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Inhibition of histone methyltransferase EZH2 suppresses endometriotic vesicle development in a rat model of endometriosis

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Abstract

Endometriosis is a painful gynecological disease with no cure and limited therapeutic options. It has been hypothesized that epigenetic drugs can be used as a non-hormonal treatment for endometriosis. This study was conducted to study the efficacy of an inhibitor of the histone methyltransferase EZH2 using an established rat model of endometriosis. We hypothesized that treatment will block or reduce the number of endometriotic vesicles in this model. We conducted a pre-clinical drug study in female rats with experimental endometriosis (uterine tissue transplanted next to the intestinal mesentery) or control sham (sutures only). Rats with endometriosis or sham surgery received either treatment with EZH2 inhibitor (5mg/kg or 10mg/kg) or vehicle (0.1%, 67% DMSO) every other day during 4 weeks. After treatment completion, the number, area, volume, and weight of vesicles were evaluated. RT^2 Profiler Arrays for Neuropathic and Inflammation, Epithelial to Mesenchymal Transition, Inflammatory Response, and Autoimmunity pathways were used to examine gene expression changes in the vesicles that developed. Treatment with EZH2 inhibitor (10 mg/kg) suppressed the development of vesicles, by significantly decreasing the total vesicle number, area, volume, and weight. In addition, EZH2 inhibition significantly increased the expression of *CACNA1B* and *FKBP1A* genes, involved in pain and proliferation, respectively. EZH2 inhibition suppresses the growth of vesicles without apparent detrimental effects to other organs. Treatment with this epigenetic inhibitor leads to upregulation

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Conflict of interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

of a limited number of genes related to endometriosis-relevant pathways. In conclusion, these data support follow up studies to evaluate its potential as a therapeutic approach for endometriosis.

Keywords

Epigenetics; Endometriosis; EZH2; Histone methyltransferase inhibitor; Animal model

Introduction

Endometriosis is defined as endometrium-like tissue located outside of the uterine cavity, and characterized by painful periods, dyspareunia, infertility, and chronic pelvic pain [1]. The etiopathology of endometriosis has not been fully elucidated, and while it has been attributed to retrograde menses flow, this hypothesis does not explain all the endometriosis clinical manifestations [2,3]. Menstrual endometrium reaching the peritoneum would need to undergo molecular and cellular alterations to be able to survive, attach, grow, and further develop into endometriotic lesions. Alterations previously observed in ectopic endometrium include altered ovarian steroid hormone biosynthesis and receptor responses, increased invasiveness and vascularization, and augmented inflammatory responses [4]. Several groups, including ours, have shown that epigenetics can be one of the underlying mechanisms responsible for molecular alterations resulting in the ectopic growth of endometrial tissue [5–8].

Previous data from our laboratory showed that endometriotic lesions are characterized by hypermethylation of Histone 3 lysine 4 (H3K4), Histone 3 Lysine 9 (H3K9), and Histone 3 Lysine 27 (H3K27) [9]. There is evidence for high positive nuclei immunostaining of trimethylated H3K27 [10] and high expression of Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2), the enzyme responsible for H3K27 methylation, in endometriotic lesions [10–12]. EZH2 is a histone methyltransferase enzyme that has recently emerged as an important regulator of tumorigenesis, epithelial to mesenchymal transition (EMT) and wound healing [13–16]. Overexpression of EZH2 has been observed in cancer (e.g., prostrate, breast, uterine, gastric, renal, and non-small-cell lung) [14,17,18]. At present, several EZH2-specific inhibitors have been developed [19–25] with proven effects decreasing proliferation and invasion rates of cancer cells and increasing the expression of pro-apoptotic associated genes in in vitro and in vivo models. In particular, GSK343, one of the most potent EZH2 methyltransferase inhibitors (HMTi), has been shown to remarkably reduce tumor growth as early as 20 days post-implantation in mice [26]. GSK343, an S-adenosyl-L-methionine competitive EZH2 methyltransferase inhibitor, is highly selective for EZH2 over several other methyltransferases [24]. Importantly, treatment with this inhibitor modulates expression of a limited proportion of the transcriptome, only 1.2% compared to over 20% of global changes by Histone Deacetylase inhibitors (HDACi). Therefore, these data suggest that this HMTi could be a more selective and thus a safer epigenetic-based therapy [27].

