Repeat Dose Rabbit Vaginal Tolerance/Toxicity Study of Diindolylmethane Cream for Topical Treatment of Cervical Intraepithelial Neoplasia

Thomas L. Horn[1], Miguel Muzzio[1], Carol Detrisac[2], Michael A. Zeligs[3], Daniel L. Boring[4], and David L. McCormick[1]

[1]IIT Research Institute, Chicago, IL; [2]Pathology Associates, Chicago, IL; [3]BioResponse Nutrients LLC, Boulder, CA; [4]National Cancer Institute, Bethesda, MD

Abstract
Final ID #2196y

ABSTRACT

Cervical Intraepithelial Neoplasia (CIN) is a premalignant condition characterized by dysplastic changes in the cervix. Because it can be identified in screening programs and may progress to invasive malignancy, CIN presents an attractive target for cancer prevention. Diindolylmethane (DIM), the condensation product of indole-3-carbinol, has chemopreventive activity in several carcinogenesis models and is proposed for study in clinical trials for cervical cancer prevention. The present preclinical study was performed to characterize the local and systemic toxicity of intravaginal administration of DIM (DIM-Vaginal-Cream, BioResponse, LLC, CO [BR-DIM-VC]). Groups of five NZW rabbits received intravaginal instillation of the maximum feasible volume (1.0 ml) of BR-DIM-VC (0% [placebo], 2%, 4%, or 6%) on alternate days for two weeks. Intravaginal administration of BR-DIM-VC induced no mortality, body weight effects, or toxicity identifiable by clinical or physical examinations. The results of clinical chemistry, hematology, and coagulation tests in all groups exposed to BR-DIM-VC were comparable to control; BR-DIM-VC had no effect on vaginal pH. BR-DIM-VC induced dose-related vaginal edema and erythema. At the 2% dose, 4/5 rabbits were normal and 1 rabbit demonstrated very slight edema without erythema. At the 4% dose, 2/5 rabbits were normal and 3/5 rabbits demonstrated very slight edema with barely perceptible erythema. At the 6% dose, 2/5 rabbits demonstrated slight edema with well-defined erythema and 2/5 rabbits demonstrated very slight edema with very slight erythema. No vaginal edema or erythema was seen in any rabbit exposed to placebo cream only. These data demonstrate that repeat-dose intravaginal administration of the maximum feasible volume of BR-DIM-VC on alternate days for two weeks induces no systemic toxicity in rabbits. BR-DIM-VC did induce very slight to slight vaginal irritation in a dose-related fashion.

INTRODUCTION

Cancer Prevention by Indoles

Epidemiologic data suggest that consumption of high levels of cruciferous vegetables such as broccoli, cabbage, cauliflower, and brussels sprouts may reduce the risk of cancer in humans. Cruciferous vegetables contain several classes of chemicals whose biological activities have been linked to efficacy in cancer prevention. Potential cancer preventive agents found in cruciferous vegetables include carotenoids, folate, ascorbate, and glucosinolates.

During food preparation, mastication, and digestion, glucosinolates are catabolized to form biologically active compounds that include isothiocyanates and indoles. Indoles such as indole-3-carbinol (I3C), and its condensation product, 3.3'-diindolylmethane (DIM), are among the most active of these compounds; both demonstrate chemopreventive efficacy in experimental models for human cancer, and DIM has advanced into clinical trials for cancer chemoprevention.

Figure 1. Structure of Indole-3-Carbinol

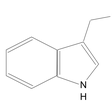
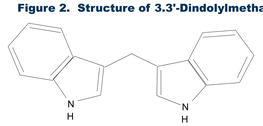


Figure 2. Structure of 3.3'-Dindolylmethane



Most early studies of cancer chemoprevention by I3C and DIM were focused on the activity of these agents as inhibitors of experimental breast cancer. Later studies demonstrated that, in addition to efficacy in breast cancer chemoprevention, I3C and DIM both have a broad range of cancer preventive activity in other sites in animal models. Significant chemopreventive activity for I3C and/or DIM has been reported in studies using *in vivo* models for cancers of the:

