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Research paper

Influence of the estrus cycle of the mouse on the disposition of SHetA2 after vaginal administration

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ABSTRACT

SHetA2 is a novel compound with the potential to treat cervical dysplasia, but has poor water solubility. A vaginal suppository formulation was able to achieve therapeutic concentrations in the cervix of mice, but these concentrations were variable. Histological analysis indicated that mice in the same group were in different stages of their estrous cycle, which is known to induce anatomical changes in their gynecological tissues. We investigated the effects of these changes on the pharmacokinetics and pharmacodynamics of SHetA2 when administered vaginally. Mice were synchronized to be either in estrous or diestrus stage for administration of the SHetA2 suppository. Pharmacokinetic parameters were calculated from the SHetA2 concentrations vs. time data. The reduction in the expression of cyclin D1 protein in the cervix was used as pharmacodynamic endpoint. Mice dosed during diestrus had a larger AUC_{cervix} (335 μg mL h^{-1}), higher C_{max} (121.8 ± 38.7 μg/g) and longer $t_{1/2,cervix}$ (30.3 h) compared to mice dosed during estrus (120 μg mL h^{-1}, 44.6 ± 29.5 μg/g and 3.6 h respectively). Therapeutic concentrations of SHetA2 were maintained for 48 h in the cervix of mice dosed during diestrus and for only 12 h in the estrus group. The treatment reduced the expression of cyclin D1 protein in the cervix of mice in the estrus to a larger extent. These results indicate that the estrous cycle of mice influences significantly the disposition of SHetA2 after vaginal administration and may also influence its efficacy.

1. Introduction

Cervical dysplasia is a precancerous condition of the uterine cervix, mainly initiated by high risk papilloma virus (HPV) infection [1]. The prevalence of HPV infection in developed countries is reported to range between 40% and 80% in young adult females [2,3]. This infection can be asymptomatic and resolved by the immune system of the individual, but if the infection persists it can progress towards invasive cervical cancer, which is second most common form of gynecologic cancer worldwide [4]. Because of this possibility, physicians may over treat the patient. Current treatments for cervical dysplasia involve invasive procedures such as ablation therapy and cold knife-conization therapy, which are expensive, cause discomfort and can lead to infertility [5,6]. Presently, there are no drug-based treatments in clinical practice for this disease.

SHetA2 (Fig. 1) is a novel, non-toxic chemotherapeutic agent [7], with strong chemopreventive activity in human cell-culture [8] and murine tumors [9]. However, it has low oral bioavailability (~10%) due to its poor water solubility [7]. To overcome these limitations, we employed Quality by Design (QbD) approaches to develop a vaginal suppository formulation for direct delivery to the cervix, the site of drug action [10]. The formulation was optimized using two consecutive designs of experiments (DoE). The composition of a hydrophilic suppository base (PEG 400: PEG3350) and the percentage of solubilizing agent (Kolliphor) were optimized in DoE1 (16 experiments). Subsequently, the optimized hydrophilic base (35% PEG 400, 60% PEG 3350 and 5% Kolliphor) was entered in a second DoE (DoE2), which compared the effect of the type of base (hydrophilic versus lipophilic base (cocoa butter)) and the % drug content on the integrity and disintegration/ melting time of the suppository. DoE2 determined that a cocoa butter base and 40% drug content produced suppositories that remained solid at room temperature and melted in 6 min. We performed proof-of concept pharmacokinetic and pharmacodynamics studies in mice to evaluate the potential of the SHetA2 vaginal

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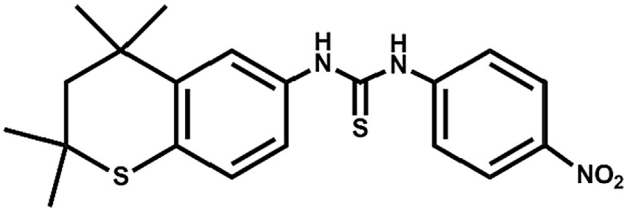

Fig. 1. Chemical structure of SHetA2.

suppositories to treat cervical dysplasia [10]. SHetA2 suppositories achieved cervix concentrations significantly higher than its predicted therapeutic concentration (4 μM) and induced a 9-fold decrease in the levels of cyclin D1 protein, a marker of efficacy associated with the prognosis in cervical cancer patients [11]. However, a large variability was observed in the SHetA2 concentrations among mice receiving the same dose, at the same time point, even though the quality control indicated that drug variation in the batch of suppositories was within United States Pharmacopoeia (USP) limits [10]. Thus, we investigated the influence of the anatomical and physiological features of the reproductive system of the mouse on SHetA2 absorption.

