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PHARMACOKINETICS AND PHARMACODYNAMICS OF ESCALATING DOSES OF SHetA2 AFTER VAGINAL ADMINISTRATION TO MICE

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Abstract

SHetA2 is a novel compound with strong potential to treat cervical dysplasia, but its low aqueous solubility limits its oral bioavailability. A vaginal suppository achieved SHetA2 cervix concentrations that were several fold above the predicted therapeutic levels. Thus, we aimed to determine the minimum dose that would achieve SHetA2 therapeutic levels while reducing cyclin D1 levels, the pharmacodynamic endpoint. The disposition of SHetA2 after vaginal administration of escalating SHetA2 doses and the corresponding reduction in cyclin D1 levels was compared to that after the conventional oral treatment. Vaginal administration of a 15 mg/kg dose achieved an AUC_{cervix} that was ~120 times larger than that after a 60 mg/kg administered orally. AUC_{cervix} and C_{max-cervix} did not increase proportionally with respect to the dose, with the 30 mg/kg dose resulting in higher AUC_{cervix} and C_{max-cervix} (1368.53 µg.mL/h and 155.38 µg/g, respectively) compared to the 15 mg/kg (334.98 µg.mL/h and 121.78 µg/g, respectively) or 60 mg/kg (1178.55 µg.mL/h and 410.38 µg/g, respectively). Likewise, the 30 mg/kg dose caused a larger reduction of cyclin D1 levels than the other doses. Thus, the 30 mg/kg dose was selected for future efficacy studies in a mouse model of cervical neoplasia.

Keywords

Mucosal drug delivery; Absorption; Pharmacokinetics; Pharmacodynamics; Cervical dysplasia; SHetA2; Vaginal drug delivery; Cancer; Vaginal suppository

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INTRODUCTION

Cervical cancer ranked as fourth among frequently diagnosed malignancies in women in 2012, and represented 19% of all gynecologic cancers worldwide ¹. Cervical dysplasia is the precursor of cervical cancer and progresses unpredictably towards cancer ². Cervical dysplasia is largely initiated by high risk human papillomavirus (HPV), which causes dysplastic changes in the stratified squamous epithelium of the uterine cervix ³. Currently, higher grades of cervical dysplasia are treated with ablative or excisional therapies ³. Ablative therapies include destruction of the tissue exposing the affected cells to either compressed nitrogen (cryogenic removal), or heat (laser ablation), but both ablative therapies are associated with pregnancy loss ⁴. Excisional therapies such as loop electrosurgical excision procedure (LEEP) and cold knife conization are associated with preterm birth (less than 37 gestational weeks) and low birth weight in women, especially who delivered within one year of therapy ^{4,5}. These unwanted effects occur because the surgery may affect the structure and functionality of the endocervical canal as well as changes in cervical volume leading to cervical incompetence ⁶. Other adverse effects of excisional therapies include vaginal bleeding, perforation of uterus, abnormal discharge, cramps and stenosis ^{6,7}. Therefore, development of a local, topical chemotherapy would be a better alternative to treat cervical dysplasia and avoid its progression to cervical cancer.

SHetA2 is a flexible heteroarotinoid compound that selectively causes apoptosis in multiple cancer cell lines and has minimal effect on healthy epithelial cells ⁸. In cervical cancer patients the elevated expression of cyclin D1 is associated with poor overall disease-free survival rate ⁹. SHetA2 can counteract this elevated cyclin D1 by causing its proteasomal degradation leading to G1 arrest and apoptosis, as has been shown in ovarian cancer cells ¹⁰. Therefore, the levels of cyclin D1 protein can be used as surrogate pharmacodynamic endpoint ^{11,12}. SHetA2 has also shown chemoprevention in the transgenic mice model of colorectal cancer, which was associated with cyclin D1 reduction ¹¹. Preclinical studies have demonstrated that SHetA2 is non-irritant to the skin, non-mutagenic and non-carcinogenic ¹³. Formal toxicological studies in rats and dogs demonstrated its safety by no observed adverse effect level (NOAEL) at doses that were 30 times higher than the therapeutic dose ¹⁴. However, the potential efficacy of SHetA2 in the prevention or treatment of cancer is limited by its low oral bioavailability caused by its poor aqueous solubility ¹⁴. Using quality by design methodology, we developed a vaginal suppository formulation that overcomes its limited oral bioavailability ¹². In a proof-of-concept study administering these suppositories to mice by the vaginal route at a 60 mg/kg dose, the resulting SHetA2 concentrations in the cervix were almost 100-fold higher than the therapeutic concentration ($4 \mu\text{M} \pm 0.4$) and reduced the levels of cyclin D1 protein by 9-fold ¹². Since these concentrations were much higher than required, the goal of the present study was to determine the smallest SHetA2 vaginal dose that can maintain therapeutic levels in the cervix for the longest time. More importantly, we sought to determine the dose that would induce a therapeutic reduction in the expression of cyclin D1 protein levels. This dose would be employed in future efficacy studies in the K14-HPV16 mouse model of cervical neoplasia ¹⁵. The disposition after a 60 mg/kg SHetA2 oral dose was also evaluated to compare with previous efficacy studies performed in other types of cancers that employed oral administration.