- colon
- oral cavity
- liver
- lung
- prostate
- endometrium
- cervix
- skin

Mechanisms of Cancer Chemoprevention by Indole-3 Carbinol and Diindolylmethane

Although their activity as chemopreventive agents varies in different organ sites and models, non-toxic levels of either DIM or I3C can inhibit both the initiation and promotion/progression phases of cancer induction. The activity of these indoles in the inhibition of tumor initiation has been ascribed primarily to modulation of the activity of carcinogen-metabolizing enzymes. For example, I3C can alter the activity of both Phase I enzymes (*e.g.*, cytochromes P4501A1, -1A2, -2B1, -2B2, -3A1, and -3A2) that catalyze the metabolic activation of procarcinogens to ultimate carcinogens, and Phase II enzymes (*e.g.*, glutathione S-transferase and quinone reductase) that participate in carcinogen detoxification and excretion.

In addition to their well-studied effects on the activities of Phase I and Phase II enzymes responsible for carcinogen metabolism, I3C, and DIM have a number of biological activities that could underlie efficacy in the inhibition of the promotion/progression phase of cancer development. Activity in tumor promotion and progression may be associated with cytostatic, pro-apoptotic, and/or other effects on transformed or incipient neoplastic cells. Representative activities of DIM through which tumor promotion/progression may be suppressed include:

- anti-inflammatory activity, as reported to underlie efficacy in the inhibition of tumor promotion in mouse skin and tumor induction in mouse colon
- inhibition of angiogenesis, mediated by inhibition of mTOR action
- inhibition of histone deactylase activity
- inhibition of cell proliferation and/or induction of apoptosis by mechanisms that may include:
  - inhibition of epidermal growth factor receptor activation
  - inactivation of NF-κB
  - modulation of Akt activity

It should be noted that, although I3C and DIM modulate numerous biological pathways through which carcinogenesis may be inhibited, DIM has activity as a topoisomerase II inhibitor. Such activity may be inconsistent with the use DIM for cancer prevention.

Clinical Studies of Cancer Prevention by Diindolylmethane

On the basis of the demonstrated chemopreventive activity of DIM and I3C in animal models for human cancer, and in consideration of the far greater stability of DIM versus I3C, DIM has been identified as a high priority candidate for evaluation in human cancer chemoprevention trials.

Pilot clinical trials of DIM have been performed in patients with cervical dysplasia or prostate cancer; the goals of these studies were to evaluate the safety of DIM in clinical populations and provide preliminary assessments of its activity. In all studies, oral administration of DIM was well-tolerated. However, oral administration of DIM was found to be ineffective in reversing preneoplastic lesions and/or modulating other markers of anticancer efficacy. Interestingly, clinically significant improvements were reported in cervical intraepithelial neoplasia (CIN) lesions in patients treated with the BioResponse formulation of DIM that was studied in this program. However, significant improvements were also seen in patients receiving placebo, so no significant evidence of DIM efficacy was found.

A potentially critical limitation to the design of these clinical trials with DIM was that all were performed using the oral route of exposure. With any agent, metabolic, tissue distribution, and/or pharmacokinetic factors associated with oral administration may limit the concentration of the agent at the target tissue. This challenge appears to particularly important with DIM as a result of its very poor oral bioavailability. As a result, local application, where logistically possible, may provide a better approach for the delivery of DIM to the target tissue.

RATIONALE

This study was performed to characterize the potential local and systemic toxicity that may result from subchronic intravaginal application of an absorption-enhanced formulation of DIM (BR-DIM-VC). This study provides a data set that will contribute to the satisfaction of regulatory requirements for the initiation of clinical trials for intravaginal delivery of the test article, and will support the design and development of clinical studies in which DIM will be administered by the intravaginal route for the treatment of cervical intraepithelial neoplasia (CIN).

STUDY GOALS

The goals of this study were to:

- evaluate the potential irritancy of intravaginal application of DIM in the vagina.
- evaluate the effects of intravaginal application of DIM on vaginal pH and microflora.
- evaluate the effects of intravaginal application of DIM on systemic toxicity, as determined by a regular schedule of clinical observations, physical examinations, body weight measurements, food consumption measurements, and effects on hematology, clinical chemistry, and coagulation endpoints.
- evaluate the effects of intravaginal application on the gross and microscopic pathology of selected reproductive tissues.
- characterize the plasma levels of DIM following intravaginal application.

