

Results from a Dose–Response Study Using 3,3'-Diindolylmethane in the K14-HPV16 Transgenic Mouse Model: Cervical Histology

Daniel W. Sepkovic¹, Johann Stein¹, Antoine D. Carlisle¹, H. Barbara Ksieski¹, Karen Auburn², Laura Raucci¹, Themba Nyirenda¹, and H. Leon Bradlow¹

Abstract

The human papilloma virus is the major cause of cervical cancer. Viral infection initiates cervical intraepithelial neoplasia, which progresses through several stages to cervical cancer. The objective of this study is to identify the minimum effective dose of diindolylmethane that prevents the progression from cervical dysplasia to carcinoma *in situ*. We document cervical histology in K14-HPV16 mice receiving different doses of diindolylmethane. Urinary diindolylmethane concentrations are reported. Diindolylmethane could enhance the efficacy of human papilloma virus vaccines, creating a new therapeutic use for these vaccines in women already infected with the virus. Five doses (0–2,500 ppm) of diindolylmethane were incorporated into each mouse diet. The reproductive tract was serially sectioned and urine was obtained for analysis of urinary diindolylmethane. The results indicate that 62% of mice receiving 1,000 ppm diindolylmethane remained dysplasia-free after 20 weeks compared with 16% of mice receiving no diindolylmethane and 18% receiving 500 ppm; 1,000 ppm of 3,3'-diindolylmethane in the diet completely suppressed the development of cervical cancer. Urinary diindolylmethane levels increased significantly as diindolylmethane in food increased. These findings imply usefulness for diindolylmethane in the search to prevent cervical cancer when used in combination with prophylactic or therapeutic vaccines. *Cancer Prev Res*; 4(6); 890–6. ©2011 AACR.

Introduction

Although cervical cancer incidence and mortality rates have declined in the United States over the past 3 decades, this disease remains a serious national and international health threat. With new vaccines against the 2 most oncogenic human papilloma virus (HPV16 and HPV18), the further incidence of cervical cancer is likely to radically decrease. Even so, it will take decades for vaccination to take effect on cervical cancer rates (1).

Incidence rates for Hispanic women are higher than those for non-Hispanic women. Even though the mortality rate for African American women has declined more rapidly than the rate for white women, the African American mortality rate continues to be more than double that of whites (2). Cervical cancer strikes approximately half a million women each year worldwide, claiming a quarter of a million lives (3).

The U.S. Food and Drug Administration has approved 2 vaccines that are effective in preventing infection with types 16 and 18, 2 "high-risk" HPVs that cause most (70%) cervical cancers, and types 6 and 11, which cause most (90%) genital warts (4). Gardasil quadrivalent vaccine (Merck) and Cevaxix (GlaxoSmithKline Biologicals) are highly effective in women with no prior exposure. Neither vaccine is effective in women already infected with the virus. There are also issues of patient compliance and duration of vaccine protection that need to be addressed.

Clinical trials have indicated that these vaccines have high efficacy in preventing persistent HPV infection, cervical cancer precursor lesions, vaginal and vulvar cancer precursor lesions, and genital warts caused by HPV types 6, 11, 16, or 18 among women who have not already been infected with the respective HPV type. No evidence exists for protection against disease caused in women infected with the HPV types at the time of vaccination. However, women infected with one or more vaccine HPV types before vaccination would be protected against disease caused by the other vaccine HPV types (5).

Gardasil and Cevaxix are prophylactic or preventive vaccines. Neither provides protection against diseases from vaccine and nonvaccine HPV types to which a woman has previously been exposed. The vaccines do not block cervical epithelial changes and have no effect on cervical intraepithelial neoplasia (CIN) in those individuals already infected with HPV. For this reason, therapeutic HPV vaccines are currently in development (6).

Authors' Affiliations: ¹The David and Alice Jurist Institute for Research, Hackensack University Medical Center, Hackensack, New Jersey and ²The Feinstein Institute for Medical Research, North Shore-Long Island Jewish Health System, Manhasset, New York

Corresponding Author: Daniel W. Sepkovic, The David and Alice Institute for Research, Hackensack University Medical Center, 30 Prospect Ave. Hackensack, New Jersey 07601. Phone: 917-673-0829; Fax: 201-457-1882. E-mail: dsepkovic@humed.com

doi: 10.1158/1940-6207.CAPR-10-0369

©2011 American Association for Cancer Research.

Data from the National Health and Nutrition Examination Survey published on February 28, 2007, have indicated that a total of 26.8% of women ages 14 to 59 tested positive for one or more strains of HPV (7). The most troubling consequence of HPV infection is the potential to cause cervical cancer.