MATERIALS AND METHODS

Materials

SHetA2 was synthesized by Cayman Chemical company, Inc. (Ann Arbor, MI) 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). Kolliphor HS15 was obtained from BASF (Germany). Sterile saline and isoflurane were obtained from Henry Schein Animal Health Inc. Acetonitrile (HPLC grade 99.5%), methanol (HPLC grade 99.5%), phosphoric acid, hydrochloric acid, crystal violet stain and sodium acetate trihydrate were purchased from Sigma Aldrich (St Louis, MO). Captiva[®] filtration equipment was purchased from Agilent Technologies Inc. for extraction of drug from tissues. Mouse cyclin D1 enzyme linked immunosorbent assay kit (ELISA) was purchased from Cedarlane lab (NC). T-PER (tissue protein extraction reagent) was purchased from Thermo-Fisher Scientific (Waltham, MA). Protease inhibitor cocktail tablets were purchased from Sigma Aldrich (St Louis, MO).

Suppository manufacturing and quality control

SHetA2 suppositories containing 15, 30 and 60 mg/kg body weight were manufactured by the fusion-molding method reported previously¹². Briefly, the drug was dissolved in melted Kolliphor HS15 and then combined with the cocoa butter. This mixture was then poured into custom-made suppository molds and allowed to solidify. All suppositories were evaluated for content uniformity (85–115% of intended content), weight variation (no more than two units having a relative standard deviation (RSD) greater than 7.8%) and softening time (less than 30 minutes) according to the guidelines specified by the United States Pharmacopoeia¹⁶.

Animals

Friend Leukemia Virus B (FVB) female mice of 7 weeks of age (National Cancer Institute Charles River Frederick Research Facility) were used in this study. Animals were housed in a facility in a constant temperature room at 22 ± 1 °C with a 12 h light/12 h dark cycle and provided continuous access to food and water *ad libitum*.

To avoid variability in drug absorption from the vaginal cavity, mice were synchronized for estrous cycle using a modified Whitten effect¹⁷. The stage of the estrus cycle was monitored daily in all mice by observing the composition of the cells obtained in the vaginal lavage of each mouse. The collected fluid was then smeared onto a microscope glass slide, the slides were air-dried and stained with crystal violet. Slides were examined with an Olympus FV microscope (Olympus Scientific Solutions Americas Corp., Waltham, MA) to determine the morphologies of the cells that were present in the smear and the stages of the cycle determined by the types of cells observed, as described by McLean *et al*¹⁸. All animal experiments were approved by University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (IACUC).

Pharmacokinetic study

Mice in the diestrus stage of their estrous cycle were assigned to a treatment group to receive either a 15, 30 or 60 mg/kg dose. Mice in the 15 and 30 mg/kg groups received a single suppository containing that dose, whereas mice in the 60 mg/kg dose received two 30 mg/kg suppositories, as previously employed in the proof-of-concept studies¹². To facilitate suppository administration, each mouse was lightly sedated with isoflurane. Mice were maintained under sedation for 3 minutes in horizontal position, as preliminary studies determined that this time was sufficient for absorption¹². Mice were then transferred to a clean cage lined with paper and observed for 10 additional minutes to account for any possible leakage of the yellow colored suppository. In preliminary studies, there was no visual evidence of melted suppository leaking during the subsequent 10 minutes after the mouse was awake and moving around¹². After dosing, mice (n = 5, per group and per time point) were euthanized at 0.5, 1, 4, 8, 12, 24, 36, 48 and 72 hours. For oral treatment, a SHetA2 suspension was prepared by homogenizing a weighed amount of the drug in a 30% Kolliphor solution to achieve 60 mg/kg dose¹⁴. Mice (n = 5, per group and per time point) were dosed with the suspension by oral gavage, and euthanized at 0.5, 1, 4 and 8 hours post-dosing. This reduced sampling period was adopted for mice dosed orally, after a pilot study with 2 mice revealed that drug concentrations were below the limit of detection in samples beyond 4 hours. Blood was collected by cardiac puncture into heparinized tubes and the plasma separated by centrifugation (12,000 rpm for 10 minutes). The gynecologic tissues (cervix, uterine horns and ovaries) of all mice receiving vaginal and oral treatments were collected and thoroughly cleaned with saline solution to remove unabsorbed yellow drug. Plasma and gynecologic tissues were snap-frozen in liquid nitrogen to prevent additional *ex vivo* diffusion of the drug to adjacent tissues. Samples kept frozen at –80°C until analysis.