We and others have recently shown that treatment with an EZH2 inhibitor reduced nuclear enrichment of H3K27me3, as well as migration and proliferation of endometriotic cells [11,28]. H3K27me3 is a well-known transcriptional regulator that results in silencing of target genes enriched with this histone mark [29]. Our laboratory confirmed enrichment

of H3K27me3 in the promoter regions of candidate genes in some, but not all, human lesions compared to endometrial samples from healthy controls [7]. Specifically, we observed a high proportion of lesions with H3K27me3 enrichment in genes known to be downregulated in lesions, such as the Estrogen receptor 1 (*ESR1)* promoter (69%, 9/13), the Cadherin 1 (*CDH1)* promoter (46%, 6/13) and the Progesterone Receptor (*PGR)* promoter (31%, 4/13) [30–32]. Taken together, these results suggest that H3K27me3 is involved in regulating the expression of endometriosis-relevant pathways such as ovarian hormone responses and epithelial to mesenchymal transition. Based on our previous observations that pharmacological blockage of EZH2 decreased migration and proliferation of endometriotic cells in vitro, the present study was designed to assess efficacy of an HMTi in an in vivo model and to elucidate the molecular mechanisms induced by histone methylation inhibition.

Methods

Animal Model:

The Institutional Animal Care and Use Committee (IACUC) from Ponce Health Sciences University approved all animal model procedures (IACUC protocol #223). Experiments using experimental animals were carried out to a high ethical standard. Female virgin Sprague Dawley rats weighing 160–180 grams were pair-housed at 23°C in a 12-hour light/dark cycle with food and water ad libitum. Animals were handled (5 minutes/day) for 7 days prior to beginning the experiments to reduce manipulation stress, and vaginal cytological smears were carried out daily before and after surgery to verify effects on reproductive cycles [33,34]. Experiments were carried out at the same time of day (9 am to 12 pm) to minimize the influence of circadian rhythms.

Induction of endometriosis:

Endometriosis was induced surgically under isoflurane anesthesia accompanied by a warm-water circulating pad, based on the model by Vernon and Wilson [35]. For endometriosis induction, the distal right uterine horn (2 cm) was removed and immersed in Roswell Park Memorial Institute culture media (RPMI) pre-warmed at 37°C. Four uterine implants were sutured around four mesenteric vessels of the small intestine of the rats to induce endometriosis (Endo). For the sham group (Sham), four silk sutures were attached around four mesenteric vessels and the uterus was massaged for two minutes.

Drug treatment:

Rats were randomly assigned to 1 of 5 groups: Sham-Drug, Sham-Vehicle, Endo-Drug, Endo-Vehicle, and Endo-Control. Endo-Drug (n=7, one rat died before starting treatment) and Sham-Drug (n=8) rats were treated with HMTi (GSK343, MedChem Express, New Jersey, USA) at 5 mg/kg or 10 mg/kg dissolved in 67% Dimethyl sulfoxide (DMSO) by intraperitoneal injections every other day for four weeks starting at 14-day post-surgery [26] (Figure 1). Typically, the injection site was in the animal's lower right quadrant of the abdomen to avoid damage to the urinary bladder, cecum, and other abdominal organs. Since the injections were every other day for multiple days, we varied the side injected between right and left so as not to cause bruising or hematoma in area. Sham-Vehicle (n=8) rats

received 0.1% DMSO (vehicle); Endo-Vehicle rats received 0.1% DMSO (n=8) or 67% DMSO (n=4). Control groups were sham surgery with drug (Sham-drug, n=8) or vehicle (Sham-vehicle, 0.1% DMSO, n=8), and an Endo no treatment group (Endo-Control) (n=8). Vaginal smears were done before, during and after drug treatments to assess effect of the HMTi on estrous cycle. After a four-week treatment period, the animals were euthanized using an overdose of sodium pentobarbital at the stage of diestrus (to avoid differences in lesion size based on physiological estrogen levels).

Evaluation of treatment efficacy:

A laparotomy was performed to examine the peritoneal cavity for the presence of vesicles and original sutures. Classification of vesicles in grades of growth was done in experimental and vehicle-treated rats as previously described [34,36]. Briefly, vesicles were assigned the following grades: 0.01–1.99 mm in length= grade 2; 2.0– 4.49 mm= grade 3; 4.5– 5.99 mm= grade 4; 6.0 mm or larger = grade 5. Grade 1 indicates that a vesicle did not develop. Endometriotic vesicles, colon, adrenals, ovaries, liver and kidneys were collected at the time of sacrifice and preserved in liquid nitrogen or formalin for further analysis.