3,3'-Diindolylmethane (DIM) effectively inhibits CIN lesions in women with cervical dysplasia (8–10). This has also been shown in the K14-HPV16 mouse model used in this study (11–13). DIM is an acid condensation product of indole-3-carbinol (I3C). I3C is formed enzymatically from the indole glucobrassicin which is found in cruciferous vegetables and spices. When I3C contacts the acid environment of the stomach, several acid condensation products are formed. DIM comprises at least 70% of these compounds. The conversion is also common in tissue culture studies. In studies that use I3C, the active biological agent is DIM. This has been reviewed previously (12).

DIM alters the phase I metabolism of estradiol and does not increase C-16 hydroxylation. C-16 hydroxylation produces 16 α -hydroxyestrone, an endogenous carcinogen and a promoter of HPV16 and HPV18 proliferation or lytic phase (14–17). DIM, by altering the directional pathway of estrogen metabolism, and through other nonestrogenic effects, suppresses viral oncogene expression. DIM also suppresses pathology in recurrent respiratory papillomatosis, an HPV-caused disease state (14, 15). The change in estrogen metabolism is important because both primary cervical cells and foreskin cells transformed by HPV16 alter estrogen metabolism toward 16-hydroxylation (18).

Tiwari and coworkers (19) have shown that the chemopreventive and antitumor effects of I3C (DIM) depend on the species and tissue type. They studied the mechanism of action of I3C in estrogen-responsive (MCF-7) and estrogen-nonresponsive (MDAMB-231) human breast cancer cell lines. I3C inhibited the growth of estrogen-responsive cell line MCF-7 but had little effect on estrogen-nonresponsive cell line MDA-MB-231. Specific C-2 hydroxylation of estrogen and induction of cytochrome P-4501A1 was enhanced in the MCF-7 but not in the MDA-MB-231 cells. Their conclusion was that the inhibitory effects of I3C may involve selective induction of estradiol metabolism and the related cytochrome P-450 system that may be limited to estrogen-sensitive cells.

DIM directly disrupts various stages in HPV proliferation in both the initiation and progression from cervical dysplasia to cervical cancer. It has been shown that DIM specifically inhibits proliferation of viral transcripts E6 and E7 in CaSki cells (9, 17). DIM can induce a G₁ cell cycle arrest in MCF-7 cells that is accompanied by inhibition of cyclin-dependent kinase expression (20). DIM inhibits cell adhesion, spreading, and invasion associated with the upregulation of *PTEN* (a tumor suppressor gene) and E-cadherin (a regulator of cell-cell adhesion) in T-47-D human breast cancer cells (21). Additionally, DIM prevents *PTEN* loss *in vivo* in the K14-HPV16 transgenic mouse (22). This mouse model is used in our research. DIM also elevates several key cytokines *in vivo*: IFN- γ , granulocyte

colony stimulating factor, interleukin-12 (IL-12), and interleukin-6 (IL-6). IFN- γ is responsible for overall immune response system (23, 24).

We have shown a reduction in the severity of CIN and in some cases the complete reversal of cervical dysplasia in women taking DIM for 4 weeks. This may indicate that HPV16 viral oncogene expression is suppressed after continued DIM administration (8, 10). By utilizing the K14-HPV16 mouse model, we can determine how long DIM administration is necessary for viral oncogene inhibition.

The long-range goal of this research is to test the theory that DIM acts to augment the efficacy of preventive and therapeutic HPV vaccines. An additional benefit may be that vaccine administration after DIM suppression of viral oncogenes will allow virus-like particle antibodies to form and thus prevent reinfection from new HPV exposure. Before the research using DIM with vaccines can begin, the most effective minimum dose of DIM needs to be determined. Here we present some of the results of a DIM dose-response study. Histology of the cervical epithelium from each dose group is presented. Urinary DIM concentrations are assessed for the first time in this model.

Methods

This study employs a well-known transgenic mouse model commonly used in studies involving cervical dysplasia and cervical cancer (11–13, 25, 26). Cervical neoplasia and cancer are linked to persistent infection of "high-risk" HPV viral types, where E6 and E7 oncogenes have enhanced affinity for cellular proteins controlling a collection of functions necessary for neoplastic progression or growth and spread of malignancies. The K14-HPV16 mouse model is transgenic for the entire HPV16 early region under control of the human keratin-14 promoter, and expresses HPV16 E6 and E7 oncogenes in basal squamous epithelial cells. Tumorigenesis in this model is hormone-dependent. Chronic estradiol treatment at 0.1 to 0.25 mg/60 days induces invasive squamous cancers in the vulva, vagina, and outer cervix of K14-HPV16 mice. None of the tumors are metastatic. At low estradiol dose (0.05 mg/60 days), squamous cancers are almost exclusively localized to the transformation zone situated between the upper cervix and lower uterus. Epithelial dysplasia and metaplasia are observed after 4 months of estradiol treatment, leading to high-grade dysplasia and multifocal carcinomas by 6 months (26). We have found that high-grade dysplasia and multifocal carcinomas occur as early as 3 months. The mouse model and preliminary data that we have obtained are more fully described elsewhere (12, 13).