Additional mice were dosed with a placebo suppository to serve as additional controls for the pharmacodynamic endpoint. Placebo vaginal suppositories were made of cocoa butter and Kolliphor only, whereas the placebo for the orally-dosed group consisted of 30% Kolliphor solution in deionized water. These mice were euthanized at 0.5 h (n = 3, per group) and at 72 h (n = 3, per group) after being dosed and their cervix tissue collected, washed with saline solution and kept frozen at –80°C until analysis.

Pharmacodynamic endpoint

The effect resulting from vaginal and oral administration of the different SHetA2 doses was quantified by the corresponding reduction in the levels of cyclin D1 protein. The expression of cyclin D1 protein in the cervix of mice treated with SHetA2 suppositories, placebo suppositories and untreated mice was determined by Enzyme Linked Immunosorbent Assay (ELISA). The cervix of each mouse was homogenized in T-PER reagent (10 µL per each mg of tissue) containing protease inhibitor cocktail. Homogenization was performed in an ice bath using an OMNI-GLH general laboratory homogenizer. Homogenates were centrifuged at 4 °C for 5 min at 10,000 x g, the supernatants were collected and the assay was performed according to manufacturer's instruction. Each standard blank and test specimen was evaluated in duplicate. The replicates with less than 15% coefficients of variance were eliminated from the analysis. The average of the test samples was compared to the standard curve to derive the cyclin D1 concentration.

Sample analysis

To determine SHetA2 concentrations, a known amount of tissue (either cervix, uterine horns, fallopian tubes/ovaries or a piece of the left lobe from lung samples) was homogenized by adding 13 μL of saline per mg of tissue. Then 45 μL of the tissue homogenate were placed in polypropylene micro-centrifuge tubes and 45 μL of 4% H_3PO_4 was added. After vortexing for 10 sec, 110 μL of ACN was added to the tube for a final volume of 200 μL . This homogenate mixture was filtered using a Captiva® filtration plate (Agilent Technologies). The filtrate was directly injected into the HPLC system. Plasma samples were prepared using the same process as tissues. Determination drug concentration in tissues was performed with a Waters Alliance HPLC system equipped with Waters X-bridge C_{18} column and matching guard column. SHetA2 was eluted after 5.35 and 6.14 min for tissues and plasma, respectively. Recovery of SHetA2 from biological samples was more than 95% of the spiked amount in tissues and more than 80% of the spiked amount in plasma. The limit of detection (LOD) was 0.005 $\mu\text{g/mL}$ and the limit of quantitation (LOQ) was 0.025 $\mu\text{g/mL}$.

The levels of cyclin D1 protein in the cervix of mice was determined by Enzyme Linked Immunosorbant Assay (ELISA). A known portion of the cervix of each mouse ($n=5$, per each time point) was homogenized in T-PER reagent (10 μL per mg of tissue) containing protease inhibitor cocktail. Homogenates were centrifuged at 4 $^{\circ}\text{C}$ for 5 min at 10,000 $\times g$, the supernatants were collected and the assay was performed as per manufacturer's instructions.

Data analysis

Pharmacokinetic parameters to characterize the vaginal disposition of SHetA2 were determined as follows: The $C_{\text{max-cervix}}$ = maximum concentration in cervix and $T_{\text{max-cervix}}$ = time to achieve maximum concentration in cervix, were determined from the cervix concentration versus time plot. The $\text{AUC}_{\text{cervix}}$ = area under the cervix concentration versus time curve, $t_{1/2\text{-cervix}}$ = half-life in cervix, V_z/F = apparent volume of distribution/ F , $\text{Cl}_{\text{cervix}}/F$ = Clearance/ F , and $\text{MRT}_{\text{cervix}} = \text{AUMC}_{\text{cervix}}/\text{AUC}_{\text{cervix}}$ were determined by non-compartmental analysis using Phoenix WinNonlin® software. The average SHetA2 cervix concentration of the 5 mice in each time point was used in the calculation. $C_{\text{max-cervix}}$ of different doses was analyzed by one-way ANOVA multiple comparison (Tukey's test). The percentage of dose absorbed into the gynecological tissues (cervix, uterine horns, fallopian tubes and ovaries) was calculated using the following equation:

$$\left[\frac{\text{SHetA2 concentration in the tissue} \left(\frac{\text{mg}}{\text{g}} \right) \times \text{Total weight of the cervix (g)}}{\text{Total Dose Delivered (mg)}} \right] \times 100$$

Where the SHetA2 concentration refers to the AUC of each organ. For example, using $\text{AUC}_{\text{cervix}}$; the total weight of the cervix (g), in average 0.11g; and the total dose delivered calculated by multiplying the average weight of a mouse (23 g = 0.023 kg) by the dose (either 15, 30 or 60 mg/kg).