Colonic Macroscopic Damage:

We have previously shown that endometriosis induced macroscopic and microscopic changes in the colon [37]; thus, we next verified effects of treatment on the distal colon. Evaluation of the colon was examined for macroscopic damage following previously described protocol [38]. In brief, the presence of adhesions (0, 1, or 2 for none, minor, or major, respectively) or diarrhea (0 or 1; absent or present, respectively) was noted, and the thickness of the colon wall was measured in millimeters using a digital caliper. The mucosal surface of the colon was examined for ulceration (0 for no damage, with increasing scores up to 10 depending on the extent of inflammation and ulceration). These were added to give a total damage score with a possible maximum of 14 to 15 points depending on the thickness of the colon.

RNA Extraction and cDNA:

Total RNA was extracted from 3 pooled endometriosis vesicles from 3 rats treated with 5 mg/kg of the HMTi or from 3 rats treated with 0.1% DMSO using the RNeasy Mini kit (Qiagen, Valencia, CA) and following the manufacturer's protocol. Briefly, 30 mg of tissue were homogenized using Bullet Blender® Green with Buffer RLT (Next Advance, Hilden, Germany). RNA quantification and purity assessment were done by using the spectrophotometer NANODrop™ 2000 (Thermo Scientific, Wilmington, USA). After reverse transcription using the kit RT^2 First Strand Kit (Qiagen, Valencia, CA), we conducted qPCR as described next.

RT^2 Profiler PCR Array:

To identify the molecular mechanisms activated by EZH2 inhibition, we evaluated expression level changes in the following gene panels: including Neuropathic and Inflammation, Epithelial to Mesenchymal Transition, Inflammatory Response, and Autoimmunity pathways (RT^2 Profiler PCR Arrrays, Qiagen, Valencia, CA). Briefly,

following the manufacturer's protocol we first synthesized cDNA from 200 ng of RNA isolated from vesicles treated with either drug (5 mg/kg of GSK343 in 67% DMSO) or 0.1% DMSO. cDNA was then amplified using the RT^2 Profiler PCR master mix and following recommended cycling conditions on the Mastercycler Realplex 2S (Eppendorf, California, USA). Results, expressed as threshold cycle (Ct), were exported to an Excel® spreadsheet and analyzed using the Qiagen Web-based PCR Data Analysis software (https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/).

Statistical Analysis:

Graphs were generated using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA) and presented as mean difference ± SEM. A P value <0.05 was considered statistically significant. Data was analyzed using one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. For the RT^2 Profiler PCR Arrays, data analysis and graphical representation were done using the ΔΔCT method using the Qiagen PCR Array Data Analysis Web portal.

Results

Treatment with 5 mg/kg of HMTi did not decrease the size or number of endometriotic vesicles

At the time of the sacrifice, the peritoneal cavity was examined for the presence of endometriotic vesicles and the original sutures. We did not observe any significant differences between the number nor size (volume, weight, and area) of vesicles treated with 5 mg/kg of HMTi compared to vehicle (Figure 2A). There were no differences in the % vesicles that developed in the rats treated with 5 mg/Kg of HMTi (71.9%) compared to 0.1% DMSO (62.5%) or Endo Control (no treatment) (90.6%) (Figure 2B). In addition, treatment with HMTi or vehicle did not affect the weight (Figure 2C) or the estrous cycle of the rats (data not shown).

Treatment with 10mg/kg of HMTi significantly decreased endometriotic vesicle development

By increasing the HMTi concentration to 10 mg/kg (n=7), the volume, area, and weight of the vesicles significantly decreased compared to rats treated with 0.1% DMSO (n=8) and untreated (n=8) (Figure 3A). Moreover, most of the vesicles (67.9%) treated with the HMTi did not develop and showed a grade 1, compared to 21.7% for 0.1% DMSO, 18.7% for 67% DMSO, and 9.4% for untreated rats (Figure 3B). More importantly, EZH2 inhibition did not affect the weight of the rats (Figure 3C) or estrous cycle (Figure 3D). The missing proestrus stage in endo-controls could be explained by the timing of smear collection (proestrus has a longer length of ~14hr in rats) [33].