The mice arrived at Hackensack University Medical Center's Department of Biological Resources and were quarantined for as long as deemed necessary by the veterinarian, based on serologic testing and results. Once received from the NCI Mouse Repository (MMHCC, Frederick, MD), the mice were housed as breeder pairs. The cages have sterile microisolator tops. Mice were fed irradiated AIN-93M diet (Purina TestDiets) and given sterile water via sterile water

bottles. The animals were given daily health checks, which included a pain scale form.

During quarantine breeding of the animals began. The hemizygote K14-HPV16 was bred with the Friend leukemia virus B strain (FVB) wild-type to produce the first litter of pups. At weaning the pups were genotyped (13). Those mice exhibiting the E6–E7 exon, (K14-HPV16 positive) were further bred with FVB wild-type to generate progeny for the experimental protocols. All females (K14-HPV16 and FVB wild-type) were trochar implanted with E₂ pellets (0.250 mg/90 day release; Innovative Research of America, Sarasota, FL) under anesthesia. E₂ pellets were replaced at 3-month intervals.

The animals were fed AIN-93M mouse diet until they were divided into respective groups. One group received AIN-93M mouse diet without DIM added and other groups received AIN-93M mouse diet enriched either 500, 1,000, 1,500, 2,000, or 2,500 ppm of DIM (LKT Laboratories).

Histology

Mice were anesthetized and euthanized. Fresh tissue from the reproductive tract and surrounding soft tissue was acquired from each animal. The uterus and cervix were immediately dissected and frozen at -80°C . Each specimen was serially sectioned at a 12 μm thickness by using a Cryostat Leica CM 1850, and 10 to 15 sections were collected at 20- μm intervals for hematoxylin and eosin staining. Examination of serial sections was done with a Leica CME binocular light microscope. The microscope has $\times 4$, $\times 10$, and $\times 40$ objectives. Images were obtained by a Zeiss Axioplan microscope with a Sony color digital camera DXC-S500 attached. The calibration bar represents 0.1 mm and was measured by a Reichert-Jung micrometer of 2 mm divided into units of 0.01 mm.

A histopathologic grading system for this transgenic mouse model regarding cervical squamous carcinogenesis developed by Riley and colleagues (16) was used to classify histologic samples. The grading system is further described by Sepkovic and colleagues (12, 13) and is based on the established criteria for classification of human cervical neoplasia or malignancy accounting for differences between the mouse model and patients.

Determination of DIM in urine

The following protocol was used to extract DIM from urine. Urine (500 μL) was combined with 1 mL of sodium acetate buffer (pH 4.8) and 20 μL of glucuronidase (110,200 units/mL; Sigma). The solution was incubated at 40°C for 24 hours. The internal standard used previously was 4,4-dichlorodiindolylmethane (dichloro-DIM; generously provided by Dr. Stephen Safe). This internal standard is no longer available to us so we modified the procedure by using [2,4,16 α ,16 β -²H₄]estradiol as an internal standard. Recovery data using the new internal standard in urine are presented in Table 1.

The internal standard was added and the sample was extracted with chloroform. Dry pyridine (20 μL) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (80 μL) catalyzed

Table 1. Percent recovery of DIM from urine

	DIM added (ng)	Concentration (ng/mL)	Percent recovery
A1	0	0	*
A2	0	1	*
A3	0	1	*
Mean (SD)		1 (0)	*
B1	54	55	102
B2	54	58	107
B3	54	59	110
Mean (SD)		57 (2)	106 (4)
C1	270	270	100
C2	270	275	102
C3	270	274	102
Mean (SD)		273 (3)	101 (1)
D1	540	508	94
D2	540	509	94
D3	540	512	95
Mean (SD)		510 (2)	94 (0)
E1	1,080	1,069	99
E2	1,080	1,095	101
E3	1,080	1,066	99
Mean (SD)		1,077 (16)	100 (1)

NOTE: Known concentrations of DIM were aliquoted in triplicate to urine in each group. The internal standard was added and each sample was extracted and derivatized. *No DIM added and no DIM and no DIM recovered.

with 1% trimethylchlorosilane (Pierce Chemical) were added, and the sample was heated to 100°C for 1 hour. The sample was injected without further treatment. Gas chromatography–mass spectrometry conditions were the same as those described by Sepkovic and colleagues (10). Creatinine determinations were done colorimetrically by using an ELISA kit provided by Assay Designs.