MATERIALS & METHODS

Animal Welfare: Prior to the initiation of experimentation, the study protocol was reviewed and approved by the IIT Research Institute Animal Care and Use Committee. All work was performed in full compliance with NIH Guidelines for the Care and Use of Laboratory Animals.

Study Design: Five female rabbits/group each received seven intravaginal doses of placebo cream or the appropriate concentration of BR-DIM-VC on Study Days 1, 3, 5, 7, 9, 11, and 13. The study was terminated on Study Day 14 with the conduct of a complete gross necropsy, with primary focus on the vagina and uterus.

Table 1. Study Design
Vaginal Tolerance/Toxicity Study of BR-DIM-VC in Rabbits

Group	BR-DIM-VC Concentration	Number of Rabbits (Female only)	Dosing Days
1	0% (placebo cream)	5	1, 3, 5, 7, 9, 11, 13
2	2% DIM	5	1, 3, 5, 7, 9, 11, 13
3	4% DIM	5	1, 3, 5, 7, 9, 11, 13
4	6% DIM	5	1, 3, 5, 7, 9, 11, 13

Experimental Animals: Female New Zealand White (NZW) rabbits used in the study were obtained from Harlan, Indianapolis, IN. Rabbits weighed approximately 3 kg at the time of receipt, and were held in quarantine for approximately 3 weeks prior to the initiation of dosing.

Animal Husbandry:

Housing: Singly, in suspended stainless steel cages over cage boards.

Basal Diet: Certified Rabbit Chow #2031C (Harlan/Teklad, Madison, WI).

Drinking Water: City of Chicago, supplied by automatic watering system.

Animal Room Light Cycle: 12 hours light/12 hours dark per day.

Test Article: Placebo cream (containing 0% DIM) and formulations of BioResponse Vaginal Cream (BR-DIM-VC) containing 2% DIM, 4% DIM, and 6% DIM were supplied by BioResponse, Boulder, CO. Vaginal creams were used as received from BioResponse.

Test Article Storage and Administration: Placebo cream and BR-DIM-VC were stored at controlled room temperature and were protected from light prior to use. Vaginal creams were administered at a total volume of 1.0 ml per dose; in a preliminary study, this dosing volume was determined to be the maximum feasible deliverable volume.

Evaluations and Observations:

Mortality/Moribundity Checks: Twice daily

Cage-side Clinical Observations: Twice daily during the dosing period

Body Weights: Once during quarantine, and on Study Days 1, 8, 13 (final day of dosing), and 14 (prior to the terminal necropsy)

Food Consumption: Twice weekly during the dosing period

Vaginal Irritation (Draize scoring): Prior to dosing (each dosing day)

Vaginal pH: Study Days 7 (prior to dosing) and 14 (prior to the terminal necropsy)

Vaginal Microflora: Study Day 7 (prior to dosing) and 14 (prior to the terminal necropsy)

Clinical Pathology (Hematology, Clinical Chemistry, Coagulation): Terminal necropsy

Plasma Drug Level Analysis: Plasma samples were collected on Study Days 1 and 13 during pre-dose and at 2, 8, and 24 hours post-dose. Plasma samples were analyzed for DIM using an LC-MS-MS method validated at IITRI. The quantitation limit was 5 ng/mL.

Gross Pathology: Complete necropsy with tissue collection, all animals

Microscopic Pathology: Two regions of the vagina (lower and upper), the uterine horns with cervix, and ovaries from all animals were evaluated histopathologically. Microscopic evaluations included scoring for leukocyte infiltration and epithelial ulceration and disruption.

Statistical Evaluations: Continuous data were compared via analysis of variance, with *post-hoc* comparisons made using Dunnett's test. Incidence comparisons were compared using X^2 analysis. A minimum significant level of $p < 0.05$ was used in all comparisons.