Treatment with 10 mg/kg of HMTi did not produce any significant colonic macroscopic damage

Macroscopic damage was evaluated in the colons of rats in each group. Anova analysis showed that there are significant differences among groups. As expected, animals with endometriosis without treatment (Endo control) exhibited significantly higher macroscopic

colonic damage scores than sham-operated animals treated with vehicle (Sham-Vehicle) ($P < 0.05$). There were no statistically significant differences between Endo-Drug (10 mg/kg) vs. Endo-Control nor sham-operated animals (Figure 4A). These data suggest that treatment with HMTi did not worsen the damage caused by endometriosis induction. In addition, we measured the colon weights and did not find any statistical significance between groups (Figure 4B).

Treatment with 10 mg/kg of HMTi did not cause any significant changes in peripheral tissue weight

In order to assess effects of the drug in other organs, we measured the weights of the adrenals, ovaries, liver, and kidney and did not find significant differences between groups (data not shown).

HMTi regulation on Neuropathic and Inflammatory, Epithelial to Mesenchymal Transition and Inflammatory Response and Autoimmunity associated genes

For these analyses, we compared pooled endometriotic vesicles (n=3 vesicles per rat) treated with the 5mg/kg of HMTi with vesicles from rats treated with 0.1% DMSO. Since few vesicles developed with the treatment with 10 mg/kg, we were unable to perform these experiments with the higher dose. In total, we evaluated 252 candidate genes, approximately 84 genes for each selected pathway. Analysis of data from 3 arrays each in duplicate included determining if fold expression changes were statistically significant when comparing drug treatment with vehicle. Graphical representation of these results included plotting the 84 candidate genes for each pathway in Volcano Plots showing fold-change expression and statistical significance. The Neuropathic and Inflammatory Pathways (Figure 5A) showed statistically significant up regulation of Calcium Voltage-Gated Channel Subunit Alpha 1 B (*Cacna1b)* in the HMTi treated group. For the Epithelial to Mesenchymal Transition Pathway, (Figure 5B) FK506 Binding Protein 1A (*Fkbp1a)* was statistically increased by the HMTi (Table 1). We did not observe statistically significant changes for the genes represented in the Inflammatory Response and Autoimmunity Pathway array (Figure 5C).

Discussion

Our laboratory has previously shown that H3K27me3, a well-known repressive histone mark, is aberrantly overexpressed in endometriosis as it is in various cancer types [14,23,26,28,39,40]. In this pre-clinical study, the efficacy of an EZH2 inhibitor for the treatment of endometriosis was examined. According to our review of the literature, this is the first pre-clinical study using this HMTi on rats with experimentally induced endometriosis. The results of the current study demonstrate that treatment with 10 mg/kg of HMTi every other day for 28 days significantly decreased the weight, area, and volume of the vesicles in a rat model of endometriosis. Moreover, we report that HMTi treatment did not affect the weight nor estrous cycle of the rats, nor did it cause macroscopic changes to the colon or weight changes in the peripheral tissues (ovaries, adrenal glands, liver, kidney) in the treated rats. Based on these initial positive results, we next aimed to dissect the molecular pathways that

are being regulated by the mechanism of action of this inhibitor, namely the reactivation of gene expression.

Several studies have demonstrated that abnormal levels of H3K27me3 represses the expression of tumor suppressors in cancer, including genes related to cell cycle inhibition, apoptosis, senescence and differentiation. Some of the tumor suppressor genes that are inhibited by H3K27me3 include *CHD1*, Cyclin Dependent Kinase Inhibitor 2a (*p16)*, Cyclin Dependent Kinase Inhibitor 1A (*p21)*, and Phosphatase and Tensin Homolog (*PTEN)* [27,41–43]. Therefore, we proposed that the reversion of gene expression profiles by HMTi will provide positive therapeutic outcomes in endometriosis. As expected, our results show that EZH2 inhibition regulates only 0.8% of the genes studied (2 out of 252), selected based on pathways known to be involved in the pathogenesis of pelvic endometriosis; neuropathic inflammatory signaling, EMT and immune response [44]. At the dose of 5 mg/kg EZH2 inhibition upregulated *Fkbp1a*, a member of the immunophilin protein family, that encodes the 12-kDa FK506-binding protein FKBP12. This protein plays a role in immunoregulation, protein folding and trafficking. FKBP12 is a specific cytoplasmic inhibitor of TGF-beta type I serine/threonine kinase receptor (TGFBR1) signaling [45]. Inhibition of EZH2 also upregulated *Cacna1b* that codes for a presynaptic neuronal voltage-dependent N-type voltage-gated calcium channel Cacna1b. Predominantly expressed in brain and peripheral nervous system, CACNA1B functions to regulate neuropathic pain [46]. This pore-forming subunit plays a critical role in controlling pain signals at many synapses by modulation of intracellular calcium concentration [47]. In fact, there has been an interest in developing drugs that target N-type channel functions for the management of pain [48]. Future studies should include *in vivo* experiments to assess whether HMTi leads to changes in pain perception in the rat model to discern the physiological outcomes of increased Cacna1b levels. At the lower GSK343 dose, EZH2 inhibition did not change the expression of any inflammatory response and autoimmunity associated genes in the array, suggesting that immune system regulation is not involved in the observed treatment effects. Unfortunately, we were unable to conduct these analyses with the higher dose of 10mg/kg because few vesicles developed and there was not enough RNA available for the assay. It could be argued that even if we are able to get enough RNA, vesicles that developed at the 10mg/kg dose likely do not have the same transcriptome as the ones that actually responded to treatment and disappeared. At the minimum, the results with the 5mg/kg provide some light regarding the molecular changes caused by the drug in the vesicles, in particular the magnitude of changes (less than 2% of the genes assayed).