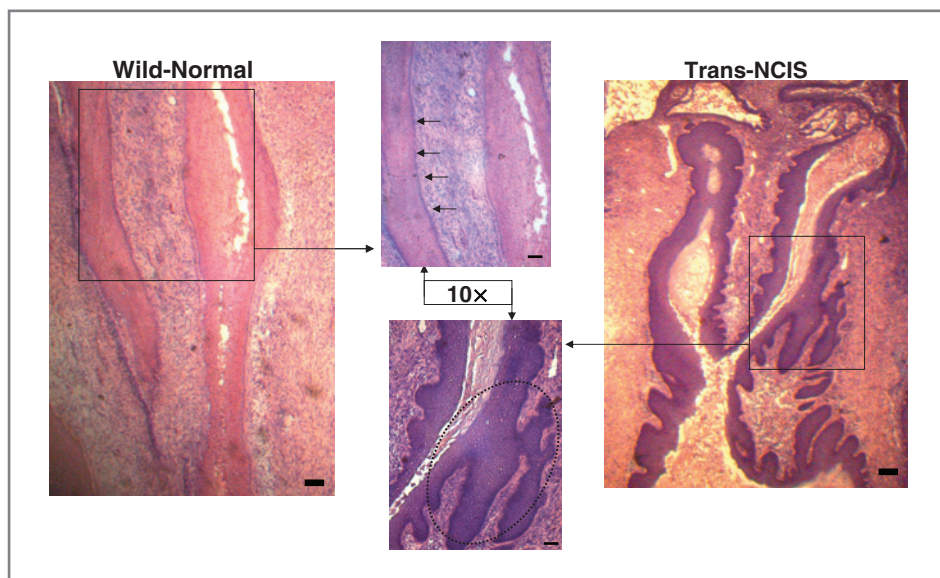
Statistical methods

Treatment of cervical histology data. The outcome of interest is the incidence rate of mice that were CIN-free at each dose level at 20 weeks. Because normal histology (CIN-free rate) at the end of the study period is desirable, we collapsed the ordinal response into CIN-free (normal) and CIN to yield a binary response.

To examine evidence of a dose–response relationship, a Cochran–Armitage test (27, 28) was conducted. Further, the minimum effective dose was determined by using pairwise Fisher exact tests for comparisons of treated mice and 0 DIM dose groups. The realized raw pairwise test *P* values were adjusted for family-wise error rate (FWER) by the step-down Hochberg procedure (28–31) before identifying the smallest DIM dose with an effect on CIN freedom (29).

Treatment of urinary DIM data. DIM concentrations per milligram creatinine were examined for evidence of DIM dose-dependent trend. A test of normality (Shapiro–Wilk)

Figure 1. Histopathologic analysis at no DIM. Comparison of the cervical epithelium in wild-type and transgenic mice receiving 0 ppm DIM in the diet for 20 weeks. The wild-type (Wild; left) shows a normal cervical epithelium. In the transgenic mouse (Trans; right) NCIS is shown. Middle, top, arrows indicate no projections of the epithelium into the stroma, epithelial cells forming a single layer of basal cells; bottom, oval indicates cytological squamous epithelial cell progression and severe irregularity of the epithelial-stromal border. Magnifications: left and right, $\times 5$; middle, $\times 10$.



was conducted on each characteristic to ascertain the underlying distribution of the concentrations. If the data were found to be normally distributed, the mean (SD) was used to summarize the realized measurements, otherwise median (IQR: 25th to 75th percentiles) was utilized. Urinary DIM concentrations reported a significant Shapiro-Wilk test P value of less than 0.05. Thus, nonparametric methods for summarizing the observed values and testing for trend in response along with comparisons with the control group (0 ppm DIM), were sought. Trend was examined by carrying out a Jonckheere-Terpstra test, and by Dunn's tests for pairwise comparisons with the concentration at the control group (0 ppm DIM dose) to adjust for multiple testing by Dunn's method as described in ref. 30. To examine whether each successive incremental DIM dose yielded a statistically different DIM concentration per mg creatinine, a modified Mann-Whitney described in ref. 31 was used. This test compared the concentration at each level to the cumulative DIM concentrations observed in all DIM groups with a lesser DIM dose including the control group. This approach maintained the FWER at 5% for all successive 5 DIM dose level tests. To provide a graphical presentation of the results obtained in the examination of urine DIM concentrations, we used box plots. The box represents the middle 50% of the dataset. The upper boundary (Q3) locates the 75th percentile of the dataset whereas the lower boundary (Q1) indicates the 25th percentile. The line in the box that indicates the median. The vertical lines extending from the box, indicate the minimum and maximum values in the dataset. The diamond indicates the mean. The small circles indicate outliers.

