A Phase I Study of Intravaginally Administered Artesunate In Women with High Grade Cervical Intraepithelial Neoplasia (CIN2/3)

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STATEMENT OF COMPLIANCE

The study will be carried out in accordance with Good Clinical Practice (GCP) as required by the following regulations and guidelines:

- United States Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46; 21 CFR Part 50, 21 CFR Part 56, and 21 CFR Part 312)
- ICH E6; 62 Federal Register 25691 (1997)

All key personnel responsible for the design and conduct of this study have completed Human Subjects Protection Training.

SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and ICH guidelines.

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LIST OF ABBREVIATIONS

AE	Adverse Event
CFR	Code of Federal Regulations
CIN	Cervical Intraepithelial Neoplasia
CRO	Clinical Research Office
CRC	Clinical Research Review and Monitoring Committee
CRF	Case Report Form
CTC	Common Toxicity Criteria
DHA	Dihydroartemisinin
DNA	Deoxyribonucleic Acid
E7	Human Papillomavirus E7 antigen
FDA	Food and Drug Administration
GCP	Good Clinical Practice
HSIL	High grade squamous intraepithelial lesion
HCG	Human Chorionic Gonadotrophin
HIPAA	Health Insurance Portability and Accountability Act
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
IC_{50}	Concentration providing 50% inhibition
IM	Intramuscular
IND	Investigational New Drug Application
IRB	Institutional Review Board
IV	Intravenous
JHH	Johns Hopkins Hospital
IND	Investigational New Drug
LEEP	Loop Electrocoagulation Excision Procedure
LSIL	Low grade squamous intraepithelial lesion
PCR	Polymerase chain reaction
SAE	Serious Adverse Event
TID	Three Times A Day
WHO	World Health Organization

1.0 SYNOPSIS

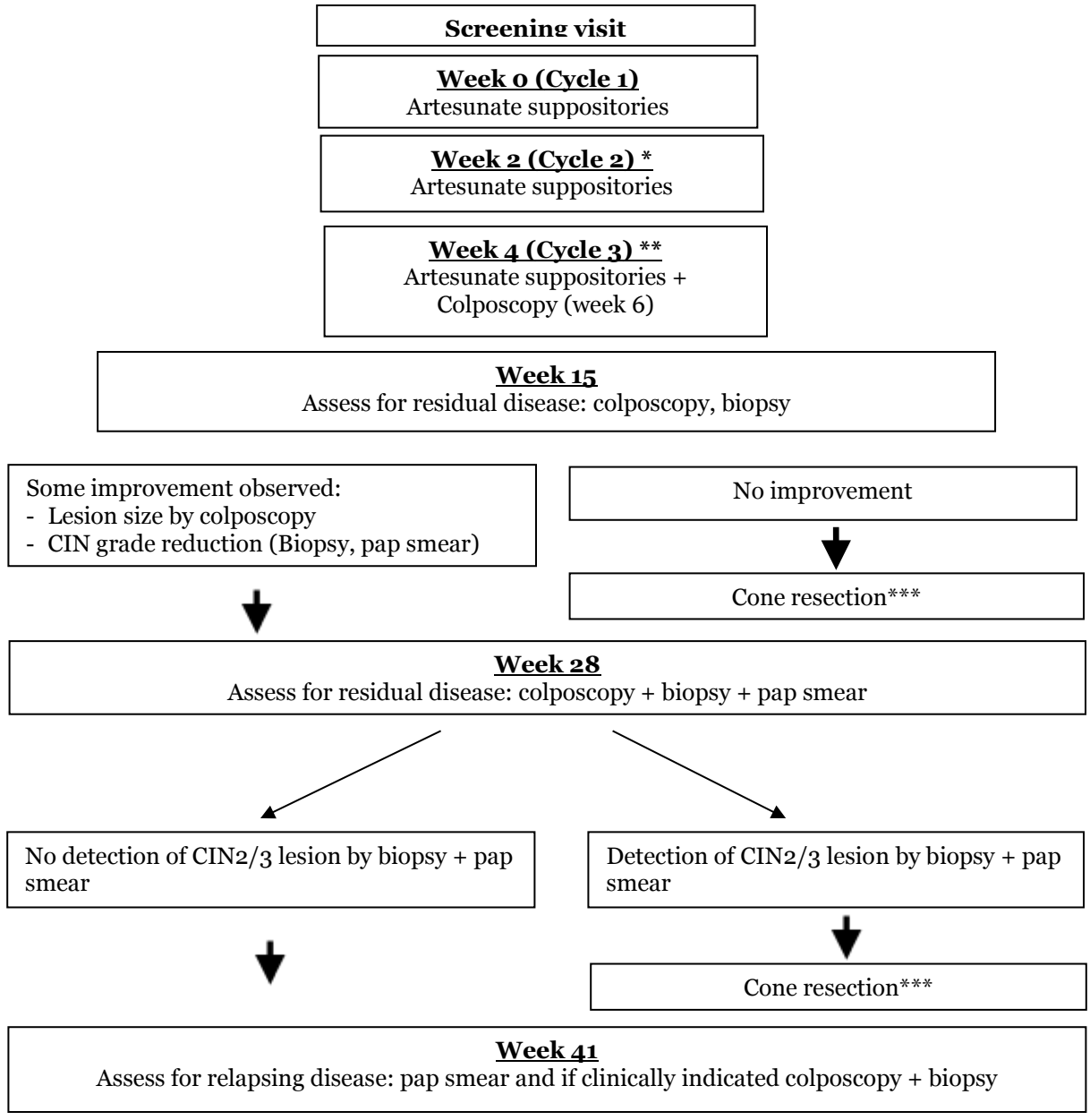
Study drug	Artesunate
Title	A Phase I study of intravaginally administered Artesunate In women with High Grade Cervical Intraepithelial Neoplasia (CIN2/3)
Indication	Female patients, 18 years and older, with CIN2/3
Study population	Women with biopsy-confirmed CIN2/3 with a visible lesion after biopsy
Rationale	Despite the approval of prophylactic vaccines which prevent infection with HPV types 16 and 18, the genotypes most commonly associated with squamous cancers, disease remains very common, even in high-resource settings, and is likely to remain so for decades. Infection with a high-risk HPV type is necessary but not sufficient for the development of squamous cervical cancer (SCCx) and its precursor lesion, high grade cervical intraepithelial neoplasia (CIN2/3). Both SCCx and CIN2/3 are associated with functionally obligate expression of two viral proteins, E6 and E7. Epithelial cells that express either or both of these oncoproteins also overexpress the transferrin receptor, and have increased levels of intracellular iron, compared to normal cells [1]. This observation raised the possibility of treating preinvasive HPV disease (i.e., CIN2/3) with an effective anti-malarial drug, Artesunate. Artesunate contains an endoperoxide bridge that reacts with intracellular ferrous iron to generate free radicals, leading to cell death [2, 3]. Artesunate, formulated as a suppository, is WHO-approved for first-line treatment for acute malaria in children who are remote from health care settings [4]. The toxicity profile of this formulation is well-documented. Preclinical data suggest that Artesunate mediates cytotoxic effect on HPV-infected cells, while having no effect on normal epithelium. Thus, it is proposed to investigate the use of Artesunate formulated in suppositories applied intravaginally to treat CIN2/3.
Evaluation Criteria	Safety will be assessed by the absence of related dose limiting adverse effects. Subjects will be monitored at each visit for the appearance of erythema; itching, burning, pain; infection; desquamation; tenesmus; bleeding; dizziness; or other unexpected AEs. Circulating blood cells will be monitored weekly for any quantitative changes.

	Efficacy will be determined by the presence/absence of HPV, by viral load, and by regression of lesions. The presence of HPV will be determined by DNA tests before pre- and post-treatment. Histologic endpoints will be assessed with colposcopically-directed biopsies obtained before and after treatment with topical Artesunate. In this population, it is feasible to directly visualize, serially quantitate, and perform therapeutic resection of premalignant lesions. By comparing immunologic parameters in the peripheral blood (systemic) compartment with measures obtained from the site of the lesion, pre- and post-drug administration, we expect to derive insights which will inform immune therapeutic strategies for incipient malignancies.
IND Phase	Phase I
Objectives	Primary Objective: • To determine and evaluate the safety, tolerability, and feasibility of intra-vaginal administration of Artesunate, administered via vaginal suppository, in healthy women with cervical intraepithelial neoplasia (CIN2/3) Secondary and Exploratory Objectives: • To measure the effect of intra-vaginal topical Artesunate administration on histology, based on the regression of CIN2/3 at study week 15, assessed as either CIN1 or no CIN lesion detected by colposcopy/biopsy and cytology. • To evaluate clearance of HPV as assessed by Hybrid Capture 2 DNA testing of cytologic specimens • To evaluate the local tissue immune response • To correlate measures of immune response with clinical response
Study Population	Women, 18 years and older, with biopsy-confirmed CIN2/3, and a visible lesion after biopsy.
Inclusion/ Exclusion Criteria	<u>Inclusion</u> • ≥ 18 years • Capable of informed consent • HPV-positive by DNA test • Histologically confirmed CIN2, CIN3, or CIN2/3 • Body weight ≥ 50 kg • Immune competent

	Exclusion • Pregnant and nursing women • HIV seropositive • Active autoimmune disease • Taking immunosuppressive medication • Evidence of concurrent adenocarcinoma in situ • Concurrent malignancy except for nonmelanoma skin lesions
Investigational Treatment	Intra-vaginal administration of Artesunate suppositories, one to three multi-day dosing cycles; 50-200 mg, once a day for 5 days per treatment cycle; prior to scheduled LEEP or cone excisional procedure (standard of care for CIN2/3 patients)
Clinical Study Sites	Johns Hopkins Hospital Greater Baltimore Medical Center (Johns Hopkins Clinical Research Network Site)
Study Design*	Phase I open-label dose escalation study of intravaginal Artesunate, formulated in suppositories, in adult females with biopsy-confirmed CIN2/3. Thirty (30) subjects will undergo up to a total of three cycles of intravaginal Artesunate. The first cycle will be initiated on Day 0, the second at Week 2, and the third and final cycle at week 4. The lowest dose cohort that demonstrates most evidence of clinical efficacy as determined by clinical endpoints will be expanded. A schematic overview of the study design is provided on the next page, including study arms, sample size and schedule of interventions. An outline of scheduled visits and assessments is presented below.
Evaluation Criteria	Safety: Subjects will be monitored for local and systemic adverse events. Efficacy: Histology, cytology, and HPV PCR at study week 15.
Number of Subjects	A total of 30 patients will be enrolled in this study
Estimated Patient Enrollment Period	12 months after the approvals of IND and JHH IRB

Subject Participation Duration	41 weeks from the first dosing
Statistical Analysis Plan	Statistical analysis will be primarily descriptive due to the small sample size. Treatment related safety and efficacy attributes will be analyzed using the Chi-square test. Any exploratory inferences with a p-value of ≤ 0.10 will be considered for further study in subsequent trials.

Schematic of Study Design:



There will be 4 treatment cohorts, which will be filled sequentially

Cohort	Number of patients	Dose	Treatment cycles
I	3	50 mg	1 (5 days)
II	9	200 mg	1 (5 days)
III*	9	200 mg	2 (5 days each)
IV**	9	200 mg	3 (5 days each)

***According to the standard of care clinical practice, a postoperative visit will be done 4 weeks after the cone resection.

2.0 BACKGROUND AND RATIONALE

2.1 HPV-associated malignancies of the cervix

Virtually all squamous cervical cancers (SCCx) are caused by a common virus, human papillomavirus (HPV). Exposure to HPV occurs with the onset of sexual activity. While most persons clear their infection without intervention, and without sequelae, a subset do not. Persistent infection with a high-risk HPV type is the proximate cause of SCCs and their precursor intraepithelial lesions, cervical intraepithelial neoplasia (CIN2/3). Other factors contribute to the development of SCC, as viral integration into the host genome is necessary but not sufficient for the initiation and persistence of malignant transformation. Currently there are no available treatments that can eradicate HPV infection. Successful prevention of SCC in infected individuals is based on early detection of preinvasive lesions prior to the development of cancer.

Despite the availability of several, inexpensive, noninvasive screening strategies to detect precursor lesions, the incidence of HPV disease (SCC and CIN 2/3) in women remains high. An effective topical intervention such as current proposal of an intravaginal Artesunate treatment would obviate the need for surgery and prevent progression to SCC. Globally, ten percent of malignancies in women are caused by HPV. Cervical cancer is the second leading cause of cancer death in women worldwide, and in the United States, cervical cancer remains the sixth most commonly diagnosed malignancy among women. Over the past decade, SEER data have documented a 17% increase in incidence in the U.S., normalized for population growth, with a disproportionate increase among young women [5] . Although preventative vaccines that protect against infection with HPV types 16 and 18, the genotypes most commonly associated with squamous cancers, and have been introduced for preteen and teen girls and boys since 2006, their impact has not yet realized as the rates of vaccination remain low in the United States. Recent data indicate that the rate of preventive vaccination in eligible girls, in 2012, was only 33% [6].

The primary strategy to decrease disease burden in HPV-infected individuals is to intervene when premalignant cervical disease, CIN2/3, is detected prior to the development of SCCs. All treatments for CIN2/3, including cryotherapy, laser vaporization, or cone excisions, are ablative and require repeated visits to health care

providers. Because they involve tissue destruction, all current therapeutic options have the potential for adverse sequelae, and furthermore, are not always curative. Among treated CIN2/3 in immunocompetent women, the overall risk of recurrence is less than ten percent when all surgical margins are clear. This risk increases to approximately 25% in women with positive endocervical margins. Women who have undergone cervical conization have three times the risk of cervical stenosis as those who have not [7]. Several authors have documented a significantly increased risk of premature delivery in pregnancies subsequent to cervical conization, and subsequent neonatal low birth weight. [7, 8] Moreover, tissue destruction from therapeutic interventions can make subsequent detection and treatment of recurrent disease more difficult, as the healing process tends to draw the transition zone of the cervical epithelium proximally, into the endocervical canal [9]. While these complications are not insurmountable, an effective local immunotherapy such as intravaginal Artesunate suppositories is clinically meaningful to obviate the need for surgeries.