The high selectivity observed in this preclinical study is consistent with previous studies showing that this HMTi only affects 1.2% of the transcriptome [27]. This contrasts with the broader genomic effects associated with another type of epigenetic treatment, histone deacetylase inhibitors (HDACi). HDACi is associated with multitude adverse effects in clinical trials due to its poor selectivity [49]. Reports from clinical trials of EZH2 inhibitors suggest that these drugs will have a safer side effect profile. Recently, a phase 2 clinical trial using Tazemetostat, an EZH2 inhibitor, demonstrated that this drug had a favorable safety and tolerability in adults with Regulator of Chromatin Subfamily B, Member 1 (INI1) negative epitheliod sarcoma [50]. Additional clinical trials, studying Tazemetostat in other diseases have also supported the safety profile of EZH2 inhibitor

[51,52]. Furthermore, investigators are conducting ongoing phase II studies of tazemetostat in follicular lymphoma, diffuse large B-cell lymphoma, mesothelioma, and certain molecularly defined solid tumors, including epithelioid sarcoma and other INI1-negative tumors[2].

In conclusion, we demonstrate here that pharmacological inhibition of EZH2 by HMTi could be a potential novel, non-hormonal and safe approach for treating endometriosis as evidenced by substantially reduced vesicle development and no significant macroscopic effects in peripheral tissues in the rat model. We also report that HMTi treatment does not cause substantial changes in the transcriptome of the vesicles that developed in the treated rats. However, further research is required to fully understand the underlying molecular mechanisms activated by the drug and to assess potential off target effects such as fertility outcomes and other long-term side effects such as malignant transformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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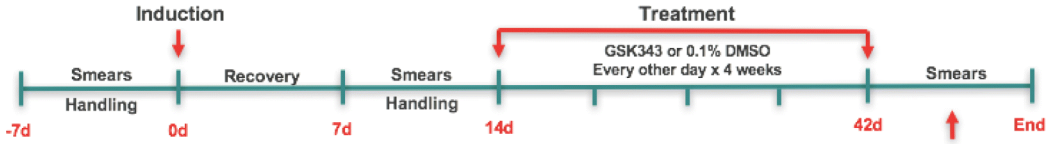

Group	Treatment
Endo Drug	GSK343 5 or 10 mg/kg/day i.p. every other day
Endo Vehicle	0.1% or 67% DMSO i.p. Every other day
Sham Drug	GSK343 5 or 10 mg/kg/day i.p. every other day
Sham Vehicle	0.1% DMSO i.p. Every other day
Endo Control	None

Figure 1. Experimental design.

At day 0, endometriosis induction and sham surgery were carried out. Rats were allowed to recover for 1 week and then the smears and handling continued. At day 14, HMTi and vehicle treatments started for 4 weeks every other day. At the end of the treatment, smears were performed in order to sacrifice at diestrus.