Unlike humans that have a menstrual cycle, the reproductive cycle of the mouse, known as estrous cycle, has four different stages: proestrus (P), estrus (E), metestrus (M) and diestrus (D) [12]. Due to differential hormonal regulation, the composition, thickness and structure of the stratified squamous and mucosal epithelium in the gynecologic tissues of mice are variable across the estrous cycle [13]. Histological examination of the uterine horns of the mice treated with the same SHetA2 dose in preliminary studies revealed that mice within the same time point group were in different stages of their estrus cycle.

Currently, there are no published reports describing the effects of anatomical and physiological changes due to estrus cycle on drug disposition after vaginal administration of compounds to mice. A single report from Hsu et al. [14] using an excised vaginal membrane in a diffusion chamber indicated that the permeability coefficients for vidarabine, an antiviral drug with activity against herpes, were 10–100 fold higher during the diestrus stage compared to those in the estrus stage. Therefore, the objective of the present study was to evaluate the influence of the anatomical and physiological changes due to the estrus cycle of the mouse on SHetA2 disposition after vaginal administration, and SHetA2 pharmacodynamic endpoint, the reduction in the levels of cyclin D1 protein.

2. Materials and methods

2.1. 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). Kolliphor HS-15 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 ThermoFisher Scientific (Waltham, MA). Protease inhibitor cocktail tablets were purchased from Sigma Aldrich (St Louis, MO).

2.2. Methods

2.2.1. Suppository manufacturing and quality control

SHetA2 suppositories containing 15 mg/kg body weight dose were manufactured by the fusion-molding method [10]. Suppositories were evaluated for content uniformity (85% to 115% of intended content), weight variation (no more than two units having a relative standard deviation (RDS) > 7.8%) and softening time (less than 30 min) as outlined by the USP [15].

2.2.2. 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 because they are the wild type species for the K14-HPV16 mouse model of cervical neoplasia [16] that will be used in efficacy studies. 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 access to food and water *ad libitum*. All animal experiments were approved by University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (IACUC).

2.2.3. Monitoring estrus cycle by the visual method

The vaginal openings of mice were monitored every 24 h to determine their stage in the estrous cycle as described by Byers et al [17]. According to this method, during proestrus the vaginal opening remains swollen, moist and pink; during estrus, the opening becomes less moist and less swollen; during metestrus, a distinctive white cellular debris is observed, but the tissue is not swollen; whereas during diestrus, the vaginal opening is very narrow and not swollen.

2.2.4. Synchronization of estrous cycle

The estrous cycle in mice was synchronized by the Whitten effect, where female mice are exposed to male pheromones from their urine, which induce them to enter the estrus stage on the third day of exposure [18,19]. Since urine from male mice was not readily available, we used the soiled bedding from male mice cage for a modified Whitten effect [18]. To verify the stage of the estrus cycle in these mice, vaginal lavage was performed every 24 h on the third, fourth and fifth day of exposure as described below.

2.2.5. Monitoring estrus cycle by observation of vaginal cytology

The vaginal lavage was performed in each mouse under light sedation with isoflurane as described by McLean et al [20]. A sterile pipette tip was filled with approximately 10 μL of sterile saline and inserted gently into the vaginal cavity of the mouse, followed by gentle aspiration, and the procedure repeated three to five times. The collected fluid was then smeared onto a microscope glass slide and air-dried. The slides were stained with crystal violet after drying.

Slides were examined with a microscope to determine the type of cells that were present in the smear. The stages of the estrous cycle were determined according to the percentage of anucleated cornified cells, nucleated epithelial cells and leukocytes presented in the smear as follows [21]. A mouse was determined to be in the: (1) pro-estrus stage when nucleated epithelial cells were predominant; (2) estrus stage when mainly anucleated cornified cells were present; (3) metestrus stage when three types of cells, leukocytes, cornified, and nucleated epithelial cells, were present; and (4) diestrus stage when the majority of cells present were leukocytes [21].

2.2.6. Histology of the uterine horns

A piece of a uterine horn from each mouse was collected, fixed in 10% neutralized buffered formalin and embedded in a paraffin block. Afterwards, sections were cut perpendicularly to the transverse axis from the paraffin blocks, so that the lumen and stromal areas were included in each section. Each tissue section was fixed onto a microscope slide and stained with hematoxylin and eosin (H&E) for