Statistical analysis of changes in the expression levels of cyclin D1 in the cervix treated mice was performed by Graphpad Prism software. To determine cyclin D1 expression for grouped studies, P values were obtained by one way ANOVA multiple comparison (Tukey's test). A P value less than 0.01, 0.001, 0.005, 0.05 were considered to be statistically significant.

RESULTS

Suppository quality control

The mouse size suppositories prepared for this study 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 ¹². The batches of SHetA2 suppositories employed in the present study had the following characteristics: (i) suppositories containing the 15 mg/kg dose had a $101.3 \pm 9.9\%$ content uniformity with a RSD of 7.1% and a softening time of 5.6 ± 0.1 minutes; (ii) suppositories containing the 30 mg/kg dose had a $105.4 \pm 0.4\%$ content uniformity with a RSD of 3.3% and a softening time of 6.0 ± 0.1 minutes.

Pharmacokinetic study

Figure 1 depicts the SHetA2 concentrations in the cervix of mice versus time after vaginal administration of SHetA2 in suppositories, or in suspension by oral gavage. All treatments achieved the predicted therapeutic level of the drug (4 μ M) in the cervix of mice within 30 minutes of administration.

Predicted therapeutic levels were sustained for 48 h in mice treated vaginally with the 15 mg/kg dose and for 72 h in mice treated vaginally with the 30 and 60 mg/kg doses, but fell below therapeutic levels within 30 minutes of administration and were below the limit of detection of the assay after 1 h in orally treated mice. For clarity, the inset in figure 1 shows SHetA2 cervix concentrations versus time profiles focused on the region of the scale for the groups dosed vaginally. In general, the first part of the profile exhibits a dose dependency, with the $C_{\text{max-cervix}}$ after 60 mg/kg dose being significantly higher ($P < 0.05$) than any other treatment. However the second part of these profiles does not exhibit this pattern, as the 60 mg/kg dose appears to be cleared more rapidly than the 30 mg/kg dose. Interestingly, the cervix concentration versus time profiles of animals dosed vaginally with the 15 mg/kg dose exhibited two extra peaks in the terminal phase, at 12 and 36 h. Likewise, mice dosed with the 30 mg/kg dose exhibited extra peaks at 12 and 48 h, whereas mice dosed with the 60 mg/kg dose exhibiting only one extra peak at 36 h.

The SHetA2 concentrations in the uterine horns versus time profile for all experimental groups are shown in figure 2. Administration of SHetA2 in all groups achieved predicted therapeutic concentrations in the uterine horns of all treated mice for up to 8 h, but after this time, only groups dosed vaginally with the 15 or 30 mg/kg doses maintained therapeutic levels of the drug until 48 h, respectively. In contrast, only the vaginal administration of the 30 mg/kg dose was capable of sustaining predicted therapeutic concentrations in the fallopian tubes and ovaries of treated mice for 72 h compared to all other treatments (figure 3). The differences in drug concentrations in these organs is to be expected given the

proximity of the uterine horns to the cervix and their large surface area, and the remoteness of the fallopian tubes and ovaries and their smaller size.

It is important to note that the SHetA2 concentration in plasma, if any, after oral or vaginal administration of all doses and at all time points was below the limit of detection (LOD=0.005 µg/mL) of the validated HPLC assay¹⁹; thus, it is unlikely that there is systemic exposure to the drug after vaginal administration.

The disposition of SHetA2 as characterized by its pharmacokinetic parameters is shown in table 1. The AUC_{cervix} after vaginal administration of the 15 mg/kg dose was 334.98 µg.mL.h⁻¹ and when the dose was increased by two-fold (30 mg/kg) the AUC increased by approximately four-fold to 1368.53 µg.mL.h⁻¹. However, when the dose was again increased by two-fold (60 mg/kg) the AUC_{cervix} remained comparable (1178.55 µg.mL.h⁻¹) to that after the 30 mg/kg dose. Notably, vaginal doses achieved AUC_{cervix} that were 150–600-fold larger than that after oral administration of 60 mg/kg dose (AUC_{cervix} = 2.25 µg.mL.h⁻¹). Likewise, the maximum concentration (C_{max-cervix}) of SHetA2 achieved after vaginal administration of all doses was not proportional to the dose increase and was 90–300-fold larger than that after oral administration of 60 mg/kg dose (C_{max-cervix} = 1.3 µg/g), which was below the predicted therapeutic level.