2.2 Noninvasive topical therapy for cervical dysplasia

The development of a topical therapy for preinvasive CIN2/3 lesions that could be self-administered would be a game-changing treatment option for women with preinvasive HPV disease. A topical treatment option would decrease adverse sequelae related to ablative procedures, decrease visits to specialized health care providers, and result in considerably decreased health care costs. In addition, in many cultures, HPV disease, particularly of the lower genital tract, carries a social stigma [10]. The development of a topical therapy that could potentially be self-administered, would be empowering for women with preinvasive lesions. A topical therapeutic also has the potential to obviate the need for surgery.

2.3 Rationale for study drug

Artesunate is a semisynthetic derivative of artemisinin, a plant compound extracted from the leaves of sweet wormwood, *Artemisia annua,* an herb used in Chinese traditional medicine for antimalarial treatment for over two thousand years, with marketed formulations since the late 1980s [11]. The safety profile of Artesunate and related compounds has been established based on millions of malaria patients, ranging from infants to adults, over decades [12]. Artesunate has been shown to be safe and well-tolerated when administered orally, intravenously, intramuscularly, or intra-rectally, as a suppository [4, 5, 13-15]. Reports submitted to the FDA by both the WHO and by Novartis summarize many of the safety studies conducted with artemisinin and its derivatives, including Artesunate [16-18].

Recently, Artesunate compounds have been shown to have antitumor activity against several human solid tumor cell lines, including breast, colon, ovarian, prostate, renal, and non-small cell lung cancer [13]. Of particular relevance, artemisinin derivatives have also been shown to have a cytotoxic effect on HPV-immortalized epithelial cells [13] .

Artemisinin and its derivatives, including Artesunate, have been shown to decrease cell proliferation, reduce angiogenesis and trigger apoptosis in cancer cells [14, 15, 19]. Although the mechanisms of action are incompletely understood, in human solid cancers, sensitivity to Artesunate has been reported to be associated with expression of angiogenesis-related gene transcripts [20]. Tumor susceptibility has also been reported to be correlated with cell surface overexpression of transferrin receptor [21, 22] and with intracellular ferrous iron [1]. It has been demonstrated that artemisinin and its derivatives contain an endoperoxide bridge that reacts with intracellular ferrous iron to generate free radicals, leading to cell death [3,4].

Both SCC and CIN2/3 are associated with functionally obligate expression of two viral proteins, E6 and E7. Epithelial cells that express either or both of these oncoproteins also overexpress the transferrin receptor, and have increased levels of intracellular iron, compared to normal cells [1]. Because cervical squamous cancers and their precursor, intraepithelial lesions (CIN) overexpress the transferrin receptor [21, 22], these observations prompted a subsequent study of the cytotoxic effect of dihydroartemisinin (DHA), the bioactive form of Artesunate, on papillomavirus-expressing epithelial cells [23] . *In vitro* studies demonstrated that, while DHA had little effect on normal cervical epithelial cells, it had a significant cytotoxic effect on HPV-immortalized cervical cells [23]. In addition, formulated as a local treatment in an oral mucosal canine nonclinical model, DHA has been reported to inhibit papillomavirus- induced tumor formation [23].

Together, these findings suggest that topical administration of an Artesunate compound may provide therapeutic benefit for intraepithelial HPV disease. Given the unmet need for a local treatment for CIN2/3 lesions and that Artesunate has demonstrated a favorable safety profile for decades in both children and adults, a prospective, dose-escalation, Phase 1 study of an Artesunate suppository, administered intravaginally to achieve local, mucosal distribution of study drug, is proposed.

2.4 Nonclinical safety literature review

Extensive reviews of the effects of Artesunate on the central and peripheral nervous system, and on cardiovascular, respiratory and gastrointestinal systems of various animal species (mice, rabbits, guinea pigs, dogs, and monkeys) have been compiled [18, 24] . There are also some recent sub-chronic and chronic toxicological studies of Artesunate in dogs [25, 26] . These reports contain data on Artesunate toxicity when administered via different routes, including oral, intravenous, intramuscular and intra-peritoneal injections at doses of up to 640 mg/kg, which is well above therapeutic levels. In general, minimal pathological changes were observed in animal studies at doses under 60 mg/kg [18, 25, 26]. The toxic effects would not therefore be considered to be of clinical concern in regard to human therapeutic use [18, 24] . In this clinical study, we propose to evaluate intravaginal suppository doses up to 200 mg (approximately ≤4 mg/kg for subjects who weigh ≥50 kg, an inclusion criterion of this study) that are considerably less than doses associated with toxicities in animals (>60 mg/kg) and the

rectal suppository doses used in children (approximately 10 mg/kg) and adults (approximately 8.3 mg/kg) for malaria treatment [18].

2.4.1 Acute (Single Dose) Toxicity

In single-dose toxicity studies of Artesunate administered intravenously or intramuscularly in mice, LD_{50} values of 520 and 475 mg/kg were determined respectively [18]. In another mice study, Artesunate exhibited a higher intravenous LD_{50} of 699 mg/kg [18]. Rats exhibited a slightly lower intravenous LD_{50} at 488mg/kg [27]. Maximum tolerated intravenous doses for guinea pigs, rabbits, dogs and monkeys were 240, 160, 80 and 60 mg/kg, respectively, and the minimum lethal doses for these species were found to be 480, 640, 480 and 160 mg/kg, respectively [18]. Toxicity symptoms observed included CNS effects (depression, unstable gait, tremor, convulsions) and respiratory suppression [18, 28] . Oral administration of Artesunate was better tolerated and resulted in LD_{50} values of approximately 1000-1300 mg/kg in mice, and 600-900 mg/kg in rats. In dogs, reticulocyte counts were decreased at doses of ≥80 mg/kg, while histopathology showed some liver damage at ≥120 mg/kg [18].

2.4.2 Sub-acute (Repeated Dose) Toxicity

In repeated-dose toxicity studies, intravenous administration of Artesunate to dogs at doses of 10 and 40 mg/kg for 14 days did not produce clinical symptoms or significant hematological, biochemical or histopathological changes [18]. Similar results were obtained in monkeys given daily 10 and 32 mg/kg intravenous injections for 14 days [18]. In rats, intravenous administration of Artesunate for 3 consecutive days at doses of up to 240 mg/kg induced a dose-dependent decrease in reticulocyte numbers, as well as decreases in erythrocytes, haematocrit and haemoglobin [18]. The decrease in reticulocyte numbers was generally the most sensitive measure of Artesunate effects, since statistical significance of the difference to the treatment controls was reached in females at 7.5 and in males at 15 mg/kg. At doses ≥60 mg/kg, body weight was also depressed [18]. Lastly, when Artesunate was administered intramuscularly to rats at a daily dose of 50 mg/kg for 7 days, food and water consumption declined starting from the first or second dose. Body weight also decreased over the treatment time. However, all these effects were reversible upon cessation of treatment [18].

2.4.3 Subchronic Toxicity

A 3-month study of orally administered Artesunate (6mg/kg) in dogs revealed no neurotoxicity [25]. Nor were any gross pathological changes observed. There was, however, evidence of some damage to the mitochondrial ultra-structure when studied using an electron microscope [25].

2.4.4 Organ and system toxicities

Artemisinins exhibit a distinctive embryotoxicity that has a very steep dose-response relationship [18]. In rats, treatment with Artesunate and other artemisinin derivatives results in large embryonic losses at modest doses . In mice, 100% resorptions were observed at a dose of 21.4 mg/kg, while in rats and rabbits, resorptions were observed in

over 90% of rats and rabbits at doses of 10.7 and 2.7 mg/kg, respectively [29]. An embryo-fetal no-observable-adverse-effect level (NOAEL) of 8mg/kg was recently established in rats [30]. Importantly, no artemisinin-induced embryotoxicity has been reported in humans [24]. This is remarkable because malaria treatment in much of tropical Africa is often dispensed on the basis of fever rather than the confirmation of parasitemia [31]. The parasite is widely thought to have a protective effect against artemisinin-induced decrease in reticulocyte count which is a marker for erythropoietic toxicity [31]. In any case, the use of Artesunate and other artemisinins in the first trimester of pregnancy is contraindicated based on observations of embryotoxicity between gestation days 9–11 in mice, 6–15 in rats, 7–18 rabbits, and 26-36 in monkeys [32].

Neurotoxicity associated with artemisinins appears to be not only dependent on the dose, but also on the route of administration and the particular artemisinin derivative [24]. The vehicle used to deliver the drug is also important with the water-soluble Artesunate showing markedly less neurotoxicity in laboratory animals than the oil-soluble arteether and artemether presumably because of the slower release and distribution of the latter compounds in blood plasma [24]. In mice, neurotoxic effects of Artesunate and DHA in a 28-day oral study were observed at a dose of >200 mg/kg/day [33]. Other effects included sedation at doses ≥200 mg/kg in mice and lower body temperature in rats at a dose of 450 mg/kg, and in rabbits and dogs at doses of >160 mg/kg. In rabbits, respiratory depression was observed at doses of ≥320 mg/kg, without any changes in the blood gases and blood pH [18, 24] . In mice, artemether in doses of 30, 50, 75 and 100 mg/kg, and Artesunate in doses of 30, 50 and 100 mg/kg, administered daily as intramuscular injections for 28 days, resulted in greater mortality in the high dose artemether cohort, compared to mice that received Artesunate at the same dose [34]. No significant effects on body weight were observed at the lowest dose. In the high-dose cohorts, differences in body weight were apparent between artemether and Artesunate [34]. Neurological observations at the high dose showed a clear distinction between Artesunate, which had practically no ill effect, and artemether, which elicited neurologic effects in 90% of treated animals. Lastly, olfactory responses, scored as the time needed to find food, were significantly impaired in the artemether but not in the Artesunate-treated groups [18, 34]. Overall, artemether appeared to have greater impact on neurological parameters than Artesunate, and intramuscular injection appeared to elicit more side effects than oral formulations [18, 24, 33, 34] .

Hematopoietic toxicities are either erythropoietic or leukopoietic. In animal experiments, artemisinins effects on erythropoiesis are frequently encountered [24, 31] . The effects are however mild and reversible in humans [31]. The data on artemisinin effects on leukocytes are contradictory, with some authors describing suppressed immune response, while others reported enhancements such as increased phagocytosis and interferon production [24].

The generation of reactive oxygen species (ROS) and carbon-centered radicals by artemisinins raises the possibility of cardiotoxicity. But whereas there have been reports

of cardiotoxicity in dogs, no impairment of heart function has been observed in numerous malarial therapy trials in humans [24].

Finally, at an elevated dosage of 240mg/kg/day (i.v. for 3 days), Artesunate causes renal failure and tubular necrosis in rats that were not infected with malaria [35]. The nephrotoxic effects were however reversible.

2.5 Nonclinical studies of Artesunate on HPV-infected epithelial cells

Our research collaborators at Georgetown University have conducted several nonclinical studies to evaluate the cytotoxic, apoptotic and anti-tumor activity of Artesunate and its active metabolite dihydroartemisinin (DHA). The effects of artemisinin, Artesunate, and DHA were studied on a panel of cell lines including primary, HPV-immortalized, and cervical tumor cell lines. Both HPV-immortalized and cervical tumor cell lines are sensitive to DHA and Artesunate. These cells express higher levels of transferrin receptor and intracellular iron as compared to normal ectocervical cells. Iron is required for the drug-induced formation of reactive oxygen species (ROS), the activation of caspases, and subsequent apoptosis [23].

Table 1: List of *In Vitro* and *In Vivo* Nonclinical Pharmacology Studies of Artesunate and DHA

Study Description	**Species/ Cell line**	**Dose**	**Method**	**Primary Findings**
In vitro cytotoxic activity study	-*HPV expressing cell lines* -*Normal cervical epithelial cells* -*Ectocervical epithelial cells* - E6 or E7 transformed cervical cells -normal cervical epithelial cells that expressed either E6 or E7	5-50 µmol/L artemisinin, Artesunate or DHA	Treatment of cell line for 3 days and measurement of viability with neutral red staining	Artesunate and DHA were cytotoxic to cervical cancer cell lines and to HPV-transformed cells, but not to normal cervical epithelium cells. DHA had a cytotoxic effect on all cells expressing E6 or E7, regardless of whether they were malignant.
In vitro apoptotic activity study	*HPV-infected andnon-infected HeLa cells*	0, 25 or 100 µmol/L DHA	HeLa cells treated with 5 µmol/L 6-carboxy-2V, 7V-dihydrofluoresceindiacetate and DHA for 3 hours and then analyzed by flow cytometry.	Cytotoxic effect on HPV-infected cells mediated by DHA is iron-dependent, and occurs via apoptosis.
In vivo activity of DHA	*Canine*	2.22 mg DHA dissolved in 100 µL	Beagles challenged with purified canine oral	Topical application of DHA inhibits the formation of virally

		DMSO (route-topical)	papillomavirus on the gum and buccal mucosa were administered topical DHA	induced papilloma *in vivo*

Note: Both DHA and Artesunate show similar *in vitro* activity against HPV-infected cells; however, DHA was selected for the *in vivo* study due to its favorable solubility in dimethyl sulfoxide (DMSO). HPV (Human papillomavirus).