RESULTS

Mortality: No mortality was seen in any dose group during the study.

Clinical Observations: No gross clinical evidence of DIM toxicity was identified in any animal.

Body Weights and Food Consumption: Administration of seven doses of DIM by intravaginal instillation had no effects on mean body weight and mean food consumed in any dose group.

Vaginal Irritation Scoring:

In animals with positive findings, vaginal irritation was generally observed within one to two days after the first dose of BR-DIM-VC. All evidence of vaginal irritation resolved during the study period; vaginal irritation scores in all animals returned to 0 (normal) by Study Day 13.

- **Placebo Control Group:** All rabbits receiving placebo cream had vaginal irritation scores of 0 (normal; no erythema or edema) at all times during the study (Table 2).
- **2% DIM Group:** Four of five rabbits receiving 2% DIM demonstrated vaginal irritation scores of 0 (normal) at all times during the study. One rabbit demonstrated an edema score of 1 (very slight/barely perceptible edema) without erythema.
- **4% DIM Group:** Two of five rabbits receiving 4% DIM demonstrated vaginal irritation scores of 0 (normal) throughout the study. Three of five rabbits in this group had erythema and edema scores of 1 (very slight [barely perceptible] edema and erythema).
- **6% DIM Group:** One rabbit receiving 6% DIM demonstrated vaginal irritation scores of 0 (normal) throughout the study. Two animals in this group had erythema and edema scores of 1 (very slight [barely perceptible] edema and erythema). Two rabbits in this group demonstrated edema and erythema scores of 2 (slight edema, well-defined erythema).

Table 2. Vaginal Irritation Scores (Draize Scoring)

DIM Dose (%)	Edema Scores					Erythema Scores				
	0	1	2	3	4	0	1	2	3	4
0 (Placebo)	5/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5
2%	4/5	1/5	0/5	05	0/5	5/5	0/5	0/5	0/5	0/5
4%	2/5	3/5	0/5	0/5	0/5	2/5	3/5	0/5	0/5	0/5
6%	1/5	2/5	2/5	0/5	0/5	1/5	2/5	2/5	0/5	0/5

Definitions of Vaginal Irritation Scores:

0 = no edema or erythema (normal)
1 = very slight edema (barely perceptible); very slight erythema (barely perceptible)
2 = slight edema (raised edges); well-defined erythema
3 = moderate edema (raised ~ 1 mm); moderate to severe erythema
4 = severe edema (raised > 1 mm and extending beyond area of exposure); severe erythema (beet redness)

Vaginal pH: Administration of DIM by intravaginal instillation had no effect on mean vaginal pH. The mean pH in all groups on Study Days 7 and 14 ranged from 8.0 to 8.4.

Vaginal Microflora: There was no change in the composition of natural vaginal flora in rabbits treated with DIM at concentrations of 2%, 4%, or 6%. Vaginal bacteria identified in all groups included *Bacillus*, *Staphyloccus*, or *Micrococcus* species; a fourth organism (likely a species of *Pseudomonas*) was also identified.

Clinical Pathology (Hematology, Clinical Chemistry, Coagulation): No statistically significant or toxicologically significant findings were noted for any clinical pathology endpoint evaluation performed on samples collected prior to study termination (Study Day 14).

Plasma Drug Levels: Intravaginal administration of DIM resulted in dose-related increases in plasma drug levels on Study Days 1 and 13 (Figures 3A and 3B).

Figure 3. Plasma DIM Concentration after Intravaginal Administration

Figure 3A. Study Day 1

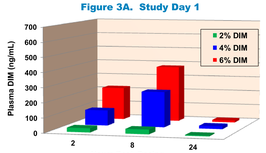
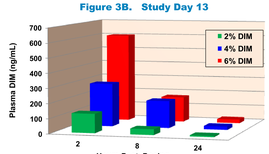