The clearance from cervix (Cl_{cervix}/F) after 15 mg/kg and 30 mg/kg dose was 0.94 and 0.46 mL/h respectively, but the clearance after the 60 mg/kg vaginal dose was unexpectedly faster (Cl_{cervix}/F = 1.06 mL/h). Oral administration of a 60 mg/kg SHetA2 suspension had a Cl_{cervix}/F of 307.46 mL/h. This faster clearance after oral administration correlated well with the short half-life of SHetA2 in cervix of 0.44 h. Vaginal administration of SHetA2 resulted in a much longer half-life, as shown by the t_{1/2-cervix} = 30.32 h after the 15 mg/kg dose and 23.32 and 17.01 h after the 30 and 60 mg/kg doses, respectively. The MRT_{cervix} followed a similar trend as the half-life with 40.98 h after the 15 mg/kg dose; 35.85 h after the 30 mg/kg dose and 16.55 h after the 60 mg/kg dose. In contrast, MRT_{cervix} after oral administration of the 60 mg/kg dose was only 5.21 h.

In order to assess the efficiency of SHetA2 delivery, the percentage of the SHetA2 dose absorbed per tissue according to the AUC of each tissue was calculated with respect to the dose delivered. Considering an average mouse weight of 21 g, then the amount of SHetA2 delivered to mice treated with the 15, 30 and 60 mg/kg doses was 315, 630 and 1260 µg, respectively. Table 2 shows the percentages of SHetA2 absorbed in each part of the gynecological tract as calculated from their respective AUCs using equation 1. As expected after vaginal administration, the largest fraction of SHetA2 was absorbed in the cervix compared to the other tissues for all doses, and the fraction of drug absorbed in the cervix after oral administration was 500–1200-fold lower than after vaginal administration. On the other hand, the fraction of the dose absorbed in the uterine horns and fallopian tubes/ovaries did not follow the same pattern as the cervix, but unexpectedly the largest fraction of drug absorbed after oral administration was in the fallopian tubes/ovaries. The efficiency of the vaginal route to deliver SHetA2 is demonstrated by the sum of the fractions absorbed in each organ, which is 25–63 times larger over oral delivery.

Pharmacodynamic endpoint

Figure 4B shows the comparison of cyclin D1 protein levels after the vaginal administration of suppositories with the different SHetA2 doses. Vaginal administration of a 15 mg/kg dose of SHetA2 induced a 50% reduction in the levels of cyclin D1 protein at the $C_{\max\text{-cervix}}$ (30 min post administration) with respect to the untreated controls ($P < 0.05$) and this reduction was sustained through the treatment time for the $C_{\min\text{-cervix}}$ (24 h). At both the $C_{\max\text{-cervix}}$ and $C_{\min\text{-cervix}}$ time points, the levels of cyclin D1 were significantly lower ($P < 0.05$) than that of mice treated with placebo suppositories. The 30 and 60 mg/kg doses also significantly decreased cyclin D1 levels at their $C_{\max\text{-cervix}}$ time point and sustained the reduction through their $C_{\min\text{-cervix}}$ time point. Notably, there were no significant differences between the extents of cyclin D1 reduction elicited by the three doses.

Figure 4A shows the reduction in cyclin D1 levels induced by administration of SHetA2 suspension by the oral route and their respective controls. A significant reduction of 43% and 37% in the levels of cyclin D1 protein is observed after 0.5 h administration ($T_{\max\text{-cervix}}$) of drug ($p < 0.005$) and placebo ($p < 0.05$) suspension groups, respectively. Even though the levels in cyclin D1 in both groups increased at $C_{\min\text{-cervix}}$, these levels were similar in both groups indicating that the effect was caused by the Kolliphor in the suspension and not the drug.

DISCUSSION

Although cervical cancer is one of the most preventable cancers due to availability of immunization, as well as established screening and intervention methods, it remains a significant public health burden in non-industrialized nations²⁰. An inexpensive and non-invasive treatment strategy may be able to reduce the cost and prevent the adverse obstetric outcomes caused by the current excisional and ablation therapies, especially in women of child bearing age.