2.5.1 *In vitro* cytotoxicity assay

The anti-tumor activity of artemisinin, Artesunate, and DHA was evaluated using a well-characterized HPV-transformed cervical cancer HeLa cell line. HeLa cells, at a concentration of 5 x 10^4, were cultured for 3 days with each test compound at concentrations ranging from 5 to 50 µM (Figure 1). Viability was measured using the neutral red uptake assay [36].

As illustrated in Figure 1, artemisinin had little effect on HeLa cells. The *in vivo* activity of artemisinin may rely on its rapid conversion into the active metabolite, DHA in the liver. Consistent with this hypothesis, DHA was highly effective in killing HeLa cells.

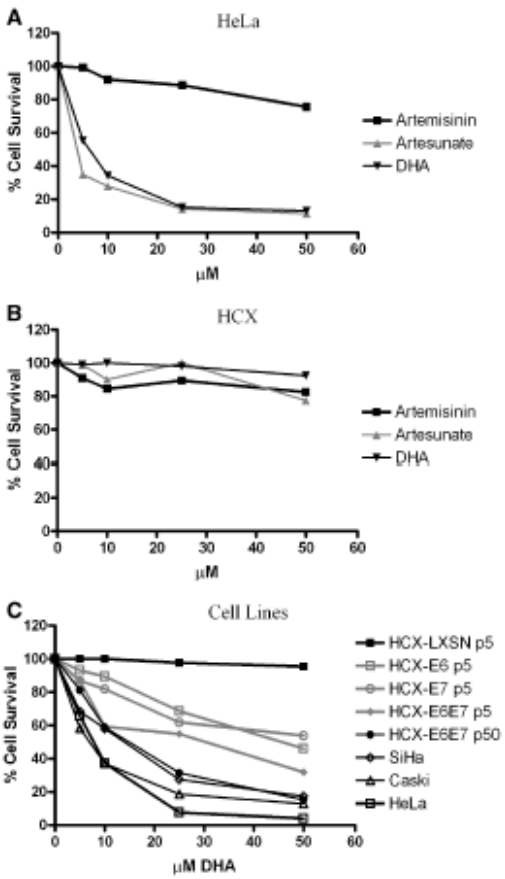


Figure 1. Effects of artemisinin, Artesunate and DHA on normal, immortalized and HPV-transformed cervical epithelial cells. **(A)** HeLa cells were cultured for 3 days with concentrations ranging from 0-50 µM of artemisinin, Artesunate, or DHA. The IC_{50} of Artesunate and DHA was 5 and 7.5 µM, respectively. **(B)** Viability of normal cervical epithelial cells (HCX) cultured as in (A) was shown. **(C)** Ectocervical cells transduced either with empty retrovirus (HCX), with either HPV-16 E6 or E7, cervical cells transduced with HPV-16 E6/E7, pre-immortalization (early p5) and post-immortalization (late p50), and cervical cancer cells (HeLa, SiHa, and Caski) were cultured for 3 days with DHA. All tumorigenic and HPV-immortalized cell lines were sensitive to DHA, with HeLa showing the greatest sensitivity. In all culture conditions, cell viability was measured using the neutral red assay. Each data point represents the mean of two wells from experiments carried out in triplicate.

The IC_{50} measurements for Artesunate and DHA on HeLa cells were determined to be 5 and 7.5 μM, respectively. Most importantly, DHA and Artesunate had little or no cytotoxic effect on normal cervical epithelial cells (Fig. 1B), even at 50 μM concentrations. As anticipated from its lack of effect on HeLa cells, artemisinin also had no effect on normal cervical cells. Because DHA is the active metabolite of Artesunate, it was used for further studies and screened for its activity against tumorigenic and HPV-infected cervical cell lines.

In the clinical setting of SCCx and its precursor lesion, CIN2/3, expression of both the E6 and E7 viral proteins is constitutive, and functionally required to maintain the transformed state [37, 38]. Cell surface expression of the transferrin receptor is increased in cells expressing these oncoproteins [21]. Because internalization of DHA is mediated in part by this receptor, we tested the effect of DHA on normal cells, on E6- and E7 -transformed cells, on epithelial cells that expressed either E6 or E7, but were not transformed, and on HPV cancer cell lines SiHa, CaSki, and HeLa.

As shown in Figure 1C, DHA had a strong cytotoxic effect on all cells expressing E6 or E7, regardless of whether they were frankly malignant. On the other hand, DHA had no effect on the viability of normal cervical epithelial cells (Figure 1C).

2.5.2 *In vitro* apoptosis assay

Correlation of transferrin receptor level with DHA sensitivity

It is known that the antimalarial activity of Artesunate and DHA depends on the high intraparasitic iron content [11]. The same panel of cells as in Figure 1C was examined for transferrin receptor level and cellular iron content. Cells, at a concentration of 1×10^6, were cultured for 24 hours and harvested for Western blot analysis (with anti-transferrin receptor antibody, 1:1000) to determine the level of transferrin receptor. As shown in Figure 2A, the blot shows a clear increase in the amount of transferrin receptor in HPV-immortalized and tumorigenic cell lines (1.7- to 2.5-fold) than corresponding normal cells (HCX). The increased level of transferrin receptor *in vitro* correlates with *in vivo* data showing increased transferrin receptors in high-grade cervical lesions and cancers as measured by immunohistochemistry [21, 22, 39].

Transferrin receptor overexpression correlated with total intracellular iron as measured by inductively coupled plasma optical emission spectrometry [23]. HeLa cells contained 50% more iron than normal cervical epithelial cells, i.e. 1.3 versus 0.88 pg per cell. Whereas the absolute level of transferrin receptor and total cellular iron in HeLa cells were elevated, they did not correlate linearly with the dramatic increase in sensitivity to DHA. This discrepancy could be explained by several possibilities. For example, total cellular iron was measured because we have not been able to quantify the level of ferrous iron, the form that reacts with the DHA endoperoxide bond to generate ROS [23]. It is possible that there is a greater difference in the level of ferrous iron in the normal and HPV-expressing cells. In addition, because HPV-expressing cells are sensitized to apoptosis by the E6 and E7 proteins, it is conceivable that slight increases in iron content may amplify toxicity of DHA in HPV-infected cells.

Abrogation of DHA-induced cytotoxicity with an iron chelator

To provide more direct evidence for the role of iron in DHA-induced cytotoxicity, a study was conducted involving an iron chelator - desferrioxamine (DFOM). HeLa cells, at a concentration of 2 x 10^4, were incubated for 6 hours with DFOM at concentrations ranging from 0 to 200 μM. Media containing DHA at concentrations of up to 150 μM and DFOM at the original concentration were subsequently added and incubated for 24 hours before cell viability was analyzed. As shown in Fig. 2B, DHA cytotoxicity was reversed in a dose-dependent manner, indicating an essential role for iron. To circumvent potential toxicity associated with prolonged exposure of cells to desferrioxamine [40], these experiments were repeated with increased amounts of DHA, and decreasing exposure from 3 days to overnight. Under these conditions, there was no detectable effect of desferrioxamine on cell viability.

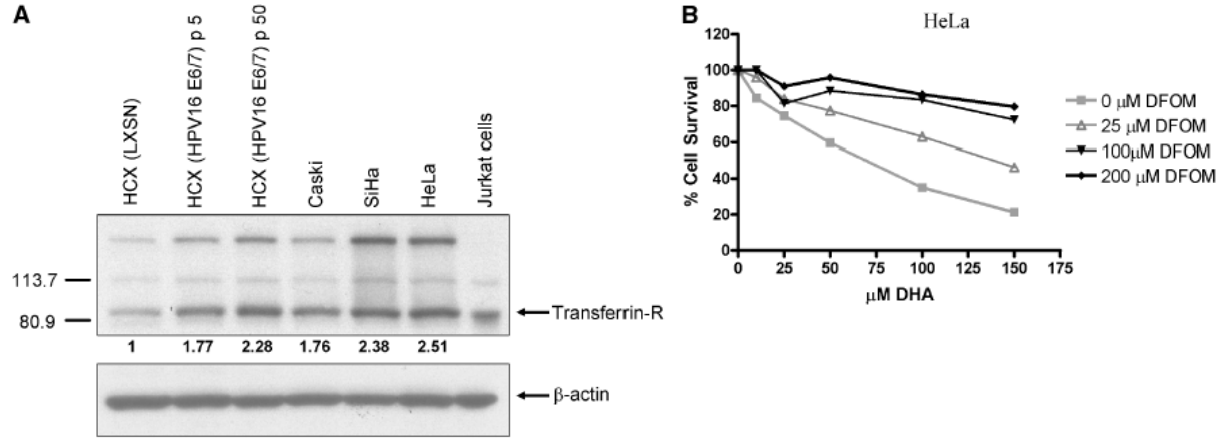


Figure 2. Transferrin receptor levels correlate with DHA sensitivity and iron-dependent toxicity in HeLa cells. **(A)** The same panel of cells as in Fig. 1C was analyzed by Western blotting for transferrin receptor level. A clear increase in the amount of transferrin receptor in immortalized and tumorigenic cell lines as compared with normal cells (HCX) was observed. Densitometry was done on the scanned image; numbers below the lanes, increase in transferrin receptor levels when HCX was set at 1. Jurkat cell extract was included as a positive control. **(B)** Viability of desferrioxamine pre-treated HeLa cells exposed to DHA. DFOM antagonized the cytotoxic effects of DHA and increased cell survival in a dose-dependent manner with >85% cell survival when the cells were pretreated with 200 μM desferrioxamine. Each point represents the mean cell viability of three wells from experiments carried out in triplicate.

DHA dose-dependent increase in presence of iron-dependent reactive oxygen species

To determine whether the DHA cytotoxicity observed in HeLa cells was a function of generation of iron-dependent ROS, the induction of ROS in HeLa cells was measured by a non-fluorescent probe, 6-carboxy-2V, 7V-dihydrofluorescein-diacetate. This probe is oxidized by ROS and converted into a fluorescent compound, 2V, 7V-dichlorofluorescein to provide a measurable indicator for the level of ROS. HeLa cells, at a concentration of 1 x 10^6, were incubated for 1 hour in 5 μM 6-carboxy-2V, 7V-dihydrofluoresceindiacetate in Dulbecco's modified eagle medium (DMEM). Subsequently, the probe was removed by rinsing in phosphate buffered saline. Cells were incubated with either fresh DMEM, DMEM + DHA (25 or 100 μM) or DMEM containing 150 μM DFOM and DHA (25 or

100 μM) for 3 hours. Cells were then analyzed by fluorescence-activated cell sorting (FACS) analysis.

As shown in the left panel of Figure 3A, The DHA-treated HeLa cells demonstrated a dose-dependent increase in fluorescence (2-fold and 4.2-fold at 25 and 100 μM doses, respectively), indicating an increase in production of ROS in response to DHA treatment. This increase in ROS was abrogated when the cells were pretreated with DFOM as shown in the right panel of Figure 3A. Altogether, these data underscore the essential role for intracellular iron in mediating the cytotoxic effects of DHA, via generation of ROS.

DHA-induced apoptosis

We further hypothesized that the cytotoxicity mediated by formation of ROS could be due to the induction of apoptosis. This is based on the observation that DHA-treated cervical cancer cells ceased proliferating, rounded up, and detached from the plate, suggesting an apoptotic process.