All statistical testing were carried out such that $P < 0.05\%$ indicated statistical significance. All data analyses in this study were carried out by SAS version 9.2 (SAS Institute Inc.; refs. 30, 31).

Results

Food consumption and body weights were measured weekly throughout the experimental period. Over the 20-week study, no significant differences in body weight were observed between wild-type controls and transgenic mice in any DIM dose group. There were also no significant differences when the transgenic dose groups were compared. All groups had dietary food consumption levels that were consistent with animal feeding recommendations for mice on custom purified diet (3–6 g/animal/day; ref. 32). There were no significant differences in food consumption within or between groups.

Histology

Each figure compares wild-type and transgenic mouse cervical epithelia from the same dose group shown side by side. Figure 1 illustrates carcinoma *in situ* (NCIS) frequently found in transgenic mice that receive estradiol pellets without DIM administration (0 ppm DIM transgenic). Commonly, cytologic squamous cell progression and severe irregularity of the epithelial-stromal border are observed. In Figure 2, the cervical epithelium of a transgenic mouse receiving 1,000 ppm in the diet is normal.

Table 2 summarizes the histologic comparison of the cervical transformation zone in each dose group. Results from the wild-type mice that were assigned to the same dosing pattern as the transgenic mice are also presented. The endpoint in this analysis is the cervical histology examined at 20 weeks and the outcome of interest is the incidence rate of mice that were CIN-free at each dose level.

To analyze the dose response, we first established whether there was a trend effect of the DIM dose on the binary response CIN-free versus CIN. On the basis of the 128 transgenic mice that were randomly assigned to the

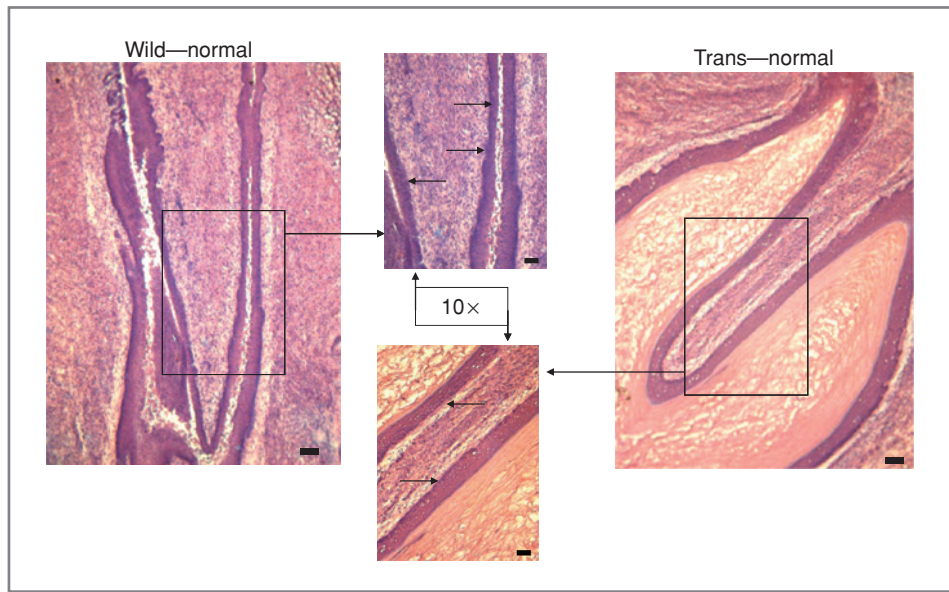


Figure 2. Histopathologic analysis at 1,000 ppm DIM. Comparison of the cervical epithelium in wild-type and transgenic mice receiving 1,000 ppm DIM in the diet for 20 weeks. The wild-type (Wild; left) shows a normal cervical epithelium. In the transgenic mouse (Trans; right) normal cervical epithelium is shown. Middle, arrows indicate no projections of the epithelium into the stroma, epithelial cells forming a single layer of basal cells. Magnifications: left and right, $\times 5$; middle, $\times 10$.

control and 5 doses, this test indicated that there is significant positive linear trend ($P < 0.0001$) such that as the DIM dose increased the proportion that were CIN-free at 20 weeks also increased. With regard to the wild-type mice the dose effect was not significant ($P = 0.5327$).

In transgenic mice, pairwise comparisons between the 5 dose levels and the 0 ppm DIM dose were done to determine which doses were significantly from the control. There was no difference between the 0 ppm DIM group

and the 500 ppm DIM group. At 1,000 ppm, there was a significant increase in normal mice ($P = 0.0123$). This difference is reflected in the higher DIM dose groups.

The results show that DIM dose of 500 ppm was not significantly different from the 0 ppm DIM ($P = 1.00$) with respect to the rate of CIN-free animals. Our analysis indicated that 1,000 ppm of DIM was the smallest dose that reported a response significantly different from the control ($P = 0.0123$), yielding the minimum effective dose for the study.

