ administered vaginal suppositories containing 100 mg of danazol daily for 30 days to patients receiving treatment for endometriosis and compared this treatment to oral administration of 400 mg of danazol. After treatment, patients were hysterectomized and drug concentrations were measured in tissues, revealing that danazol concentrations in the cervix of patients receiving 100 mg of danazol vaginally were similar to cervical concentrations in patients receiving 400 mg danazol orally. These studies confirm that in humans, the vaginal route can also achieve higher local drug concentrations compared to oral delivery. This finding is important as it suggests that local delivery of drugs may improve the bioavailability of drugs that have poor aqueous solubility and high permeability such as those in the Biopharmaceutics Classification System class II⁵¹.

The levels of cyclin D1 in tissues were evaluated as pharmacodynamic end point to confirm if SHetA2 concentrations in tissue were indeed able to induce an effect at tissue level⁹. SHetA2 induces phosphorylation, ubiquitination and proteasomal degradation of cyclin D1 that leads to G1 cell cycle arrest in multiple cancer cell lines^{6, 18, 52}. It is also reported that, as cervical dysplasia progresses to invasive cancer, the expression of cyclin D1 increases. Therefore, cyclin D1 levels often correlate with a prognosis of cervical cancer. This proof-of-concept, single point evaluation determined that vaginal administration of SHetA2 suppositories resulted in approximately 9-fold reduction of cyclin D1 levels. Thus, confirming a relationship between drug concentration and pharmacodynamic effect in the present study.

CONCLUSION

The SHetA2 vaginal suppository formulation optimized using QbD methodology met quality control parameters outlined for suppositories for human use. The proof-of-concept *in vivo* studies suggested that these suppositories are capable of achieving therapeutically

effective SHetA2 concentrations at the cervix, the site of drug action. Detailed pharmacokinetic studies evaluating different SHetA2 doses are underway and will be followed by efficacy studies in the mouse model of disease, to establish the potential use of SHetA2 vaginal suppositories in the treatment of cervical dysplasia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ishikawa diagram for the optimization of the SHetA2 vaginal suppository formulation. The upper segment corresponds to the optimization of the suppository formulation having a hydrophilic base consisting of a mixture of PEG 400 and PEG 3350 performed in DoE1. This base composition was entered in DoE2 as the hydrophilic base to be compared with the lipophilic base in the optimization of the SHetA2 suppositories for vaginal administration used in *in vivo* studies.

Figure 2.
Influence of formulation factors on product CQAs as determined by DoE software.

Figure 3.
Optimized mouse size suppositories (inset) showing actual length of the suppository 4.95 mm.

Figure 4.

Release of SHetA2 from cocoa butter suppositories in water (pH 6.93) and simulated vaginal fluid (pH=4.25). (An unpaired t-test was performed between the two profiles. At 0, 10, 20 minutes, the dissolution rates of SHetA2 in SVF and water media, were significantly different with a $p < 0.001$, $\alpha = 0.05$).

Figure 5. SHetA2 concentration in gynecologic tissues (µg/g) of FVB mice after vaginal administration of the optimized suppository formulation.

Figure 6.

Effects of SHetA2 on cyclin D1 protein levels in cervix-uterine tissue after vaginal administration of the optimized suppository formulation.

A) Western blot of cyclin D1 protein levels in cervix-uterine tissue extracts collected 30 min after the final administration of either SHetA2 or placebo suppositories. B) Densitometric quantitation of the Western blot bands and comparison of the average relative cyclin D1 levels (cyclin D1 / β actin band density ratios).

Table 1

Design of Experiments 1 (DoE1): Optimization of the proportion of PEG 400 and PEG 3350 for suppository base

Experiment No.	Component A: PEG 400	Component B: PEG 3350	Component C: Kolliphor	Response1 Fully formed (yes/no, appearance)	Response2 Integrity at room temperature
1	0.197	0.503	0.300	Yes, Solid	Solid
2	0.197	0.503	0.300	Yes, Solid	Solid
3	0.300	0.400	0.300	Yes, Solid	Solid
4	0.100	0.600	0.300	Yes, Solid	Solid
5	0.100	0.600	0.300	Yes, Solid	Solid
6	0.368	0.400	0.232	Yes, Solid	Solid
7	0.253	0.524	0.222	Yes, Solid	Solid
8	0.253	0.524	0.222	Yes, Solid	Solid
9	0.326	0.477	0.197	Yes, Solid	Solid
10	0.222	0.600	0.178	Yes, Solid	Solid
11	0.222	0.600	0.178	Yes, Solid	Solid
12	0.400	0.448	0.152	Yes, Solid	Solid
13	0.309	0.557	0.134	Yes, Solid	Solid
14	0.395	0.520	0.086	Yes, Solid	Solid
15	0.395	0.520	0.086	Yes, Solid	Solid
16	0.350	0.600	0.050	Yes, Solid	Solid

Table 2
Design of Experiments 2 (DoE2): Optimization of the suppository formulation for human size

Experiment No.	Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3
	A: % drug	B: base	C: Kolliphor	Fully formed? (yes/no, appearance)	Integrity at room temp	Disintegration/ melting time (minutes)
1	20	CB	5	Yes, Solid	Solid	7.4
2	40	CB	5	Yes, Solid	Solid	6.0
3	20	PEG	5	Yes, Solid	Solid	37.0
4	40	PEG	5	Yes, Solid	Solid	95.0
5	20	CB	30	No, Poor	--	--
6	40	CB	30	No, Brittle	--	--
7	20	PEG	30	Yes, Solid	Solid	31.0
8	40	PEG	30	Yes, Solid	Solid	65.0