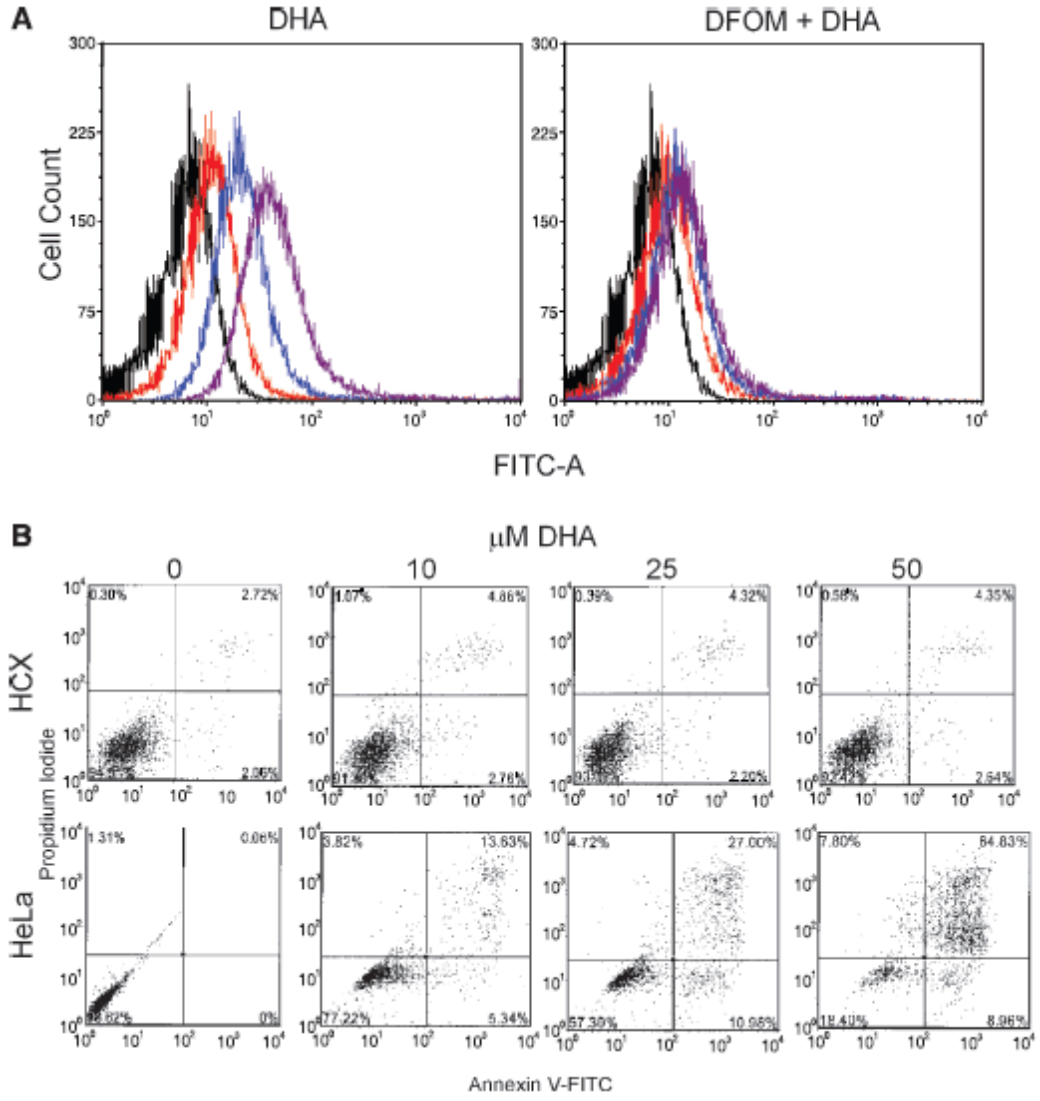


Figure 3. DHA-induced apoptosis is caused by iron-dependent ROS formation. **(A)** HeLa cells were treated with a fluorescent probe for ROS, and simultaneously with 0 μM (red line), 25 μM (blue line), or 100 μM (purple line) DHA for 3 hours (left). The black lines represent untreated cells in both panels. HeLa cells cultured with DHA demonstrated a dose-dependent increase in fluorescence (2-fold and 4.2-fold at 25 and 100 μM doses, respectively), indicating an increase in production of ROS in response to DHA treatment. The production of ROS was markedly inhibited by pretreating the cells with the iron chelator DFOM (right), indicating that the formation of ROS in response to DHA treatment is iron-dependent. **(B)** Control cervical cells (HCX) and HeLa cells were treated at 0, 10, 25, 50 μM DHA for 3 days, and stained for apoptosis with FITC-conjugated Annexin V and propidium iodide. HCX showed only a minor increase (from 2.7% to 4.4%; top) in stained cells while HeLa cells showed a significant increase in Annexin V and propidium iodide (from 0.1% to 64.8%; bottom).

To determine the mode of DHA-induced cell death, HeLa cells and control normal cervical epithelial cells were stained with fluorescent-conjugated Annexin V for early apoptotic detection, and with fluorescent-conjugated propidium iodide for late apoptotic events. The frequency of stained cells was quantified by flow cytometry.

As shown in Figure 3 (top), control normal cervical epithelial cells (HCX) showed only a marginal increase of either Annexin V-stained or propidium iodide-stained cells following treatment with increasing concentrations of DHA for 3 days (increase from 2.7% to 4.4% at the highest DHA concentration of 50 μM). In contrast, DHA-treated cervical cancer cell lines stained with Annexin V and rapidly became propidium iodide positive, indicating irreversible loss of membrane integrity. After 3 days in 50 μM DHA, approximately 65% of the cervical cancer cells were stained positively with propidium iodide, with an additional 9% staining positive for Annexin V.

2.5.3 *In vivo* activity of DHA in a canine oral mucosa model

To determine whether *in vitro* activities of DHA shown in previous sections were applicable *in vivo*, a canine oral papillomavirus (COPV) model was used. This animal model emulates the effects of HPV on the mucosal sites in human [41] and has been previously used for efficacy evaluation of papillomavirus vaccines. In this model, 100% of challenged animals become infected and develop tumors. Canine oral papillomas have an incubation period of 4–8 weeks following inoculation, and typically undergo spontaneous regression in the ensuing 4-8 weeks [42].

The maxillary buccal mucosae of six 10-week-old, female beagles were abraded with a sterile wire brush. Wart homogenate containing 500 ng (based on L1 concentration) of oncogenic COPV was then applied to the excoriated mucosa with a cotton swab. After 24 hours, the infected areas of three beagles were treated topically with 2.22 mg DHA dissolved in 100 μL dimethyl sulfoxide (DMSO) (78 mM). The infected areas of the other three beagles were treated with 100 μL DMSO as the placebo control. Treatment was applied once daily, five times a week, for 6 weeks.

Figure 4A depicts a schematic of viral challenge, drug treatment, tumor appearance, and tumor regression. In all control animals, tumors appeared approximately 3 weeks after the COPV challenge. Figure 4B is a photograph of virus-infected dogs treated with DMSO (left) or DHA (right) at 6 weeks post-challenge. The tumor shown in the DMSO-treated dog is representative of the size of tumors observed in all tumor-positive dogs and was taken at the pinnacle of tumor growth. The DHA-treated dog is free of tumor. In addition, the oral mucosa treated with DHA shows no evidence of ulceration or inflammation, consistent with the lack of DHA cytotoxicity for normal epithelial cells *in vitro*. All tumors were regressed by 8 weeks following initial viral challenge. Study animals were observed for another week after regression to ensure that no additional tumors were formed. Peripheral blood was obtained 1, 3, and 5 weeks after the onset of tumor formation.

Figure 5A summarizes the results of tumor formation in the six study dogs. All virus-infected dogs treated with DMSO developed tumors at 3 to 4 weeks post-challenge. In contrast, DHA treatment abolished tumor formation in two of three infected animals. The lesions that developed in the DHA-treated animal (dog 5) underwent regression 2 weeks earlier than those in the animals treated with DMSO alone, although the mass was of comparable size to those in control animals.

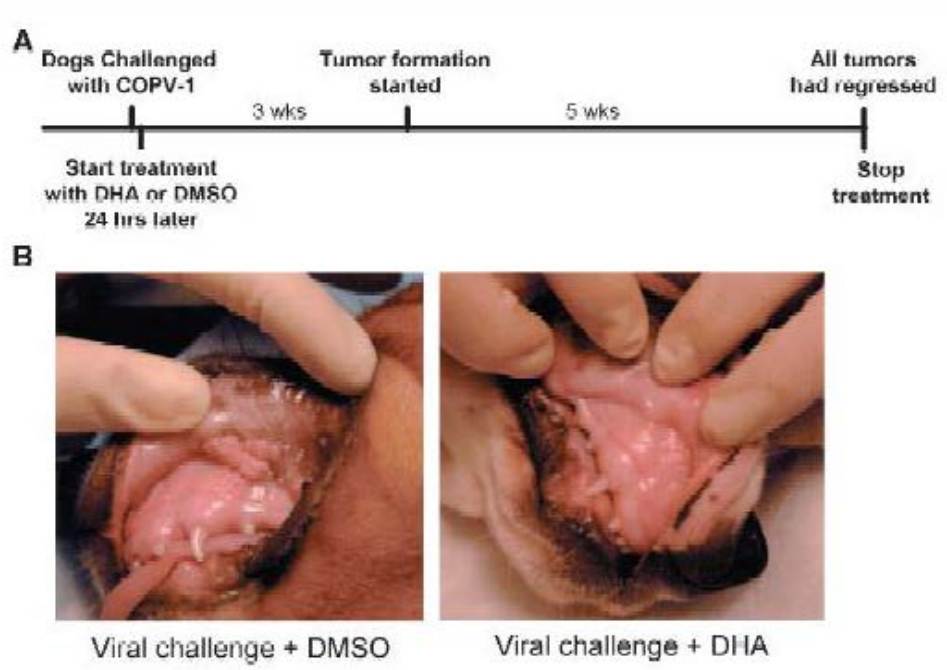


Figure 4. Topical therapeutic effect of DHA in a preclinical papillomavirus model. **(A)** a schematic representation of the experimental protocol. Treatment was initiated 24 hours after the COPV challenge and stopped when all tumors had regressed. For this COPV model, tumors appeared approximately 3 weeks after viral challenge and all tumors had regressed 8 weeks post-challenge. **(B)** Representative images of dogs with and without tumor formation.

Although the study sample size was small, precluding statistical analysis, it should be emphasized that the COPV model resulted in a 100% tumor formation rate during decades of working with this model by the group. Thus, the absence of tumors in 2 of 3 treated animals is biologically significant, suggesting that DHA is effective in the prevention of tumor formation.

Visual observations of the oral mucosa approximately 3 weeks post-challenge identified a similar mucosal "roughness" in all of the challenged dogs, including the DHA-treated animals, indicative of early papilloma formation. We postulated that DHA might be inhibiting tumor formation rather than virus infection.

To determine whether the DHA-treated dogs had a subclinical viral infection, serum samples obtained at the end of the trial were used to detect and quantify antibody responses to the L1 viral capsid protein. Serum samples were analyzed for papillomavirus antibody using an ELISA immunoassay. The results are shown in Figure 5B. Despite the finding that only one DHA-treated dog developed a tumor, all DHA-treated animals developed antibody titers against the L1 protein, suggesting that the tumor-free animals indeed had been infected with virus and that the virus had replicated and synthesized significant amounts of L1 protein. These data suggest that DHA inhibits tumor formation rather than viral replication. As anticipated, all of the

control animals also developed antibodies to L1, which is normally observed during the natural regression of these tumors.

A Tumor Formation in Dogs

Dog ID	DMSO or DHA	Right Side	Left Side
1	DMSO	+	+
2	DMSO	+	+
3	DMSO	+	+
4	DHA	-	-
5*	DHA	+	+
6	DHA	-	-

*Tumors regressed two weeks earlier than the DMSO-treated animals

B Anti-L1 capsid protein antibody titers

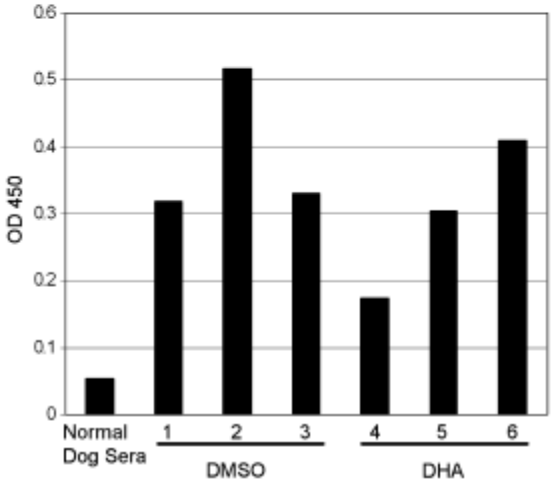


Figure 5. Topically applied DHA inhibits papilloma formation by COPV. **(A)** Tabulation of tumor formation in dogs. All 3 control animals and 1 DHA-treated animal developed tumors that reached a similar size to those shown in Figure 4B, left. The tumor in the DHA-treated dog regressed 2 weeks sooner than the tumors in DMSO-treated dogs. **(B)** ELISA results from dog serum collected during the last week of the study. All dogs were positive for antibodies against L1, indicating an initial infection in all dogs. Dogs 4 and 6 without the presence of tumors showed antibodies against L1, suggesting DHA can inhibit tumor formation without inhibiting the initial infection or replication of the virus.

In summary, and taking into account the large body of clinical experience garnered from the use of systemically administered Artesunate for the treatment of acute malaria, these data provide rationale for the use of a topical formulation of Artesunate in the treatment of incipient, intraepithelial neoplasia caused by HPV.

3.0 CLINICAL SAFETY AND TOLERABILITY

Commercially available Artesunate has been used for the treatment of acute malaria for decades using systemic dosing that is at least 5-fold higher than the suppository dosing proposed in this clinical trial. Rectal suppository formulations are approved for use as a second-line treatment for acute malarial infections in children, and used off-label in adults, who cannot tolerate oral administration and are distant from a hospital setting [4]. For severe malaria during pregnancy, there is less certainty about the safety of Artesunate during the first trimester, but Artesunate is recommended as first-line therapy during the second and third trimesters [43]. Two reports submitted to the FDA by the WHO and Novartis summarize many of the safety studies conducted with artemisinin and its derivatives, including Artesunate [4, 17, 18, 29].

For young children of less than 5 years of age, rectal Artesunate suppository administered at a single dose of 10 mg/kg is the only WHO recommended safe pre-referral treatment for severe malaria. Rectal suppository dosing has also been tested in adults, including regimens of 500 mg/day for 5 consecutive days [44]. No local or serious systemic side effects have been reported at these doses. The initial starting intravaginal suppository doses proposed, 50 mg (≤1 mg/kg in individuals who weigh ≥50 kg, a subject inclusion criterion for this study) and the highest proposed dose, 200 mg (≤4 mg/kg) are approximately 10 and 2.5-fold lower than the approved pediatric rectal suppository dose.

3.1 Clinical Toxicology and Pharmacology

Clinical effects reported in subjects treated with artemisinin compounds have included mild gastrointestinal disturbances, dizziness, and tinnitus, albeit with weak evidence of association [45]. There have also been reports of transient reticulocytopenia, neutropenia and elevated liver enzyme values, though none has been considered to be of clinical significance [45]. Electrocardiographic abnormalities such as bradycardia and prolongation of the QT interval, have been reported but again, no impairment of heart function has been observed in numerous malarial therapy trials in humans [24, 45] . The only potentially serious adverse effects that have been convincingly associated with administration of artemisinin compounds are the type 1 hypersensitivity reactions in approximately 1 in 3000 patients [46]. In nonclinical studies in mice, CNS effects have been observed; however, such effects have not been observed in humans. Because the dosage evaluated in mice (100 mg/kg, i.m., artemotil and artemether) is at least 6 fold higher than therapeutic doses in humans, these toxicities are likely of little clinical relevance (see nonclinical toxicology section 2.4 above).