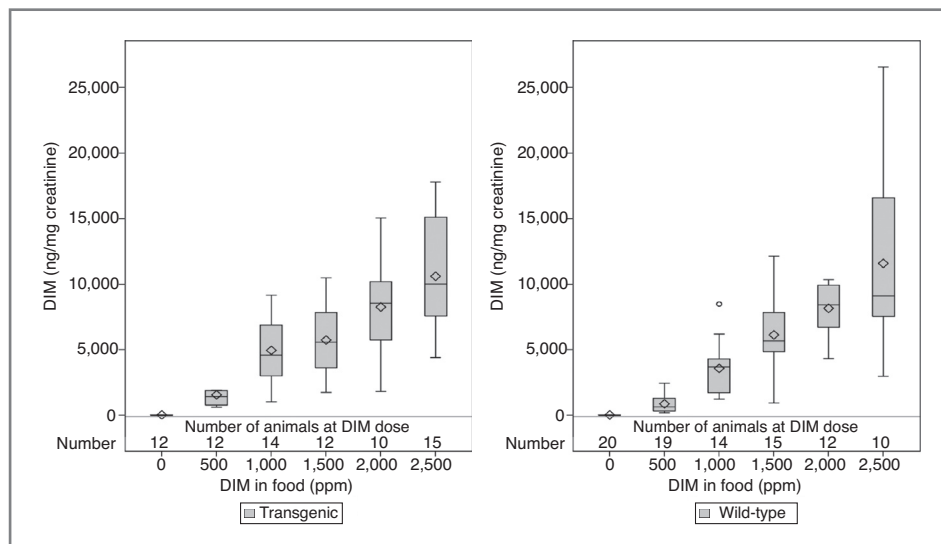
Table 2. Histopathology of wild-type and transgenic mice receiving different doses of dim for 20 weeks

Cervical epithelium	DIM					
	0 ppm	500 ppm	1,000 ppm	1,500 ppm	2,000 ppm	2,500 ppm
Transgenic						
CIN-free	3	4	13	14	13	14
CIN1	1	3	2	0	0	1
CIN2	5	9	4	5	9	5
CIN3	6	5	2	3	1	1
NCIS	2	0	0	0	0	0
SCC	2	1	0	0	0	0
Total	19	22	21	22	23	21
CIN-free/total, %	16	18	62	64	57	67
Wild-type						
CIN-free	26	27	21	18	19	20
CIN1	5	5	0	5	2	4
CIN2	3	0	0	2	4	2
Total	34	32	21	25	25	26
CIN-free/total, %	76	84	100	72	76	77

NOTE: Histopathologic response dichotomized as CIN-free or not. For the binary outcome, CIN freedom, Cochran–Armitage test for trend reported significant DIM dose effect in transgenic mice ($P < 0.0001$) and not significant in wild-type ($P = 0.5327$). Cochran–Armitage exact test with Hochberg procedure for multiple testing identified 1,000 ppm as the minimum effective dose at $P < 0.05$.

Downloaded from <http://aacrjournals.org/cancerpreventionresearch/article-pdf/4/6/890/2249913/890.pdf> by guest on 19 July 2024

Figure 3. Concentrations of DIM in transgenic and wild-type mice from each dose group. Box plots of DIM Levels at each DIM dose in transgenic and wild-type mice. Jonckheere-Terpstra trend test result: both type reported significant trends ($P < 0.0001$). Modified Mann-Whitney test was used to significant effect of each DIM dose cumulatively. Both transgenic and wild-type reported significant dose effect ($P < 0.05$) at consecutive DIM-treated groups.



Notable was the fact that 21% of mice in the 0 ppm DIM group and 5% on the 500 ppm DIM groups had either NCIS or squamous cell carcinoma (SCC). No cancers were reported at 1,000 ppm DIM or higher.

With regard to the wild-type mice, the dose effect was not significant ($P = 0.5327$), which means that based on the 163 mice, there was no DIM dose effect on the CIN freedom at 20 weeks.

Urine DIM levels

The DIM concentrations normalized by creatinine (ng/mg) were measured and summarized as median values as shown in Figure 3. There was a significant trend in this parameter ($P < 0.0001$). Urine DIM concentrations increased significantly in a linear fashion with each DIM dose group in both transgenic and wild-type mice ($P < 0.05$).

Discussion

In this study, we determine the minimum effective dose of DIM that prevents cervical dysplasia by using a transgenic mouse model. At 1,000 ppm (DIM in food) significant numbers of mice with normal cervical epithelia were obtained after 20 weeks. NCIS and SCC were completely eliminated in the 1,000 ppm DIM group compared with 21% of cancers in the 0 ppm group and 5% in the 500 ppm DIM group. This is a clear indication that DIM suppressed the oncogenic potential of the viral transgenes.