The site of cervical dysplasia and the anatomical location of the cervix makes it suitable for vaginal drug delivery because the disease is confined within the stratified squamous epithelium²¹. An extensive histological evaluation of samples from 319 patients revealed that even the highest degree of dysplasia is located within 7.60 ± 4.32 mm from the stratified squamous epithelium, and within 1.35 ± 1.15 mm depth²¹. Moreover, the lesions are located mainly in the transformation zone, ectocervix and in the endocervical canal. All these sites are accessible for treatment using vaginal drug delivery and thus, makes it ideal to treat cervical dysplasia because it will deliver the drug directly to the target site. Furthermore, an effective drug treatment could also prevent the recurrence of the disease after a primary surgical procedure²².

SHetA2 has shown *in vivo* efficacy in a colorectal tumorigenesis model, in which an oral formulation of SHetA2 reduced polyp incidence and size by 50% without adverse side effects¹¹. SHetA2 has a differential effect on cancer versus normal cells, as in cancer cell it causes mitochondrial swelling, induction of G1 cell cycle arrest and apoptosis, but only G1 cell cycle arrest in normal epithelial cells^{10, 23–26}. In a Caki-1 renal cancer xenograft model, oral formulation of SHetA2 (20, 40, 60 mg/kg) with PEG400, induced tumor inhibition

without side effects in the studied animals²⁶. However, the efficacy of SHetA2 is limited by its poor oral bioavailability due to its limited aqueous solubility¹⁴. The SHetA2 vaginal suppository formulation that we developed to overcome these limitations induced SHetA2 concentrations in cervix of treated mice that were 100–400-fold higher than the therapeutic concentration (4 μ M) and reduced cyclin D1 levels by 9-fold¹². Given that these PK and PD outcomes are larger than desired, the present study aimed to evaluate the disposition of escalating vaginal doses of SHetA2 and to establish the superiority of the vaginal route over the oral route to achieve therapeutic concentrations of SHetA2 in the cervix. The ultimate goal was to determine the smallest SHetA2 dose that can achieve therapeutic concentrations at the target site for the longest period of time and induce desired PD endpoint.

SHetA2 plasma concentrations after oral or vaginal administration of all doses were below limit of detection at all times. This outcome does not affect the goal of our study because the site to treat cervical dysplasia is the cervix; thus, PK parameters were determined based on drug concentrations in the cervix tissue. The superiority of the vaginal route over the oral route to administer SHetA2 was demonstrated by the cervix concentration versus time profile (figure 1) and the corresponding PK parameters (Table 1). All three vaginal doses were able to achieve therapeutic SHetA2 concentrations in cervical tissues of treated mice for 24 – 72 h (figure 1), whereas the SHetA2 concentration in the cervix of mice receiving an oral dose of 60 mg/kg SHetA2 fell below therapeutic levels just one hour after administration. Furthermore, the efficiency of vaginal delivery, expressed as the sum of the fractions absorbed in each part of the gynecological tract, is 25–63-fold larger than that after oral delivery (table 2). Moreover, even though oral administration of SHetA2 reduced the levels of cyclin D1 protein with respect to untreated controls, this reduction was similar to that after administration of a placebo solution indicating that the reduction was caused by the Kolliphor and not the drug (figure 4B). In contrast, all three vaginal doses were able to reduce the levels of cyclin D1 protein to a larger extent compared to untreated controls and placebo.

While the three vaginal doses achieved therapeutic SHetA2 concentrations in the cervix of treated mice, the 30 mg/kg dose was the one that maintained higher drug levels for the longest period of time (figure 1). The appearance of extra peaks in the SHetA2 cervix concentration versus time profiles was somehow unexpected but correlates well with the extra peaks observed in the dissolution profile of SHetA2 suppositories in simulated vaginal fluid¹². The mechanisms that have been postulated to explain the appearance of the extra peaks in drug concentration versus time profiles include: enterohepatic recirculation²⁷, absorption window in a specific site of absorption^{28,29}, precipitation and re-solubilization of precipitated drug³⁰, gastric emptying due to variable motility²⁹ and the administration site acting as depot³¹. For enterohepatic recirculation to occur, the drug has to reach portal circulation, be excreted in bile, and then be dumped into the intestine to be reabsorbed. This is unlikely to be the case in the present study, as the SHetA2 concentrations in plasma were below detection limits at all times. The most likely explanation for the extra peaks in the present study is the precipitation and re-solubilization of the drug. The suppository formulation is a solid dispersion of SHetA2 in cocoa butter¹². When the suppository is administered into the vaginal cavity of mice, it melts in vaginal fluid, and a fraction of the dose is rapidly absorbed into the tissue, as demonstrated by the high $C_{\text{max-cervix}}$ achieved by