With regard to strong nonclinical evidence of artemisinin compounds-induced embryotoxicity, the tight relationship to a specific period of the gestational cycle has informed the contraindication of their usage during the first trimester of pregnancy in humans [29, 32] . Importantly, in malaria treatment, there is no evidence for adverse effects on the fetus following exposure during the second and third trimesters, when these drugs are recommended depending on the safety profile of the partner drug in the artemisinin-based combination treatment [45]. This recommendation can be

sidestepped in cases of severe malaria where artemisinin derivatives are clearly superior in terms of life-saving efficacy [45]. Prospective tracking of over 1000 pregnancies did not reveal any significant adverse effects following artemisinins' use during the 2nd and 3rd trimesters [47, 48].

Finally, artemisinins are generally well tolerated and human studies with the various drugs used in combinatorial therapy against malaria have not revealed major clinical concerns regarding untoward drug interactions. However, because such interactions are peculiar to the compounds themselves, each potential drug interaction has to be considered on its own [18, 49].

In this clinical protocol, intra-vaginal Artesunate suppositories will be tested. Direct application to the mucosal surface at the doses we propose is unlikely to result in significant systemic absorption. While there are no published pharmacologic data for intra-vaginal administration of Artesunate suppositories, clinical pharmacology of Artesunate and its metabolite DHA following rectal, IV, IM, and oral administration are well documented in the anti-malaria literature [4, 18, 24, 28, 50-52]. Years of pharmacokinetic data already performed with Artesunate informed the choice of intra-vaginal dosing [49-51, 53, 54]. A review of clinical toxicology and pharmacology assessments of Artesunate and its metabolite DHA is summarized in Table 1.

Artesunate is rapidly absorbed, with peak plasma levels occurring 1.5 h, 2 h and 0.5 h after oral, rectal and intramuscular administration, respectively [3, 55-58]. It is almost entirely converted to its active metabolite DHA [59]. Elimination of Artesunate is very rapid, and its activity is determined by DHA elimination, which has a half-life of approximately 45 min [11, 52].

Table 2: Clinical Toxicity and Pharmacology of Artesunate

Route of Administration	**Dosing Regime**	**Toxicity Summary**
Oral Administration of Artenimol-R (DHA) in patients with squamous cell carcinoma stage 3 or 4 [60]	Treatment Phase: 7 days @ 100mg/day, and if no NCI CTCAE grade 3 or 4, then additional 21 days @ 200 mg/day Follow Up Phase: Four patients with relapse received 28 days of DHA at 200 mg/day	Treatment Phase: Zero incidence of grade 3 or 4 AEs; five patients with grade 1 or 2 AEs described as transient 'flu-like syndrome', headache, and abdominal pain
Rectal Artesunate – data from 1167 patients in 15 clinical trials	A single high dose of rectal Artesunate (10mg/kg) was 5X more likely to	Total incidence of AEs considered by clinicians to be possibly drug-related was approx. between 2.7% and 9.0% of all rectally

Route of Administration	Dosing Regime	Toxicity Summary
of rectal artemisinin derivative (Artesunate, artemisinin and artemether) therapy [61]	achieve >90% parasite reductions at 24 hours than were multiple lower doses of rectal Artesunate, or a single lower dose administration of rectal artemether.	artemisinin-treated patients, compared with 22% of quinine-treated patients. Majority of the possibly drug-related adverse events involved either the gastrointestinal system or were generalized and non-specific in nature and were not severe.
IV Administration of Artesunate in US Phase I safety trial [51]	Dose escalation of single dose of 0.5, 1, 2, 4, or 8 mg/kg over 2 min	No dose-limiting toxicity
IV Administration of Artesunate in a Phase 1b Study [50]	3 days @ 2, 4, or 8 mg/kg over a 2-min infusion	• No subject drop-outs for adverse events or other treatment-related issues. • Dose-related decrease in reticulocyte count noted that peaked 4 days after dosing and returned to normal by study day 7 in most cases, and day 10 in all patients. • No other clinically significant laboratory abnormalities detected. • No deleterious hemodynamic or ECG effects • A transient, reversible sensation of altered or unusual taste, lasted 30 minutes in all cases at higher doses of Artesunate
IM Administration of Artesunate in Children with Severe Malaria [54]	IM 2.4 mg/kg at 0, 12, and 24 h in children from 6 months to 11 years old	No direct report of treatment-specific complications
Comprehensive review of clinical pharmacology following administration of Artesunate by the	IV Route • High initial plasma concentration subsequently declined rapidly with a half-life of less than 15 min. Clearance and volume estimates averaged 2 - 3 L/kg/hr and 0.1 - 0.3 L/kg, respectively. • The metabolite DHA concentrations peaked within 25 minutes post-dose, and a half-life of 30 - 60 minutes.	

Route of Administration	Dosing Regime	Toxicity Summary
IV, IM, oral or rectal routes [52]	DHA clearance and volume averaged between 0.5 - 1.5 L/kg/hr and 0.5 - 1.0 L/kg, respectively IM Route • Lower peaks, longer half-life values, and higher volumes of distribution for Artesunate • Delayed peaks for DHA; other parameters were generally similar due to the high bioavailability, assessed by exposure to DHA, associated with IM Artesunate administration (> 86%). Oral Route • High bioavailability of DHA (> 80%). • Artesunate C_{max} within 1 hour, and a half-life of 20 - 45 minutes • DHA C_{max} within 2 hours and a half-life of 0.5 - 1.5 hours. • AUC values reported for Artesunate often substantially lower than those reported for DHA following oral administration Rectal Route * Similar PK results as oral administration, with the exceptions of delayed C_{max} and longer half-life	

Based on clinical safety studies of Artesunate in the U.S. [62], tests on daily IV boluses of up to 8 mg/kg of Artesunate did not result in dose-limiting toxicities [55]. At 8 mg/kg, a transient, reduced reticulocyte count had been observed, but resolved without intervention over a timeframe of 4-7 days [24]. Drug concentrations did not accumulate in subjects receiving multiple daily IV treatments, corroborating with known rapid elimination rates for artemisinin derivatives [50]. A significantly lower maximum plasma concentration after intra-vaginal administration is expected, compared to IV administration. This prediction is based on the pharmacokinetic studies of IM Artesunate [52], in which the rate of tissue absorption slows drug distribution; on average, one thirtieth and one fifth of C_{max} observed by the IV route for Artesunate and DHA, respectively. The differences between Artesunate and its principal metabolite are due to a combination of differences in solubility, and to rapid conversion of Artesunate to DHA [52]. Despite the prediction of low systemic concentration of drug, rapid elimination following intra-vaginal administration would be expected, as seen with IM administration [52].

3.2 Dosing Rationale

Although no serious adverse events are anticipated with the doses we propose, the first cohort of subjects will be a run-in of three patients receiving 50mg suppositories. If no mucosal or systemic adverse events are reported, we will proceed with 200mg dosing.

A five-day dosing regimen with a single dose of 200 mg per 24-hour period is selected based on the clinical toxicology and pharmacology database from anti-malarial and cancer trials as well as the performance data presented on the inhibition of HPV infected cells *in vitro* and *in vivo*. We anticipate that intra-vaginal administration of 200 mg Artesunate suppositories to the cervix will provide a local drug concentration well above IC_{50} of HPV-infected cells. Based on our experimental experience in nonclinical studies, we plan to assess more than one 5-day treatment cycle to explore potential dosing regimens suitable for subsequent phases of clinical studies.

The doses selected for this study are 50 mg and 200 mg. A dose level of 200 mg is equivalent to ≤ 4mg/kg based on a minimum body weight of 50 kg as a subject inclusion criterion. The proposed dose level should be extremely safe as it is well within WHO recommended pre-referral dose for parenteral Artesunate treatment of severe malaria in young children; that is a single dose of 10mg/kg body weight. The proposed dosing regimen also resembles WHO recommended multi-day dosing regimen for adults and children with severe malaria upon hospitalization; that is 2.4mg/kg IV or IM TID on day 1 (or 7.2 mg/kg) and continued daily dosing at 2.4mg/kg for a total of 7 days [43]. Due to the rapid rate of elimination *in vivo*, negligible systemic accumulation of Artesunate or DHA metabolite is expected over multi-day dosing, especially in the case of local administration.

In summary, local intravaginal administration of Artesunate as a suppository at the highest dose in this study, 200 mg or approximately ≤ 4 mg/kg is not expected to cause any systemic toxicity. Although no serious adverse events are anticipated with the proposed doses, dose escalation will only occur if no mucosal or systemic adverse events are reported in the first cohort of three subjects who receive the lowest dose. Subjects will be monitored for any unexpected adverse events locally and systemically.

4.0 OVERALL ASSESSMENT OF RISKS AND POTENTIAL BENEFITS

4.1 Potential Risks

The major risks for the study subjects are associated with surgical excision procedures that are the standard of care for patients with CIN2/3. Other potential risks posed by this clinical protocol would include local tissue irritation due to the presence of the investigational drug, and venipuncture for peripheral blood collection.

Intra-vaginal administration of Artesunate suppositories has not been approved for general use by the Food and Drug Administration (FDA). An Investigational New Drug (IND) application covering the proposed was submitted to the FDA. Approval to conduct this clinical trial was obtained from the FDA before clinical enrollment began. The IND governing this protocol is IND 124299. In parallel, the clinical trial protocol

was reviewed and approved by the Johns Hopkins Hospital Institutional Review Board, and the Johns Hopkins Cancer Center Clinical Research Committee.

4.2 Known Potential Benefits

This study is designed to assess safety, tolerability, and feasibility of using Artesunate suppositories in women with CIN2/3. Treated subjects whose CIN lesions regress during the study period would avoid the need for surgical LEEP or cone excisional biopsy procedure.

In summary, the potential to develop a non-invasive therapy for this disease is worth the minimal risk with this study.

5.0 RATIONALE FOR CLINICAL TRIAL DESIGN

This trial is a prospective, open, dose-escalation study of topical Artesunate, formulated as a suppository, administered intravaginally, prior to standard therapeutic resection of the squamocolumnar junction at study week 15. An initial dose cohort of 3 subjects will receive intravaginal Artesunate for one 5-day treatment cycle. Based on clinical experience with Artesunate, we expect that absorption in the cervical mucosa, which is stratified squamous epithelium, will be less than what is observed with rectal administration, which is a single layer of glandular epithelium. Because cervical stroma is much less vascularized than colorectal stroma, we also expect significantly lower systemic levels of Artesunate in subjects receiving intravaginal dosing. The goal of topical administration is to achieve local, mucosal distribution of study drug, as opposed to systemic levels.

If we do not observe untoward side effects, including mucosal excoriation, in the first dose cohort, we will proceed with dose-escalation. The subsequent cohort will undergo two cycles of treatment, spaced at 2-week intervals. The final cohort will undergo a total of three cycles of treatment, spaced at 2-week intervals. All subjects will undergo an interval colposcopic exam at study week 6. At week 15, if there is still a colposcopically detectable lesion, subjects will undergo standard therapeutic resection. If no lesion is obvious, a biopsy will be taken at the site of the original lesion. The cohort receiving the fewest cycles of Artesunate, and showing therapeutic effect will be expanded (see Section 12 for details). At each vaginal exam, cervical swabs will be obtained and cryopreserved. Peripheral blood will be obtained by venipuncture at baseline, and at study weeks 5, 15, and 28, 41, and the postop visit, and will be cryopreserved for assessment of biomarkers.

This clinical cohort, healthy subjects with colposcopically-directed, biopsy-confirmed CIN2/3, was selected for safety reasons. The study protocol window (15 weeks), is within the standard of care. In pregnant women who have a cervical HSIL, the standard of care is to follow colposcopically until postpartum [7]. We and others have reported that the probability that CIN2/3 will progress in this timeframe is essentially zero [63]. In fact,

we and others have also reported that in this timeframe, approximately 20-25% of CIN2/3 lesions associated with HPV16 will undergo regression [63]. Lesions that are associated with HPV genotypes other than HPV16 are even more likely to regress in this timeframe [64]. Moreover, in this population, it is feasible to visualize directly, quantitate serially, and perform therapeutic resection of premalignant lesions. Based on safety and tolerability data in pediatric populations receiving intrarectal suppositories at a dose that is approximately five-fold compared to the regimen we propose in this clinical protocol, we do not expect dose-limiting toxicities with the dosage and regimen proposed in this proof-of-principle study. The Trimble group has a decade of experience in the design and execution of prospective clinical protocols specific for this patient cohort, testing immunotherapeutic interventions prior to standard therapeutic resection of any residual lesions. [65, 66]. The parallel design of this trial will allow comparison of tissue-based endpoints in this cohort.

Despite the experience with active immunotherapy in other tumor models, it is still not clear what measures of immune response are relevant to lesion regression. In particular, it does not appear that classic measures of immune response, measured in the circulation, correlate at all with clinical response. Because the biology of HPV disease is extensively characterized; because there are known, quantifiable risk factors for persistence and progression of disease; because we have ready access to an immunocompetent population of patients at elevated risk for carcinoma development; and because we can directly visualize and subsequently therapeutically resect and analyze affected tissue; we are presented with an opportunity to rapidly evaluate therapeutic regimens, characterize relevant immune responses, and provide direction for future interventions, both in this disease and in other tumor models. The relatively long precursor phase in HPV disease also provides many opportunities for intervention, including, optimally, prophylaxis against the development of cancer.

A larger goal of these clinical trials is to correlate measures of immune response with known clinical parameters of disease. The larger implications of this effort lie in the identification of appropriate and relevant measures of the mucosal immune microenvironment. The identification of intermediate biomarkers in the tissue not only serves to further our understanding of the immune response and our efforts to manipulate it, but also has practical implications in the setting of monitoring patients at risk for disease recurrence or progression. In the case of high grade cervical intraepithelial neoplasia, lesion size and viral load are both quantifiable risk factors for persistent and progressive HPV disease. Therefore in these trials we will quantitate both outcome measures sequentially and correlate them with local tissue and peripheral measures of immune response.

6.0 STUDY OBJECTIVES

This trial is designed to test the hypothesis that intra-vaginal application of Artesunate is well-tolerated in women with CIN2/3.