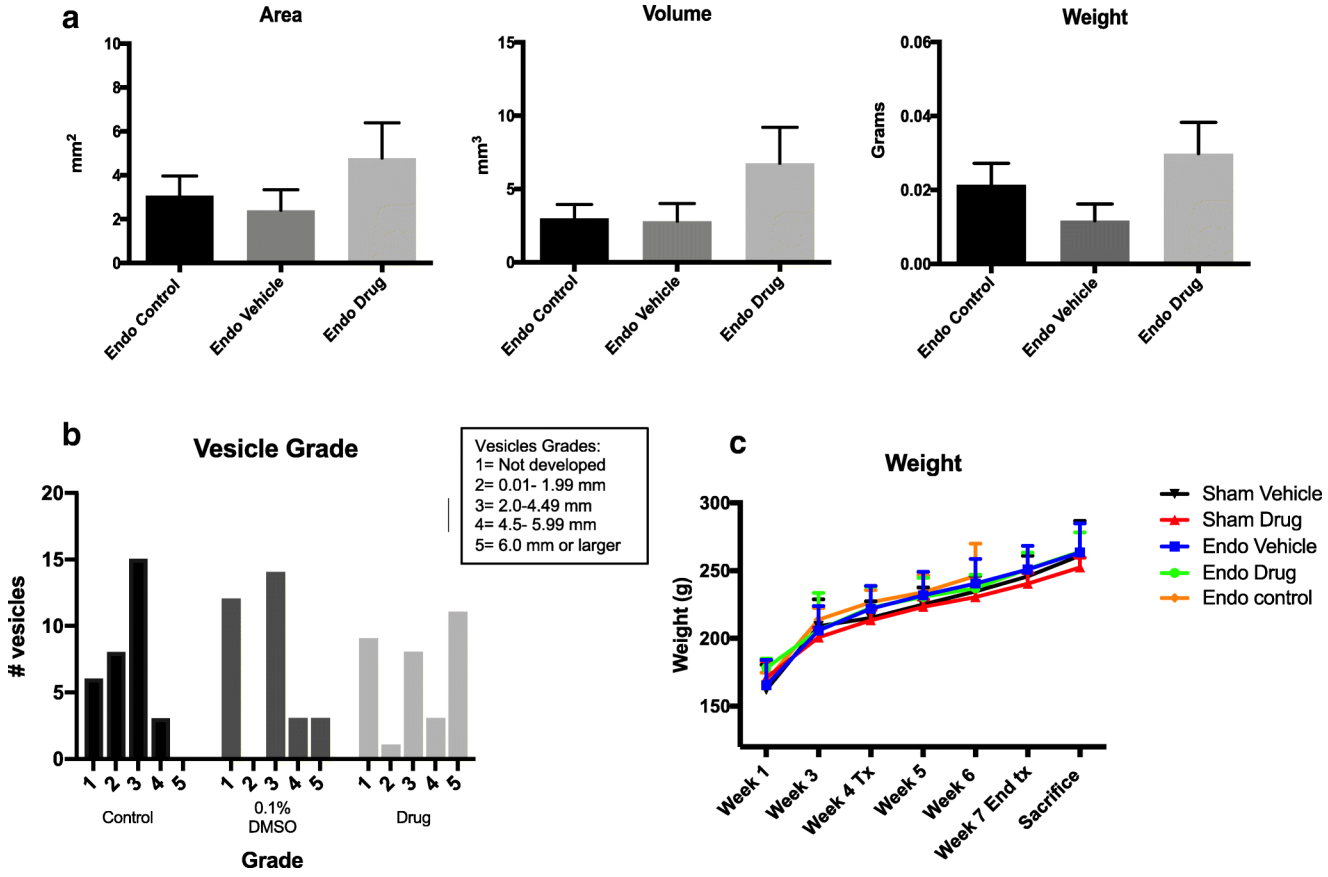

Figure 2. Treatment with 5 mg/kg of HMTi did not decrease the size or number of vesicles.

A. There was no significant difference in area, volume, or weight of the vesicles per group. B. Rat's weight did not change after treatment in any of the groups. (n=2–9 ± standard error of the mean [SEM]). Two vesicles that exceeded two standard deviations from the mean were excluded from the analysis.