Figure 3B. Study Day 13



- **On Study Day 1,** peak plasma levels of DIM were seen in all groups at 8 hours. Peak plasma DIM levels were 31.9, 241, and 379 ng/mL in groups receiving 2%, 4%, or 6% DIM.
- **On Study Day 13,** measurable levels of DIM (~10 ng/mL) were present in the plasma of mid- (4%) and high- (6%) dose rabbits prior to dosing (~48 hours after the Day 11 dose; data not shown). By contrast, plasma levels of DIM in the low dose (2%) DIM group prior to dosing on Day 13 were below the limit of quantitation (5 ng/mL).
- Peak plasma DIM levels in all groups were higher on Day 13 than on Day 1.
- On Day 13, peak plasma DIM levels were seen at 2 hours post-dosing. Plasma DIM levels at 2 hours on Day 13 (129, 292, and 601 ng/mL in the 2%, 4%, and 6% DIM groups, respectively) were 2.7 to 4.6 X the levels measured at 2 hours on Day 1.
- On Day 13, plasma DIM levels measured at 8 hours post-dosing had declined to ≤ plasma DIM levels measured at 8 hours post-dosing on Day 1.

Gross Pathology: No gross pathology attributable to the test article was seen at the terminal necropsy performed on Study Day 14.

Microscopic Pathology: Epithelial atrophy of the cranial vagina (Figure 4) was seen in all groups receiving intravaginal exposure to DIM. Neither the severity nor the incidence of this lesion was dose-related (Table 3). No other treatment-related microscopic lesions were seen.

Figure 4. Effect of Intravaginal Administration of DIM on Vaginal Epithelial Morphology

Figure 4A. Normal epithelial histology in placebo-treated rabbit (x 40)

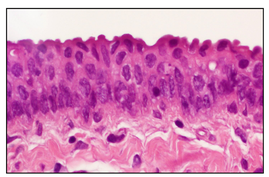


Figure 4B. Epithelial atrophy in rabbit receiving 6% DIM (x 40)

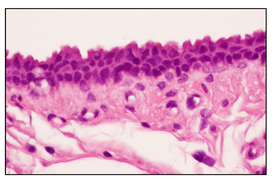


Table 3. Incidence of Epithelial Atrophy of the Cranial Vagina

DIM Dose (%)	Lesion Incidence and Severity				
	Total	Minimal	Mild	Moderate	Severe
0 (Placebo)	0/5	0/5	0/5	0/5	0/5
2%	3/5	0/5	3/5	0/5	0/5
4%	2/5	1/5	1/5	0/5	0/5
6%	2/5	1/5	1/5	0/5	0/5

CONCLUSIONS

1. Repeat-dose intravaginal administration of the maximum feasible volume of BR-DIM-VC containing 2%, 4%, or 6% DIM to rabbits on alternate days for two weeks was well-tolerated. Intravaginal administration of BR-DIM-VC induced no systemic toxicity in any animal: body weights, food consumption, and the results of clinical observations and clinical pathology evaluations in DIM-treated rabbits were comparable to those in placebo-treated controls. Intravaginal administration of BR-DIM-VC had no effect on vaginal pH or vaginal microflora.
2. Repeat-dose intravaginal administration of BR-DIM-VC induced very slight to slight vaginal irritation (Draize scores of 1 and 2) in all groups. In all groups, vaginal irritation was seen within two days after administration of the first dose; the incidence and severity of vaginal irritation were dose-related. In spite of continued dosing, gross evidence of vaginal irritation in all study animals was resolved by Study Day 13.
3. Plasma DIM levels in rabbits receiving repeat-dose intravaginal administration of BR-DIM-VC were dose-related. Peak plasma drug levels were seen at 8 hours after dosing on Day 1 and at 2 hours after dosing on Day 8.
4. The accelerated time to C_{max} and the rapid decrease in plasma DIM levels between 2 and 8 hours after dosing on Study Day 13 both suggest that DIM may induce its own metabolism.
5. Administration of BR-DIM-VC induced minimal to mild epithelial atrophy of the cranial vagina; this effect was not clearly dose-related. The vaginal epithelial atrophy resulting from DIM exposure may be the result of the induction by DIM of apoptosis in the vaginal epithelium.

ACKNOWLEDGEMENTS

This research was supported by contract HHSN261201200025I from the Chemopreventive Agent Development Research Group, Division of Cancer Prevention, National Cancer Institute.