The primary objective is to evaluate the safety and tolerability of the intra-vaginal administration of Artesunate in study subjects.

The secondary objective is to explore clinical activity, such as clearance of HPV disease, corresponding to intra-vaginal administration of Artesunate.

7.0 STUDY OUTCOME MEASURES

7.1 Primary Outcome Measures

Safety will be assessed by the findings of adverse events, including dose related limiting AEs. Subjects will be monitored for the appearance of erythema, itching, burning, pain, infection, desquamation, tenesmus, bleeding, dizziness, and any unexpected AEs.

7.2 Secondary Outcome Measures

Indicators of drug efficacy (see below) will be drawn from any evidence demonstrating a significant difference (by simple chi-square analysis; $p<0.1$) between treated subjects and the baseline data in the Johns Hopkins Colposcopy database

- Extent of lesion clearance (by lesion number, size, and/or area of coverage)
- Presence/absence of HPV DNA
- Regression of CIN stage by histopathology (comparison of initial biopsy to LEEP/cone excision-procured tissue).

Histologic regression will be judged as none (persistent CIN2/3 in week 15 resection specimen) or total (no CIN2/3 in resection specimen).

Virologic HPV viral load. Longitudinally obtained cervical swabs will be used to quantitate HPV viral load as a continuous variable.

Tissue sections from the diagnostic biopsy obtained as part of the screening process, and from the resection specimen obtained post-treatment will be assessed for expression of CD8 (effector T cells), CD71 (transferrin receptor), and cleaved caspase 3 (apoptotic cell death).

8.0 STUDY DESIGN

Prior to enrollment, patients referred for evaluation of an HSIL pap smear will undergo a full colposcopic evaluation, with biopsy confirmation. Clinical, histopathologic, virologic, and immunologic parameters will be assessed longitudinally in each study participant, as outlined below. The trial is a dose-escalation design, with a run-in cohort to assess the likelihood of mucosal irritation.

These serial assessments will directly parallel those obtained in the currently open CIN2/3 cohort study (JHMI JCCI# 99-08-27-04). Those patients who have measurable disease after biopsy will be eligible for study participation.

Cervical tissue specimens obtained as routine diagnostic and therapeutic specimens will be analyzed to characterize local tissue immune response. Diagnostic biopsies obtained at initial colposcopy will be used to confirm the diagnosis of CIN2/3. The initial diagnostic biopsy specimens will also be characterized in terms of type and quantitation of inflammatory cell infiltrates. Immunocytochemical techniques will be used to determine presence and numbers of macrophages, dendritic cells, T cells, B cells, and NK cells. We will also use standard staining techniques to detect cleaved caspase 3. As tissue availability permits, the specimens will also be analyzed for other immune cells. We will be comparing tissue profiles with those obtained on circulating PBLs in our study population. In the post- suppository administration, therapeutic LEEP/cone biopsy specimens, markers of local tissue immune response will be similarly characterized and quantitated. Dr. Trimble will review all of the histologic slides.

9.0 STUDY POPULATION AND ENROLLMENT

Patients with high-grade cervical intraepithelial lesions (CIN2/3) will participate in this study. At Johns Hopkins, patients will be recruited from the Johns Hopkins Colposcopy Service, and from referring physicians. Annually, the Colposcopy Service treats over 800 patients, approximately 15% of whom have high-grade lesions. In terms of feasibility, over the past 12 years we have recruited nearly 500 patients for screening to our cohort and vaccine studies, which consists of the same study population as the one described for this interventional trial. Of the HSIL patients approached for study entry, 80% have consented for screening. Of the patients who consented for screening, 33% were African American. This percentage is representative of our colposcopy population. It is anticipated that as therapeutic options become available through the upcoming Phase I trial, that referral volume will continue to increase.

9.1 Subject Inclusion Criteria

- Patients with high-grade cervical intraepithelial lesions (CIN2/3) confirmed by colposcopy and biopsy.
- Patients who have measurable disease after diagnostic biopsy.
- Patients who weigh ≥50 kg
- Patients who are HPV-positive by DNA test
- Patients who are age 18 or older.
- Patients who are able to give informed consent.
- Patients who are immunocompetent.
- Patients who are not pregnant; committed to using adequate contraception through week 15 if of childbearing age. Condom use will be encouraged.
- Patients who have the ability to collaborate planned follow-up

9.2 Subject Exclusion Criteria

- Patients with cytologic evidence of glandular dysplasia
- Patients with cytologic evidence of adenocarcinoma in situ

- Patients who are taking immunosuppressive medication
- Patients with concurrent malignancy except for nonmelanoma skin lesions
- Patients who have an active autoimmune disease or history of autoimmune disease requiring medical treatment with systemic immunosuppressants, including: inflammatory bowel disease, systemic vasculitis, scleroderma, psoriasis, multiple sclerosis, hemolytic anemic, or immune thrombocytopenia, rheumatoid arthritis, SLE, and Sjogren's syndrome, sarcoidosis. Asthma or COPD that does not require systemic corticosteroids or routine use of inhaled steroids is acceptable

10.0 STUDY PROCEDURES

10.1 Screening

All subjects will receive medical care that is routine to clinical practice.

Drs. Trimble (JHU) and Levinson (GBMC) and the study nurses or coordinators will recruit patients at the time they are seen for their initial clinical evaluation, or when they return to discuss their colposcopy results. All eligible patients will be asked if they would like to be screened for participation in this clinical trial. Drs. Trimble and Levinson and the study nurses or coordinators will be responsible for recruiting study participants and for obtaining written informed consent. They will allow as much time as necessary to review the consent forms with subjects, and to allow them to ask questions. Participants will receive a copy of the consent form at the time of their appointment, and consented subjects will undergo the procedures listed in Table 3 below.

Once patients sign consent, will be assigned a study ID, and their demographic and clinical information will be entered in the Cervical Dysplasia Research Program database. This information is also entered in the institutional database CRMS. To maintain confidentiality, the study-specific ID will be used to label all specimens collected and banked during the study. Only the clinical team and program manager will have access to PHI. All other members of the research team will only have access to study-specific IDs.

A schema of the data flow is outlined in Figure 1 below.

Figure 1: Data Flow Diagram

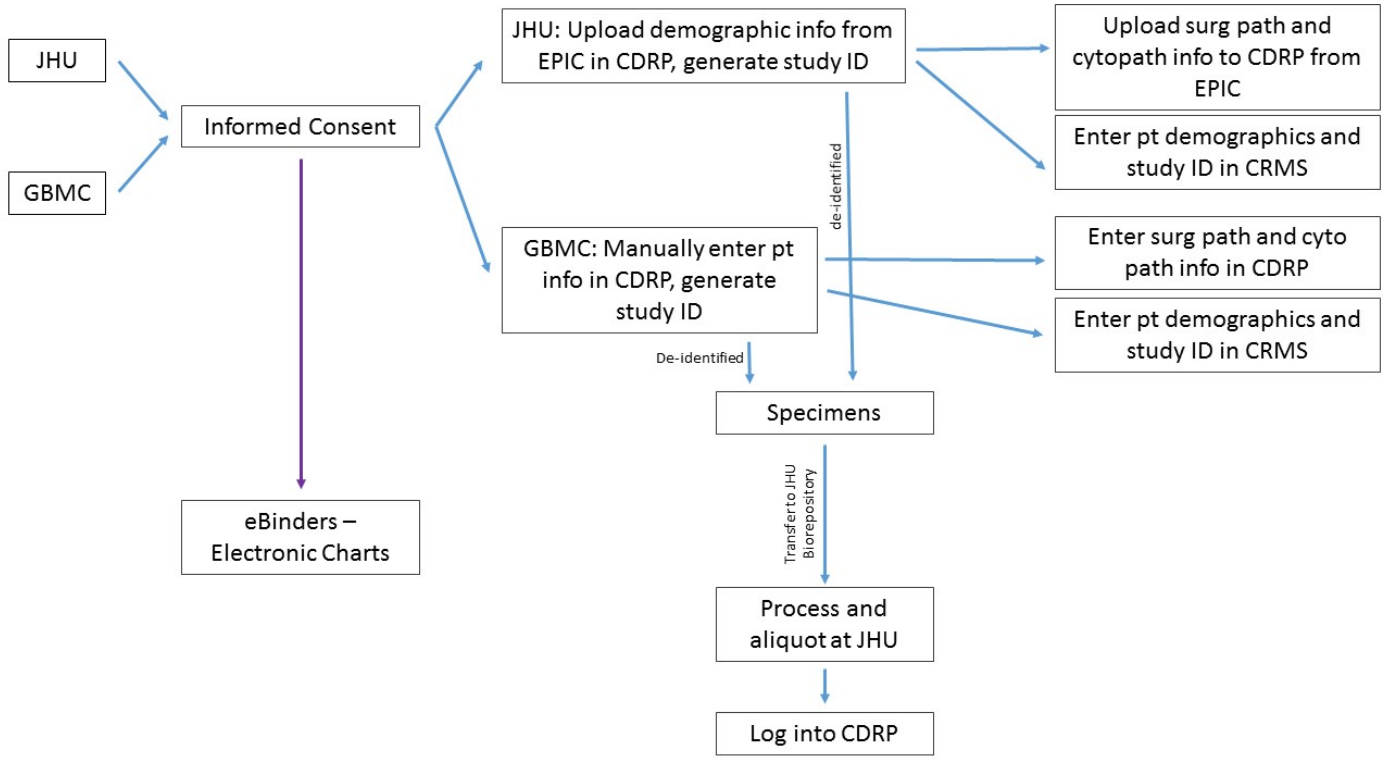


Table 3: Outline of Evaluation Procedures

	-3wks	Wk 0	Wk 2 [1,2]	Wk 4[2]	Wk 6	Wk 15	Postop **	Wk 28	Wk 41
Informed consent	X								
Artesunate dosing		X	X	X					
Vital signs	X	X	X	X	X	X	X	X	X
Cervix check			X	X	X	X			
Medical history	X								
History of disease	X								
Demographic data	X								
Diary cards (give to pt)		X	X	X		X			
Diary cards (collect from pt)			X	X		X			
Adverse events		X	X	X	X	X	X	X	X
Inclusion/exclusion criteria	X								
Colposcopy	X				X	X		X	(X)*
Pap smear	X				X	X		X	X
HPV genotyping	X				X	X		X	X
Cervical swabs	X	X	X	X	X	X	X	X	X
LEEP/Cone**						(x)		(x)	
HLA testing ***	X								
PBL for banking****	X	(X)			X	X	X	X	X
Urine HCG	X	X	X	X		X			
Optional pheresis******									

1. Treatment Group III
2. Treatment Group IV

* Colposcopy only if clinically-required at wk 41

** if residual disease is present (the procedure can happen any time after week 15, based on the physician's clinical judgment). The Postoperative visit will take place 4 +/- 1 weeks after the LEEP/Cone procedure.

*** HLA can be done at any time during the study, not an eligibility criterion

**** First blood draw can be done at either the screening visit or Wk 0, prior to dosing

***** pheresis is optional and can happen any time after week 15

10.2 Intervals Between Visits

The intervals between screening and first administration may be 3 ± 2 weeks, if necessary. A subject is considered enrolled when she receives the first Artesunate suppository at the scheduled Week 0 visit. After the first administration of Artesunate, the time window for each subject to complete 4 remaining daily doses of Artesunate suppositories may be between 4 days and 2 weeks (for example, to minimize potential wash-off of the treatment during a menstrual period). There will be a window of ± 1 week at weeks 2, 4, 6, 15, and post-operative visit, and ± 2 weeks at the week 28, and 41 visits.

10.3 Description of Study Visits

All clinically relevant data collected during the study procedures must be recorded first in the patient file. These data will then be transcribed into the CRF. All visits will be outpatient visits.

At the initial screening visit, in addition to undergoing a standard of care colposcopic evaluation, we will obtain cervical brush specimens for HPV typing, and peripheral blood specimens to determine HLA phenotype, HIV status, and immunologic studies. Subjects with biopsy-confirmed CIN2/3 will be enrolled into treatment cohorts as depicted in Table 4 below.

Table 4 – Treatment Cohorts

Cohort	Number of patients	Dose	Treatment cycles
I	3	50 mg	1 (5 days)
II	9	200 mg	1 (5 days)
III	9	200 mg	2 (5 days each)
IV	9	200 mg	3 (5 days each)

In cohort I, at the first study visit, the subjects will be given a total of five suppositories and applicators. The first suppository will be inserted in clinic, by the patient. Patients will be given diary cards, and will return 1-2 weeks after completing the regimen, for visual assessment of the cervicovaginal mucosa.

In cohorts II-IV, the first study visit will be the same as the first study visit for the run-in cohort. Subjects will return to clinic 1-2 weeks later for visual assessment of the cervicovaginal mucosa. At study week 2, patients enrolled in cohorts III and IV will undergo a visual assessment of their cervicovaginal mucosa and will be given an additional 5 suppositories and applicators. Patients enrolled in cohort III will return to clinic 1-2 weeks later for visual assessment of the cervicovaginal mucosa. At study week 4, subjects enrolled in cohort IV will undergo a visual assessment of their cervicovaginal mucosa, and will also be given five suppositories and applicators. At study week 6, **all subjects** will undergo an interval colposcopic assessment. If their lesions do not appear to be more severe than at study entry, they will return at study week 15. At study weeks 15 and 28, all subjects will undergo colposcopic evaluation. If there is any residual colposcopically abnormal tissue, it will be removed using standard excisional procedures. Peripheral blood and cervical brush specimens will be obtained at study entry, at week 5, at week 15, and at the follow-up visits: postoperative visit, and at weeks 28 and 41.