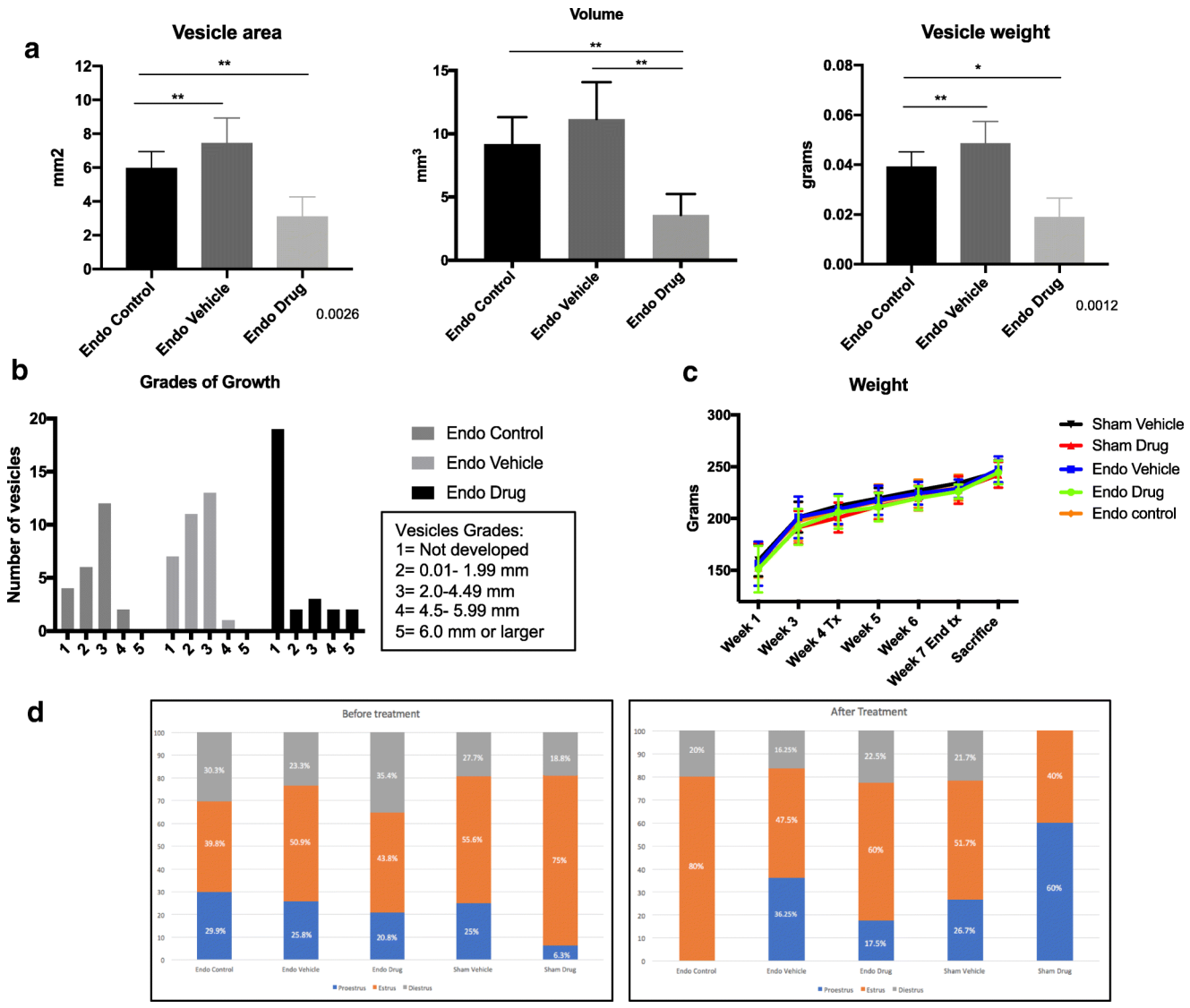

Figure 3. Treatment with 10mg/kg of HMTi significantly decreased the vesicles size and number.

A. The total area, volume and weight of all vesicles treated with HMTi were significantly smaller than the ones treated with vehicle and non-treated (endo control). B. Non-developed and developed vesicles were classified by grade based on a scale by size. C. Rat's weight did not change after treatment in any of the groups. D. Smears were collected before and after HMTi treatment to analyze drug effect on the rat's reproductive cycle. (n=6–8 ± standard error of the mean [SEM]) *P < 0.05; **P < 0.01. Two vesicles that exceeded two standard deviations from the mean were excluded from the analysis.