all vaginal doses. As the SHetA2 dissolution and absorption occur, they form a supersaturated solution of SHetA2 in the vaginal fluid that is in equilibrium with a supersaturated state of the drug in tissue. We hypothesize that once the supersaturated form surpasses the solubility in tissue it may precipitate as an amorphous form in tissue, which can be easily re-solubilized with time and absorbed as the drug that was already absorbed is cleared from the tissue. This may be a possible explanation for the two extra peaks observed in the profiles of the 15 and 30 mg/kg doses. However, when the 60 mg/kg dose is administered, it may be plausible that the dissolution of the drug in the vaginal fluid may be so high that it surpasses the supersaturation state and some of the drug may precipitate out as a crystalline form, which is more difficult to re-dissolve. A crystalline precipitate can be more easily cleared from the site of administration, which may explain the absence of a second peak. This assumption is consistent with a previously published study on Danazol³⁰, a drug with poor water solubility (0.017 mg/mL) and with a $\log P = 3.48$. These values are similar to those of SHetA2, which has a water solubility of 0.4 $\mu\text{g/mL}$ and $\log P$ of 3.84¹². After oral administration, danazol was rapidly absorbed in the stomach but precipitated in the intestine. The authors hypothesized that the precipitated danazol may be solubilized again aided by the bile salts and show a secondary peak³⁰. Previously published studies have described the solubilization of drugs poorly soluble in water by the formation of crystal seeds that can later become precipitates in different biological media^{32–34}. The extent of supersaturation influences crystallization, nucleation and crystal growth of a drug during dissolution^{33, 34}. This can ultimately lead to formation of precipitates of different solubilization properties, hence, resulting in multiple peak phenomena.

Our hypothesis of supersaturation, precipitation and re-solubilization is also supported by the PK parameters obtained after vaginal administration of SHetA2. As shown in table 1, $\text{AUC}_{\text{cervix}}$ did not increase proportionally with the dose, with the $\text{AUC}_{\text{cervix}}$ after administration of the 60 mg/kg dose being smaller than that after administration of the 30 mg/kg dose. It may be plausible that the amount of drug released from the 60 mg/kg dose into the vaginal fluid was larger than that allowed by the supersaturation concentration, with the excess drug precipitating and cleared from the vaginal cavity. This assumption is supported by the pattern shown in the $\text{CL}/\text{F}_{\text{cervix}}$ after vaginal administration (table 1), which is inverse to that of $\text{AUC}_{\text{cervix}}$ when the dose was increased from 15 to 30 mg/kg, the $\text{CL}/\text{F}_{\text{cervix}}$ was twice as slower (from 0.94 to 0.46 mL/h), but it was twice as faster (1.06 mL/h) in mice receiving the 60 mg/kg dose. These observations are attributed to precipitation rather than faster clearance. The trend in $\text{Vd}_{\text{cervix}}/\text{F}$ with respect to the dose was similar to that of $\text{CL}/\text{F}_{\text{cervix}}$. This resulted in a somewhat proportional decrease in $t_{1/2-\text{cervix}}$ and $\text{MRT}_{\text{cervix}}$ with respect to the vaginal doses, with the longest $t_{1/2-\text{cervix}}$ (30.32 h) and $\text{MRT}_{\text{cervix}}$ (40.98 h) exhibited after administration of the 15 mg/kg dose.

All three vaginal doses were able to induce reduction of cyclin D1 levels in the cervix of treated mice that was significantly different than untreated controls at $\text{T}_{\text{max-cervix}}$ and to untreated control and placebo at $\text{C}_{\text{min-cervix}}$ (figure 4). These results are in agreement with previously observed cyclin D1 reduction *in vivo*^{11, 12}. However, the reduction in cyclin D1 levels was not proportional to the dose, as the reduction in cyclin D1 levels was greater after administration of the 30 mg/kg dose, but the reduction the cyclin D1 levels after administration of the 60 mg/kg dose was similar to that elicited by the 30 mg/kg dose. This

is likely due to the similarity in AUC_{cervix} between the 30 and 60 mg/kg doses that was caused by drug precipitation.

The present study was performed in healthy FVB mice, but SHetA2 would cause a larger reduction of cyclin D1 levels in cancer cells than in healthy ones as demonstrated by Benbrook *et al.*²⁵. This is due to the fact that in healthy cells, cyclin D1 is present mainly in proliferating cells; thus, the effect of SHetA2 on cyclin D1 will depend on the percentage of cells that are proliferating, i.e. on the baseline amount of the protein.