10.4 Examinations, Evaluations, and Procedures

Vital signs

Supine blood pressure and heart rate will be measured both prior to each procedure and drug administration. Additional investigation may be added at the discretion of the investigator.

Demographic data/medical history

The patient's age, weight, height, and ethnicity will be ascertained at the screening visit. Information will be collected on the patient's past significant medical history including tobacco exposure, gynecologic and obstetric history including contraceptive use, total and current number of partners, and current medical status.

History of HPV disease

The following information will be noted at the screening visit:

- month/year of detection of CIN lesions
- Details of previous therapies

Colposcopic examination

Patients will undergo serial colposcopic examinations; at the screening visit, and at weeks 6, 15, 28. Exfoliated cell samples will be obtained using Digene swabs at the time of colposcopy, for HPV typing. Specimens for HPV genotyping will be processed by Quest Diagnostics, Nichols Institute. Specimens for quantitation of HPV viral load should be directed to the GYN biorepository, where they will be processed and cryopreserved for batch analysis. Swabs of cervical secretions will be banked in the tissue bank. Cervical lymphocyte samples will be processed directly.

LEEP/Cone Excisional Biopsy Procedure

After week 15, if there is residual colposcopic disease, the affected area will be excised using standard LEEP or cold knife cone technique. The resection specimens will be taken fresh to surgical pathology. Frozen section will be performed to confirm the presence or absence of CIN2/3. The frozen section block will be banked at –70°C, and a sliver of tissue immediately facing the frozen section will be reserved for isolation of immune cells. This will allow unhampered routine diagnostic ability as well as preserve tissue sections frozen for characterization of inflammatory cell infiltrates. Routinely processed formalin-fixed, paraffin-embedded sections will also be analyzed for the characterization and quantitation of tissue infiltrates.

Peripheral blood samples

Blood will be drawn at various visits as noted in the protocol for analysis of various parameters and markers. The volume of blood will vary depending on the tests to be performed.

HLA typing: one 10 ml yellow top tube

Immunology samples: approximately 50 mls of blood drawn in green top (heparinized) tubes to the tissue core.

These specimens will be separated by standard Ficoll centrifugation, and the serum and PBLs aliquoted and banked at –70°C.

Histologic response

We will evaluate pathologic changes in diagnostic biopsy and post-suppository therapeutic resection material, on standard hematoxylin and eosin slides.

Urine sample

A urine pregnancy test for HCG will be performed in patients of childbearing potential prior to each suppository administration.

HIV Testing

HIV testing will be performed using standard JHH procedures including the JHH consent forms.

Immune Response

We will assess peripheral blood specimens for immune responses to HPV antigens, using standard ELISA and ELISPOT assays. We will also perform quantitative digital image analyses of the tissue specimens obtained at diagnosis, and at week 15.

10.5 Compensation

Study subjects will receive a total of $200 if they participate in the entire study. A check will be mailed to their home within a month after each visit. Study subjects will receive $20 for visits at weeks -3, 0, 2, 4, 5, 28, 41 and the postop visit and $40 for the visit at week 15.

Upon completion of the postoperative visit, patients will have routine follow-up, including examinations at three-month intervals for the first year according to the standard of care.

11.0 STUDY DRUG AND ADMINISTRATION

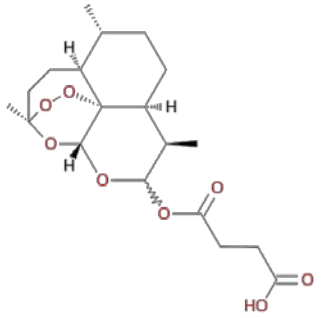
11.1 Description of Study Medication

Study Medication: Suppository Formulation of Artesunate

Drug Substance

Artesunate is a derivative of artemisinin which is the active principle of the Chinese medicinal herb *Artemisia annua*.

Nomenclature	Artesunate, Artesunic acid
Chemical Name	[3*R*, 5a*S*,6*R*,8a*S*,9*R*,10*S*,12*R*,12a*R*)-3,6,9-trimethyldecahydro-3,12-epoxypyrano[4,3-*j*]-1,2-benzodioxepin-10-ol, hydrogen succinate;
CAS Reg. No.	88495-63-0



General Properties	
Molecular Formula	$C_{19}H_{28}O_8$
Molecular Weight	384.4 g/mol
Physical Description	A fine, white crystalline powder
Solubility	Very slightly in water; very soluble in dichloromethane; freely soluble in ethanol (~ 750 g/l) and acetone
Melting Point	132-135°C
Optical Rotation	R; [α] D 20 °C = +2.5° to +3.5° in a 10mg/mL in dichloromethane
pH	3.5-4.5 in an aqueous suspension containing 10 mg/g

Suppository Formulation

Artesunate suppositories will be produced under controlled environmental conditions as prescribed in USP Chapter 795. Artesunate will be added and dissolved into a molten fatty acid base. The mixture will then be transferred into disposable pre-formed suppository molds to cool into solid suppositories.

Clinical trial supplies will be produced by Buderer Drug Co. in Perrysburg, Ohio. Buderer maintains compliance with USP Chapter 795 (Pharmaceutical Compounding – Nonsterile Preparations) and USP Chapter 797 (Pharmaceutical Compounding – Sterile Preparations) which establish practice standards and outline the responsibility of the compounder, selection and appropriate sources of ingredients, quality control, and considerations regarding the stability of compounded preparations. Two additional USP informational chapters are in effect, including, USP Chapter 1075 – Good Compounding

Practices, and USP Chapter 1160 – Pharmaceutical Calculations in Prescription Compounding.

Packaging and Labeling

Suppositories for dispensing will be packaged in standard prescription use plastic or cardboard containers. Proper labeling will be applied per state regulations.

Product Storage and Stability

The suppositories may be stored at controlled room temperature. Suppositories for dispensing will be packaged in plastic or cardboard container, and kept in their plastic mold until administration.

USP 795 designates a beyond use date for this preparation not longer than 6 months. Potency testing will be obtained to validate the preparation process.

11.2 Dosage and Administration

At each dosing visit, after a negative pregnancy test is confirmed, the study physician or nurse will provide five daily vaginal suppositories. During that visit, the subjects will be trained in the vaginal insertion of Artesunate suppositories followed by a tampon, and instructed to administer all daily doses at home at bedtime.

The first 3 study subjects (healthy volunteers with CIN2/3) will receive five (5) daily suppositories that contain 50 mg Artesunate. The 50 mg dose (or ≤ 1mg/kg for subjects weighing 50kg at minimum) is considerably lower than that used when the anal suppository is intended to deliver a systemic dose of drug for malaria – the 50 mg suppository is recommended by WHO for pediatric care. If no local irritation or systemic toxicities observed in the first 3 patients at 50mg, the dosage will escalate to 5 daily doses of 200 mg per day for the remainder of study enrollments (see treatment cohort table).

11.3 Accountability Procedures for the Study Investigational Drugs

Clinical investigators will issue a prescription for each enrolled patient per USP 1168 and State and Federal Law. Buderer Drug Co. will schedule, prepare, package, label and ship clinical trial materials to participating facilities.

The study drug will be kept in the institutional Investigational Drug Pharmacy. Upon receipt of drug orders, and documentation of negative pregnancy test, the pharmacist will dispense enough suppositories for a full cycle (5 days).

11.4 Assessment of Subject Compliance with Self Administration of Artesunate Suppositories

Eligible patients will have clinic visits scheduled at weeks 0, 2, and 4 for a urine pregnancy test and dosing per their treatment cohort. Once a negative pregnancy test is obtained, the patient will receive 5 doses of Artesunate for the respective treatment cycle. They will receive instructions on how to apply the vaginal suppositories a tampon for medication barrier at bedtime. Patients will be instructed to keep the tampon for the remainder of the night. Before the patient leaves the clinic, the study team will confirm contact information where they can be reached the following week. Patients will document any adverse events, and will undergo a cervix check in clinic when they return for the following Artesunate dosing visit.

Toxicities and Adverse events

Table 5: Solicited General Signs and Symptoms

Adverse Event	Short Name	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Allergic reaction/ hyper-sensitivity (including drug fever)	Allergic reaction	Transient flushing or rash; drug fever <38°C (<100.4°F)	Rash; flushing; urticaria; dyspnea; drug fever ≥38°C (≥100.4°F)	Symptomatic bronchospasm, with or without urticaria; parenteral mediation(s) indicated; allergy-related edema/angioedema; hypotension	Anaphylaxis	Death
Fever (in the absence of neutropenia, where neutropenia is defined as ANC <1.0 x 10^9/L)	Fever	38.0-39.0°C (100.4-102.2°F)	>39.0-40.0°C (102.3-104.0°F)	>40.0°C (104.0°F) ≤24 hours	>40.0°C (104.0°F) >24 hours	Death
Cytokine release syndrome/acute infusion reaction	Cytokine release syndrome	Mild reaction; infusion interruption not indicated; intervention not indicated	Requires therapy or infusion interruption but responds promptly to symptomatic treatment (e.g., antihistamines, NSAIDS, narcotics, IV fluids); prophylactic medications indicated for ≤24hrs	Prolonged (i.e., not rapidly responsive to symptomatic medication and/or brief interruption of infusion); recurrence of symptoms following initial improvement; hospitalization indicated for other clinical sequelae (e.g. renal impairment, pulmonary infiltrates)	Life-threatening; pressor or ventilatory support indicated	Death

Nervous system disorder	Head aches	Mild pain	Moderate pain, limiting instumental ADL	Severe pain, limiting self care, ADL	_	-
Nervous system disorder	Dizziness	Mild unsteadiness or sensation of movement	Moderate unsteadiness or sensation of movement, limiting instrumental ADL	Severe unsteadiness or sensation of movement; limiting self care ADL	_	-
Ear and labyrinth disorder	Tinnitus	Mild symptom sintervention not indicated	Moderate symptoms, limiting onstrumental ADL	Severe symptoms, limiting self care ADL	-	-

Patients will be monitored throughout the study for both known potential risks and for unexpected risks. During office visits, patients will be monitored specifically for any signs of:

- Symptoms of itching, burning, pain, warmth, or tenesmus
- Signs of skin breakdown including erythema, blistering, desquamation, ulceration or bleeding
- Neurologic symptoms
- Unexpected adverse events

Subjects will be given diary cards to record local and general signs and symptoms most likely to occur between office visits. The diary cards will be collected, signs and symptoms verified, and inserted into the CRF at each subsequent visit.

Subjects who become pregnant during study will be required to discontinue the use of suppositories. Subjects removed from the study will be replaced in the treatment group. Although not considered an adverse event, pregnancy will be reported in the same way. Whenever possible, a pregnancy should be followed to term, and the status of mother and child reported. The Pregnancy Notification form will be sent by sites to the study PI (Dr. Trimble) and to the sponsor (Frantz Viral Therapeutics) within 48 hours of discovery. Whenever possible, the Follow-up Pregnancy Outcome Form will be filled after delivery.

11.5 Management of Toxicities

All local reactions will be considered causally related to administration of the suppositories. Causality of all other adverse events will be assessed individually. We will follow up subjects with non-serious adverse events until symptoms end. Those with

related serious adverse events will be followed until resolved, until no further improvement is expected, until a non-study antitumor therapy is initiated, or for 30 days after last study treatment, whichever comes first (with the exception of neuropathy, which must be followed until resolution to ≤ grade 1 or until stable for 60 days).

Outcome will be assessed as
1=Recovered
2=Recovered with sequelae
3=Ongoing at subject study conclusion
4=Died
5=Unknown

Any serious study events that are unexpected and related to the study drug or procedure occurring during the period starting from day of administration of the first dose of Artesunate suppository to each subject and ending at the last study visit for that subject (i.e., scheduled LEEP or cone excisional biopsy procedure), will be reported by the principal investigator.

Subjects who become pregnant during study will be required to discontinue drug. Subjects removed from the study prior to week 15 will be replaced in the dose cohort. Although not considered an adverse event, pregnancy will be reported in the same way. Whenever possible, a pregnancy should be followed to term, and the status of mother and child reported. The Pregnancy Notification form will be sent by sites to the study sponsor (Dr. Trimble) within 48 hours of discovery. Whenever possible, the Follow-up Pregnancy Outcome Form will be filled after delivery.

Significant local inflammation will be treated symptomatically with cold packs and oral analgesics. Local infection or ulceration will be treated with routine wound care and antibiotics if necessary.

Subjects will be given diary cards to record local and general signs and symptoms most likely to occur at home. The diary cards will be collected, signs and symptoms verified, and inserted into the CRF at each subsequent visit. Subjects will be monitored at regular intervals to ensure that all adverse events are recognized, documented, and treated. Patients will be educated to contact medical staff should they feel that an adverse event has occurred at a time when they are not scheduled for follow-up monitoring and will have access to medical staff to follow up on any such concerns.

All unexpected and related SAEs will be reported immediately to Dr. Trimble, who will remove any at-risk patient from the study. Any subject who shows evidence of a drug-related SAE (grade III or IV) will be removed from the study and the SAE will be reported to the FDA and the JHMIRB within 7 working days of finding out.

Risks to privacy of individuals, confidentiality of data: There is a potential risk of loss of confidentiality. To ensure that all information and specimens collection remain

confidential and anonymous, all study data will be de-identified. Only the research team will have access to study participant private data. All study specimens are de-identified and cannot be linked to the subjects. Specimens will be barcoded before storage so that patient identifiers will not be available to the laboratories performing the assays proposed in this application.

To ensure that all study data remains confidential, all study data will be maintained on a password-protected database. Patient identifying information will not be associated with the study research data. Only the research investigators will be able to access a list connecting patient identifiers with the study ID, and this can only be provided upon special request from the study database managers.

The rate at which invasive cancer develops from CIN is slow and can take years and perhaps decades [49]. The fifteen-week treatment study window precedes the standard LEEP or cone excisional biopsy procedure given to the targeted study population, and therefore will not extend the normal time clinicians allow between disease detection and therapy and as such, participation in this study will not affect optimal patient treatment.