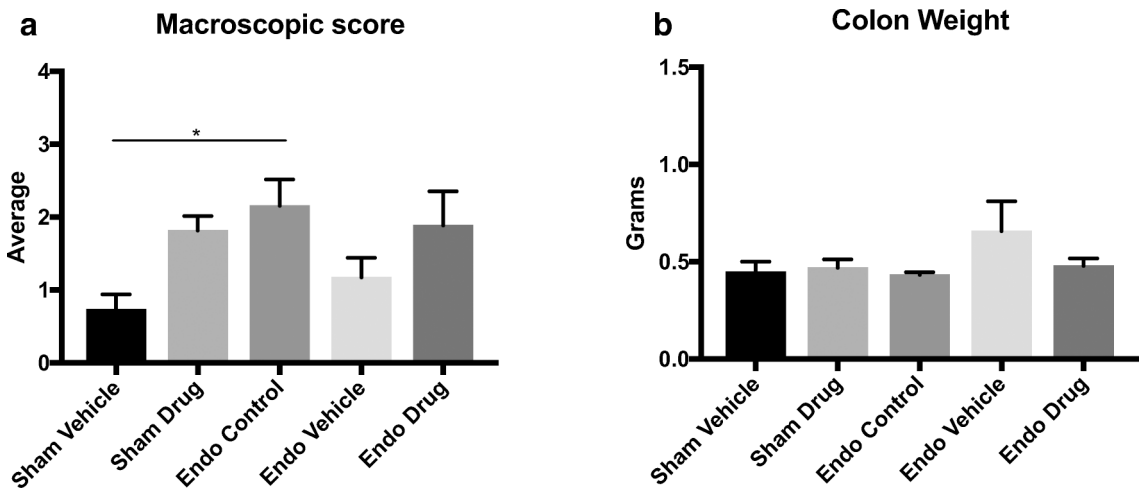

Figure 4. Colonic macroscopic damage score.

A. The macroscopic damage in the colon of the experimental induced rats without treatment (Endo-Control) was significantly increased when compared with sham-operated animals treated with vehicle (Sham-Vehicle). B. Statistically significant differences in colon weights were not found between groups. (n=6–8 ± standard error of the mean [SEM]) *P < 0.05.

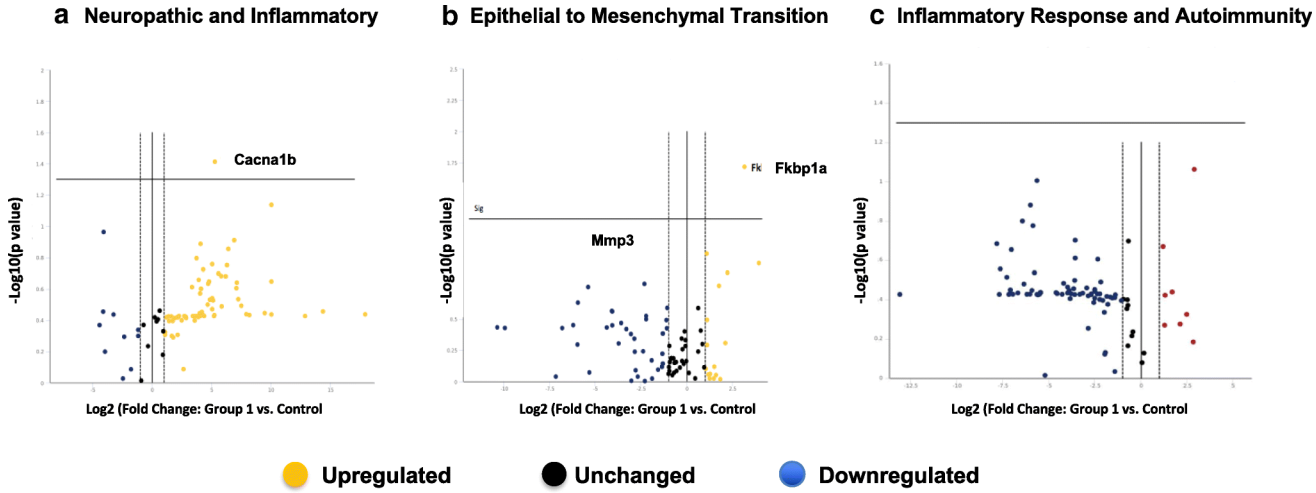

Figure 5. Effects of HMTi in different pathway associated genes.

A. In the Neuropathic and Inflammatory pathway array, we found that treatment with 5 mg/kg of HMTi significantly increased the expression of CACNA1B in vesicles when compared to vehicle. B. In the Epithelial to Mesenchymal Transition pathway array, we found that treatment with 5 mg/kg of HMTi significantly increased the expression of FKBP1a in vesicles when compared to vehicle. C. In the Inflammatory Response and Autoimmunity pathway array, we did not find any significant differences between groups.

Table 1:

Expression analysis of the significant genes by RT^2 Profiler PCR arrays.

Gene Symbol	Gene expression $2^{\wedge-\Delta Ct}$	Fold Change in Gene Expression Level	(P-value)
Cacna1b	0.915945	39.41	*0.038582* *
Fkbp1a	0.026923	9.01	*0.019191* *

* Significance was determined by $p<0.05$.