It is important to note that in the present study, the suppositories were administered to mice during diestrus, which is the longest stage of their estrous cycle (65 h) where mucosal epithelium of the reproductive tract does not change^{35, 36}. However, if suppositories would have been administered during any other stage of the estrous cycle (either proestrus, estrus or metestrus) the extent of drug absorption would likely be smaller, and as consequence the pharmacokinetic parameters would be different. The extent of this difference between diestrus and estrus stages has been reported in our previous publication³⁷, but this difference cannot be extrapolated to humans. Unlike rodents, humans experience a menstrual cycle that has three phases in which the thickness and viscosity of the human cervical mucus varies, but the thickness of the human vaginal epithelium does not drastically change across the cycle^{38, 39}. Still, the changes in mucus volume secretion during the menstrual cycle may influence the extent of drug absorption. Therefore, the effect of the variations in mucus secretion on drug absorption after vaginal delivery should be studied in a relevant animal model.

CONCLUSION

The present study supports the superiority of the vaginal route for SHetA2 delivery, to achieve predicted therapeutic concentrations and a measurable reduction of cyclin D1 levels in the cervix, the site of cervical dysplasia. The 30 mg/kg dose achieved and maintained predicted therapeutic concentrations of SHetA2 in the cervix for the longest time. Future studies will evaluate multiple vaginal administrations of SHetA2 suppositories to optimize a dosing regimen for use in efficacy studies in the K14-HPV16 mouse model of cervical neoplasia.

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Figure 1.

Comparative preclinical PK study of SHetA2 vaginal suppository escalating doses versus oral suspension. SHetA2 concentrations (µg/g) were measured over time in cervical tissue homogenates from FVB female mice treated with 15-, 30-, and 60-mg/kg suppository and oral suspension of 60-mg/kg dose (* indicates the statistically significantly higher Cmax-cervix of the 60-mg/kg dose, $p < 0.05$).

Figure 2:

Drug concentration profile in the uterine horn tissue homogenate of FVB female mice treated with 15, 30, 60 mg/kg suppository, and oral suspension of 60 mg/kg dose

Figure 3:
Drug concentration profile in the ovary-fallopian tube tissue homogenate of FVB female mice treated with 15, 30, 60 mg/kg suppository, and oral suspension of 60 mg/kg dose

Figure 4.

Cyclin D1 expression level in cervix tissue in the animals treated with 15-, 30-, and 60-mg/kg SHetA2 vaginal suppository and oral suspension treatment: Cyclin D1 expression levels were evaluated at C_{min} (minimum concentration time) and C_{max} (maximum concentration) times in cervical tissues using ELISA and compared among untreated control, placebo (P), and SHetA2 30-mg/kg suppositories; (a) 15-, 30-, and 60-mg/kg suppository treatment, (b) 60-mg/kg oral suspension treatment. C_{max} was observed at 0.5 h for all vaginal dose and oral dose, C_{min} was observed at 24, 72, and 72 h and 4 h for 15-, 30-, and 60-mg/kg vaginal dose and oral 60-mg/kg dose, respectively. Cyclin D1 expression in placebo and treatment group was normalized with the expression level in untreated control (* and ** indicate p value less than 0.05 and 0.005, respectively; ns = not significant p values were derived using 2-way analysis of variance Tukey's post-test, n=5).

Table 1:

Pharmacokinetic parameters after 15, 30, 60 mg/kg vaginal dose vs 60 mg/kg oral suspension dose

PK Parameter	15 mg/kg vaginal	30 mg/kg vaginal	60 mg/kg vaginal	60mg/kg Oral
AUC _{cervix} ($\mu\text{g.mL.h}^{-1}$)	334.98	1368.53	1178.55	2.25
C _{max-cervix} ($\mu\text{g/g}$)	121.78 $\pm$ 38.66	155.38 $\pm$ 74 *, p<0.05	410.38 $\pm$ 247	1.3 $\pm$ 0.23
t _{1/2-cervix} (h)	30.32	23.32	17.01	0.44
Cl _{cervix} /F (mL/h)	0.94	0.46	1.06	307.46
Vd _{cervix} /F (mL)	41.31	15.48	26.23	185.04
MRT _{cervix} (h)	40.98	35.85	16.55	5.21

Table 2:

Percentage of the SHetA2 dose absorbed per tissue according to the AUC of each tissue with respect to the dose delivered as per average mouse weight of 21 g.

Tissue	15 mg/kg vaginal	30 mg/kg vaginal	60 mg/kg vaginal	60mg/kg Oral
Cervix	11.69%	23.89%	10.28%	0.02%
Uterine horns	1.54%	6.53%	2.58%	0.09%
Fallopian tubes + Ovaries	6.78%	3.056%	0.37%	0.42%
Fraction of drug absorbed from total dose	20.02%	33.48%	13.24%	0.53%