The goal of this research is to find the minimum effective dose of DIM that gives a normal cervical epithelium. At 1,000 ppm DIM continuously given in the diet, several observations were apparent. Histology revealed a high percentage of mice with normal cervical epithelia. At this dose, the complete elimination of NCIS and SCC were observed. For these reasons, 1,000 ppm was established as the minimum effective dose.

These findings imply usefulness for DIM in the quest to prevent cervical cancer. Vaccination by a preventive

vaccine in combination with DIM in women after HPV infection could prevent reactivation of oncogene expression. Additionally, DIM administration could increase the effectiveness of therapeutic vaccines that currently are approximately 50% effective.

There is a positive association between new sexual partners and reinfection and that true new infections with the same types harbored earlier in life are possible in older women (33). DIM, with subsequent vaccination, could prevent reinfection in this age group and prevent reacquisition of HPV types in women who have been exposed to before and subsequently cleared.

Urinary DIM concentrations in the 1,000 ppm DIM group compare favorably with DIM measured in women taking either 200 mg or 400 mg of I3C (DIM) twice daily for 4 weeks (10). At 200 mg of DIM, urinary DIM peak concentrations were approximately 12 $\mu\text{g}/\text{mg}$ creatinine. Women receiving 400 mg had urinary DIM values of approximately 16 $\mu\text{g}/\text{mg}$ creatinine daily. In the mouse model, at 1,000 ppm DIM, urinary values were somewhat lower. Therefore, on the basis of the results of this dose ranging study, 1,000 ppm DIM in the mouse model is the minimum effective dose and a viable dose for future human studies.

Disclosure of Potential Conflicts of Interest

There are no potential conflicts of interest.

Grant Support

This study was supported by the NIH Grant 5R01CA136847.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 10, 2010; revised March 2, 2011; accepted March 2, 2011; published OnlineFirst March 7, 2011.