11.6 Definition of a Serious Adverse Event

A serious adverse event or reaction is any untoward medical occurrence that at any dose:

- results in death
- is life-threatening (an event in which the subject is at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more serious)
- requires inpatient hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability or incapacity
- is a birth defect/congenital anomaly

11.7 Recording and Follow-up of Adverse Events

The severity of each adverse event must be assessed using the NCI Common Terminology Criteria for Adverse Events, v4.0 (CTCAE 4.0).

All adverse events that occur from the time of study drug administration through 30 days after the final treatment will be recorded. Duration, severity, treatments administered and outcome for each adverse event will be recorded on the CRF for the following visit. Drug related adverse events will be followed until resolved, until no further improvement is expected, until a non-study antitumor therapy is initiated, or for 30 days after last study treatment, whichever comes first (with the exception of neuropathy, which must be followed until resolution to ≤ grade 1 or until stable for 60 days).

Adverse events beginning 30 days after the last treatment that the investigator considers to be related to study treatment will be reported to the appropriate contact person at any time such events occur.
Conditions that were present at the study start and that worsen during the study should be reported as beginning on the date the event worsened, not the date it began pre-study. Event text may include the word "worsened" or "exacerbated."
Conditions which were recorded as "intermittent" at the study start which occur during the study must be reported if they are more frequent or of greater severity.

AE Reporting
Expected AEs will be reported to the institutional IRB and reviewed at the time of the annual review. Any SAEs or multiple occurrences of an AE determined to be an unanticipated problem will be reported to Johns Hopkins Institutional Review Board, the Johns Hopkins Cancer Center Clinical Research Committee, and the Johns Hopkins Institutional Biosafety Committee. SAE's will be reported to all regulatory agencies within 7 working days. Unanticipated or unexpected AE is defined as any AE not listed in available sources including the labeling, the Investigator's Brochure, or this protocol.

11.8 Definition of Dose-Limiting Toxicity

A dose-limiting toxicity is defined as any grade 3 toxicity in any organ system, or grade 2 or higher allergic reaction/hypersensitivity reaction (allergy/immunology), as delineated in CTCv4.0.

11.9 Early stopping rules

Because this is a pilot study evaluating tolerability of a new intra-vaginal treatment, the stopping rule for safety is the presence of Grade 3 toxicity in any organ system, or grade 2 or higher allergic/hypersensitivity reaction as delineated in CTCAEv4.0. In the event of two identical unexpected related treatment-related Grade 3 toxicities, accrual will be suspended pending further review.

Subjects who become pregnant during study will be required to discontinue drug. Although not considered an adverse event, pregnancy will be reported in the same way. Whenever possible, a pregnancy should be followed to term, and the status of mother and child reported. The Pregnancy Notification form will be sent by sites to the study sponsor (Dr. Trimble) within 48 hours of discovery. Whenever possible, the Follow-up Pregnancy Outcome Form will be filled after delivery.

Finally, if there is evidence of disease progression at the interval visit regardless whether the progression may or may not be associated with Artesunate, patients will undergo resection at that point.

12.0 STATISTICAL CONSIDERATIONS

12.1 Study Design/Endpoints/Accrual

The primary statistical outcome of the trial is safety. The treatment groups will be filled sequentially in a non-blinded manner. Study dropouts will be replaced.

The dose escalation algorithm is described as follows (see Table 4 for dose levels to be tested):

1. A run-in cohort of three patients will be treated with one 5-day cycle of 50mg vaginal suppositories. If no patient has a DLT observed by the cervix check visit after the last suppository, the trial will proceed to the next level.

2. A cohort of 9 patients will be enrolled and treated with one 5-day cycle of 200mg vaginal suppositories. If no patient has a DLT observed by the cervix check after the last suppository, the dose level will be considered tolerable and the trial will proceed to the next level.

3. If exactly one of the 9 patients has a DLT by Week 1-2 after the last suppository, three more patients will be enrolled and treated at the current dose level. If none of the three patients has a DLT by the cervix check after the last suppository, the current dose level will be considered safe and dose will be escalated.

4. If two or more of the 9 or 12 patients have a DLT observed by Week 1-2 cervix check, dose escalation will be stopped. The current dose level will be considered unsafe.

5. A cohort of 9 patients will be enrolled and treated with 2 cycles of 200 mg vaginal suppositories, at 2-week intervals. If no patient has a DLT observed in Cohorts II and III, then the dose level will be considered safe, and the dose will be escalated.

6. A cohort of 9 patients will be enrolled and treated with 3 cycles of 200 mg vaginal suppositories, at 2-week intervals. If no patient has a DLT observed by the cervix check after the last suppository, then the dose level will be considered safe.

   With the dose escalation algorithm, there will be less than 9% probability to escalate to the next higher dose level if the DLT rate for the current dose level is 30%.

The maximum sample size for dose escalation is 39. Based on our experience in investigator-initiated therapeutic trials in this patient population at this institution, our anticipated accrual rate is 20-25 patients per year.

At the end of dose escalation phase, one dose will be selected for the expansion phase from dose levels II-IV that are considered safe. In general, the lowest dose level with significant within subject decreases in viral load (from baseline at Week 15) and lesion clearance to CIN1 or less will be selected (see Section 12.2 for details). If none of the safe doses achieve significance for decrease in viral load and lesion clearance, a decision will be made based on the totality of safety and efficacy profiles of the dose levels considered safe.

A cohort of 12 patients will be enrolled and treated at the selected dose level at the expansion phase to further evaluate the safety and efficacy of Artesunate.

12.2 Secondary Endpoints

Secondary endpoints include change in histology (CIN2/3 or no CIN2/3) at week 15 and changes in cervical HPV viral load. These endpoints are chosen because in other trials they have been shown to be quantifiable and reproducible. They do reflect the biology of HPV infection and cervical intraepithelial disease, and furthermore have been shown to be modulated in response to intervention in other trials.[67] The histological outcome will be determined by comparing the initial diagnostic biopsy used for protocol entry with the exit cone resection. HPV viral load will be ascertained from longitudinally obtained cervical cell samples, using quantitative PCR.[68] This data will be used to estimate the regression rate within the fifteen-week window in the exploratory analysis.

Secondary endpoints will also include change in lesion size by colposcopy, and characterization of peripheral and local tissue response to the suppository. These measures will be correlated with clinical response, and with preclinical experimental data in the exploratory analysis. In every case possible, changes in lesion size by colposcopy will be ascertained by analyzing serially obtained digital colposcopic images. Measures of immune response will be ascertained on serially obtained peripheral blood specimens, and on flash frozen tissue from the therapeutic resection specimens.

To test that HPV viral load is different during and after treatment as compared to before treatment, paired t-tests and signed rank tests will be used. Log transformation (or other appropriate transform) will be performed to symmetrize the distributions and achieve normality. For each of these variables, a paired t-test will be performed. Due to the small sample size and potential non-normality of the data, the non-parametric signed rank test will also be used. The paired t-test tests whether or not the mean HPV viral loads at baseline and follow-up are

equivalent, whereas the Wilcoxon matched-pairs signed-ranks test tests whether the distributions of HPV viral loads is the same at baseline and follow-up.

12.3 Other Efficacy Endpoints

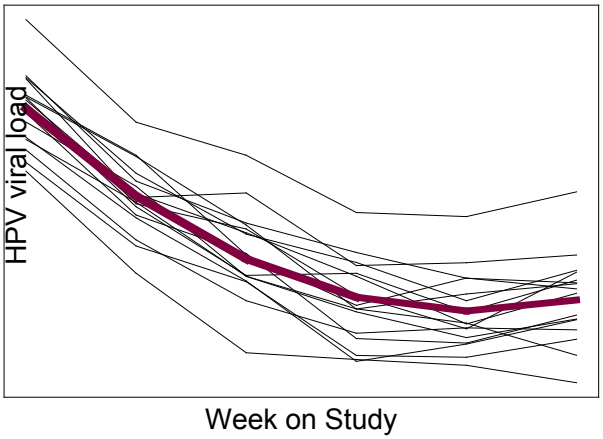
In every case possible, change in lesion size from week 0 to week 15 will be measured using serial digital colpographs. Changes in HPV16 E7-specific T-cell response (CD8) will be compared at week 0 to week 15. Differences will be calculated between baseline and follow-up and the appropriate transform performed. Point estimates, confidence intervals, and hypothesis testing (i.e. paired t-test and signed rank test) will be done.

12.4 Additional exploratory analysis

In addition to hypothesis testing, we will characterize the change in HPV viral load over the course of the trial using longitudinal data modeling. For each individual, we will have data on HPV viral load at weeks -2 (Screening visit), 9-10, and 15. A random effects model will be used to model the distribution of HPV viral load over time:

$$hpv_{it} = \beta_{0i} + \beta_1 t + \beta_2 t^2 + \varepsilon_{it}$$

where hpv_{it} is individual i's HPV viral load at week t. Correlations between observations within an individual are accounted for by the random intercept (i.e. random effect) term. A quadratic model is used to account for non-linearity in the change of HPV viral load. From the fitted model, we can estimate the effect of the vaccine treatment on viral load. The plot below is based on simulated data and shows the type of result we hypothesize: a downward trend in HPV viral load that flattens as time increases. Based on the random effects model shown above, we can estimate the average response (denoted by the thick line) and better understand the association between the vaccine and response and specifically when changes in viral load tend to occur.



Lesion size will also be described using the random effects linear longitudinal model described above to look for time trends.

The secondary efficacy endpoints will be compared with analogous data obtained in a currently open cohort study, "Evaluation of Tissue and Peripheral Blood Immune Response in Women with HPV16+ Cervical Intraepithelial Lesions" (Johns Hopkins IRB# NA_00037578). This observational cohort protocol has been open since 1999, and currently includes over 250 subjects. These subjects have the same eligibility criteria as the criteria for this trial, and are followed prospectively for the same timeframe, before undergoing standard therapeutic resection at week 15. To date, no CIN2/3 lesions have progressed to invasive disease in this timeframe, which is well within the standard of care. We will compare changes in HPV viral load and lesion size from baseline at Week 15, rates of histologic regression, and change in viral load over the 15 week window, in both cohorts.

We will correlate immune response with clinical response by looking at the association with disease regression. A logistic regression analysis will be performed with regression as the outcome and baseline and change between baseline and follow-up HPV viral load as predictors, where HPV viral load and the change have been transformed for normality. Using these two predictors, we can assess how change in viral load is associated with clinical response, adjusting for baseline level. Other predictors of interest will also be evaluated, including measures of E7-specific immune response. To explore the association between continuous outcomes of interest and time, the random effects model described above will be used.

The frequency and severity of all toxicities will be tabulated from CRFs and summarized for review in a descriptive fashion. The initial review of toxicity will be completed after the first treatment group has completed the intervention portion of the study (through week 5).

13.0 SUBJECT COMPLETION/DROPOUT

13.1 Definition of a Dropout

A dropout is defined as any subject who did not come back for at least week 15 visit foreseen in the protocol. A subject who returns for the concluding visit foreseen in the protocol is considered to have completed the study participation. Dropouts will be replaced to reach the target enrollment.

13.2 Procedures for Handling Dropouts

The investigator will make a written attempt to contact those subjects who do not return for scheduled visits or follow-up. Information gathered should be described on the Study Conclusion page of the CRF.

13.3 Reasons for Dropout

It should be specified on the Study Conclusion page of the case report form which of the following possible reasons were responsible for dropout of the subject from the study:

- serious adverse event
- non-serious adverse event
- Protocol violation (specify)
- Consent withdrawal, not due to an adverse event
- Migration from the study area
- Lost to follow-up
- Other (specify)

14.0 CLINICAL MONITORING

This trial will be monitored by the Sidney Kimmel Cancer Center data managers. Data is also reviewed on a bi-weekly basis within the Division of Immunology Clinical Trials Working Group meetings. The principal investigator, study nurse, and data manager participate in these weekly meetings. The JHU and GBMC teams will have weekly conference calls to discuss study progress, and coordinate specimen collection and study activities. The PI and Co-investigator will meet at least monthly. Johns Hopkins Comprehensive Cancer Center (SKCCC) monitoring will occur on a regular basis with the frequency dependent on the subject accrual and the rate of study's progress. This is a DSMP Level II study under the SKCCC Data Safety Monitoring Plan (12/6/12). Data Monitoring of this protocol will occur on a regular basis with the frequency dependent on the rate of subject accrual and the progress of the study. The protocol will be monitored internally at SKCCC by the Principal Investigator and externally by the SKCCC CRO in accordance with SKCCC guidelines. Trial monitoring and reporting will be done through the Safety Monitoring Committee (SMC) at SKCCC. (See Appendix A)

15.0 ETHICS AND REGULATORY CONSIDERATIONS

15.1 Institutional Review Board

This protocol and an eIRB application and any other required documents will be submitted to the Johns Hopkins IRB and CRO, and their written unconditional approval will be in the possession of the investigator before commencement of the study protocol. No deviations from, or changes to, the protocol will be initiated without prior written IRB approval. The IRB/CRO will be informed of all protocol violations, informed consent changes, or revisions of other documents originally submitted for review. The IRB/CRO will also be informed of serious adverse events occurring during the study, all subsequent protocol modifications, or new information that may affect adversely the safety of the subjects or the conduct of the study. Annual updates will be submitted.

15.2 Informed Consent

Information should be given in both oral and written form whenever possible. Subjects will be given ample opportunity to ask about details of the study. Subjects will be

informed about the expected risks and benefits, and procedures of the study protocol. They will be informed of alternative procedures. The informed consent form will be approved by the IRB/CRO. Written consent will be obtained before screening for study enrollment.

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