References

- Sundström K, Eloranta S, Sparén P, Arnheim Dahlström L, Gunnell A, Lindgren A, et al. Prospective study of human papillomavirus (HPV) types, HPV persistence, and risk of squamous cell carcinoma of the cervix. *Cancer Epidemiol Biomarkers Prev* 2010;19:2469–78.
- U.S. Department of Health and Human Services—National Cancer Institute. *Cancer incidence – Surveillance, Epidemiology, and End Results (SEER) registries research data*. Bethesda, MD: National Cancer Institute, Cancer Statistics Branch; 2010.
- Division of STD Prevention. *Prevention of genital HPV infection and sequelae: report of an external consultants' meeting*. Atlanta, GA: Centers for Disease Control and Prevention; 1999.
- Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, et al. A controlled trial of a human papillomavirus type 16 vaccine. *N Engl J Med* 2002;347:1645–51.
- Villa L, Costa R, Petta C, Andrade R, Ault K, Giuliano A, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. *Lancet Oncol* 2005;6:271–8.
- O'Shaughnessy JA, Kelloff GJ, Gordon GB, Dannenberg AJ, Hong WK, Fabian CJ, et al. Treatment and prevention of intraepithelial neoplasia: an important target for accelerated new agent development. *Clin Cancer Res* 2002;8:314–46.
- Dunne EF, Unger ER, Sternberg M, McQuillan G, Swan DC, Patel SS, et al. Prevalence of HPV infection among females in the United States. *JAMA* 2007;297:813–9.
- Bell MC, Crowley-Norwick P, Bradlow HL, Sepkovic DW, Baugh S, Howell P, et al. Placebo controlled trial of indole-3-carbinol in the treatment of cervical dysplasia. *Gynecol Oncol* 2000;78:123–9.
- Yuan F, Chen D, Liu K, Sepkovic DW, Bradlow HL, Auburn K. Anti-estrogen effect of indole-3-carbinol in cervical cancer cells: implication for prevention of cervical cancer. *Anticancer Res* 1999;19:1673–80.
- Sepkovic DW, Bradlow HL, Bell M. Quantitative determination of 3,3'-diindolylmethane in the urine of individuals receiving indole-3-carbinol. *Nutr Cancer* 2002;41:57–63.
- Jin L, Qi M, Chen DZ, Anderson A, Yang GU, Arbeit JM, Auburn KJ. Indole-3-carbinol prevents cervical cancer in human papilloma virus type 16 transgenic mice. *Cancer Res* 1999;59:3991–7.
- Sepkovic DW, Stein J, Carlisle AD, Auburn K, Nyirenda T, Bradlow HL. Early findings from a dose response study using 3, 3'-diindolylmethane in the K14-HPV16 transgenic mouse model: cervical histology. *The 2010 Proceedings of ASCO-NCI-EORTC Annual Meeting on Molecular Markers in Cancer*. (ISBN 978-1-932312-80-5). Alexandria, VA: American Society of Clinical Oncology; 2010. p. 98.
- Sepkovic DW, Stein J, Carlisle AD, Ksieski HB, Auburn K, Bradlow HL. Diindolylmethane inhibits cervical dysplasia, alters estrogen metabolism, and enhances immune response in the K14-HPV16 transgenic mouse model. *Cancer Epidemiol Biomarkers Prev* 2009;18:2957–64.
- Auborn K, Abramson A, Bradlow HL, Sepkovic DW, Mullolly V. Estrogen metabolism and respiratory papillomatosis: a pilot study on dietary prevention. *Anticancer Res* 1998;18:4569–74.
- Coll DA, Rosen CA, Auburn K, Potsic WP, Bradlow HL. Treatment of recurrent respiratory papillomatosis with indole-3-carbinol. *Am J Otolaryngol* 1997;18:283–5.
- Riley RR, Duensing S, Brake T, Münger K, Lambert PF, Arbeit JM. Dissection of human papillomavirus E6 and E7 function in transgenic mouse models of cervical carcinogenesis. *Cancer Res* 2003;15:63:4862–71.
- Carter TH, Liu K, Ralph W Jr, Chen D, Qi M, Fan S, et al. Diindolylmethane alters gene expression in human keratinocytes in vitro. *J Nutr* 2002;132:3314–24.
- Auborn KJ, Woodworth C, DiPaolo JA, Bradlow HL. The interaction between HPV infection and estrogen metabolism in cervical carcinogenesis. *Int J Cancer* 1991;2:49:867–9.
- Tiwari RK, Guo L, Bradlow HL, Telang NT, Osborne MP. Selective responsiveness of human breast cancer cells to indole-3-carbinol, a chemopreventive agent. *J Natl Cancer Inst* 1994;86:126–31.
- Cram EJ, Liu BD, Bjeldanes LF, Firestone GL. Indole-3-carbinol inhibits CDK6 expression in human MCF-7 breast cancer cells by disrupting Sp1 transcription factor interactions with a composite element in the CDK6 gene promoter. *J Biol Chem* 2001;276:22332–40.
- Meng Q, Goldberg ID, Rosen EM, Fan S. Inhibitory effects of indole-3-carbinol on invasion and migration in human breast cancer cells. *Breast Cancer Res Treat* 2000;63:147–52.
- Mei Q, Anderson AE, Chen DZ, Shishinn S, Auburn KJ. Indole-3-carbinol prevents PTEN loss in cervical cancer in vivo. *Mol Med* 2005;11:59–63.
- Riby JE, Xue L, Chatterji U, Bjeldanes EL, Firestone GL, Bjeldanes LF. Activation and potentiation of interferon-(gamma) signaling by 3,3'-diindolylmethane in MCF-7 breast cancer cells. *Mol Pharmacol* 2006;69:430–9.
- Xue L, Pestka J, Maoxiang L, Firestone GL, Bjeldanes LF. 3,3'-Diindolylmethane stimulates murine immune function in vitro and in vivo. *J Nutr Biochem* 2008;19:336–44.
- Arbeit JM, Munger K, Howley PM, Hanahan D. Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. *J Virol* 1994;68:4358–68.
- Arbeit JM, Howley PM, Hanahan D. Chronic estrogen-induced cervical and vaginal squamous carcinogenesis in human papillomavirus type 16 transgenic mice. *Proc Natl Acad Sci U S A* 1996;93:2930–5.
- Armitage P. Test for linear trends in proportions and frequencies. *Biometrics* 1955;11:375–85.
- Cochran WG. Some methods for strengthening the common χ^2 tests. *Biometrics* 1954;10:417–51.
- Dmitrienko A, Tamhane AC, Bretz F. *Multiple testing problems in pharmaceutical statistics*. Boca Raton, FL: CRC Press; 2010.
- Dmitrienko A, Chuang-Stein C, D'Agostino R. *Pharmaceutical statistics using SAS: a practical guide*. Cary, NC: SAS Institute Inc.; 2007.
- Chen YI. Nonparametric identification of minimum effective dose. *Biometrics* 1999;55:1236–40.
- Institute of Laboratory Animal Resources. Commission on Life Sciences. National Research Council. *Guide for the care and use of laboratory animals*. Washington, DC: National Academy Press; 1996.
- Munoz N, Mendez F, Posso H, Molano M, Van Den Brule AJ, Ronderos M, et al. Incidence duration and determinants of cervical human papillomavirus infection in a cohort of Columbian women with normal cytological results. *J Infect Dis* 2004;190:2077–87.