2017

Effects of Dioscin on DNA Methyltransferase 1 Protein Expression in Human Breast Cancer Cell Lines

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Effects of Dioscin on DNA Methyltransferase 1 Protein Expression in Human Breast Cancer Cell Lines

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of Sally McDonnell Barksdale Honors College

Oxford
May 2017

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ACKNOWLEDGMENTS

I would first like to thank Dr. Asok Dasmahapatra for taking the time to mentor and advise me during the writing of this thesis. I would also like to thank Olivia Dale for her countless hours spent in the laboratory teaching and assisting me during these experiments. I extend my gratitude to those at The National Center for Natural Products Research, especially to the executive director, Dr. Larry Walker, and the associate director, Dr. Ikhlas Khan. Thanks to Dr. Shabana Khan and Dr. Murrell Godfrey for their willingness to read this thesis. I am immensely grateful for Dr. Douglas Sullivan-Gonzalez, Dr. Debra Young, Dr. John Samonds, and the entire faculty at the Sally McDonnell Barksdale Honors College for inspiring me to get the most out of my education during my four years here at Ole Miss.
ABSTRACT

In previous experiments, the steroidal saponin, dioscin, which was extracted from the roots of the wild yam (*Dioscorea villosa*), was observed to have potent anti-proliferative effects on the human breast cancer cell lines MCF-7 and MDA-MB-231. Dioscin was also able to activate the transcription of tumor suppressor genes GATA3 and CDH1 in these cell lines. We hypothesize that the anti-proliferative effect of dioscin on human breast cancer cells could be mediated through the epigenome. Consequently, in order to explore this hypothesis, we have analyzed the expression of DNA methyltransferase 1 (DNMT1) protein in these cells. MCF-7 and MDA-MB-231 cells were treated with differing concentrations of dioscin, as well as a 1μM concentration of the known DNMT1 inhibitor, 5-azacytidine. Protein lysate was collected from these cells and was then subjected to Western blot analysis. Both dioscin and 5-azacytidine decreased protein expression of the DNMT isoform with a molecular weight of 145 kDa. It may be concluded that dioscin has potential DNMT1 inhibiting ability, but whether or not this ability directly contributes to dioscin’s anti-proliferative effects and the activation of the tumor suppressor genes GATA3 and CDH1 remains to be seen.
TABLE OF CONTENTS

List of Figures..................................................................................................................6
Introduction......................................................................................................................7
Materials and Methods.................................................................................................16
Results and Observations..............................................................................................19
Discussion.......................................................................................................................25
List of References..........................................................................................................27
LIST OF FIGURES

Figure 1  Methylation Mechanism..........................................................10
Figure 2  5-azacytosine and Cytosine......................................................12
Figure 3  Structure of Dioscin.................................................................14
Figure 4  Western Blot Picture 1...............................................................20
Figure 5  Western Blot Picture 2...............................................................21
Table 1   Relative Intensity Ratios 1.........................................................22
Table 2   Relative Intensity Ratios 2.........................................................22
Figure 6  Mean Expression of DNMT1 Graph..........................................23
Table 3   Percent Increase/Decrease Table..............................................24
Introduction

Breast cancer has become one of the most frequently diagnosed cancers in the United States today. The American Cancer Society estimates that 1 in 8 women will acquire invasive breast cancer in their lifetime, making it the second most prevalent cancer in women behind skin cancer. Although the mortality rate has dropped over the years, breast cancer is responsible for 40,000 deaths per year in the United States alone (Lu et al. 2009). These deaths are mostly attributed to the reoccurrence of the cancer, which frequently present with metastases (Lu et al. 2009). Metastatic breast cancer today is generally treated with a cocktail of cytotoxic drugs that can immensely reduce the patient’s quality of life and allow the cancer to develop a drug resistance quite quickly. The search for a viable anti-cancer/anti-metastatic agent is still very much alive due to the devastating side effects of most known drugs.

For many years, it was thought that the mechanisms behind breast cancer were genetic alterations. In other words, breast cancer resulted from direct changes in the DNA code, such as deletions, point mutations, and chromosome rearrangements (Aumsuwan et al. 2016). However, it is now known that breast cancer not only arises from genetic alterations, but also from epigenetic modifications (Aumsuwan et al. 2016). Epigenetics is a rapidly growing field in
cancer research, mainly because epigenetic modifications are enzyme-regulated processes; they do not affect the primary DNA sequence and are able to be reversed, making epigenetic drugs an exciting prospect for cancer therapies. The most common types of epigenetic alterations are histone modifications, DNA methylation, and noncoding RNA interface (Aumsuwan et al. 2016). While all of these epigenetic mechanisms have been found to be altered in cancer cells, we know the most about DNA methylation and will focus mainly on this process.

DNA methylation is an epigenetic mechanism where a methyl group (CH$_3$) is added to the DNA, thereby altering the function of a gene. In mammals, DNA methylation occurs mostly on C-5 of cytosine at CpG sites, which are cytosine-guanine (CG) dinucleotide sequences associated with gene promoters (Carey, 2013). When these CpG sites are not extensively methylated, the chromatin structure is loosely packed, the gene promoter region is unhindered, and transcription of the gene proceeds (Neidhart, 2016). In contrast, when CpG sites are highly methylated, chromatin structure becomes compacted, transcription activators are unable to bind to the promoter region, and gene transcription is shut off (Neidhart, 2016). DNA methylation therefore provides an efficient way to regulate gene expression, because the effects are reversible. Cancers are able to abuse this regulation pathway, however, by altering the promoter regions of tumor suppressor genes, proto-oncogenes, and other genes associated with DNA repair pathways (Carey, 2013). When the promoter regions on tumor suppressor genes and DNA repair genes are highly methylated, the genes are inactivated and tumor growth is unable to be held in check. In breast cancer alone, over 100 different tumor suppressor
genes have been shown to be hypermethylated (Hinshelwood et al. 2008). Cancers are able to accomplish this uncontrolled tumorigenesis by hijacking the enzymes responsible for DNA methylation.

It is now documented that breast cancer, along with various other cancers, is able to silence tumor suppressor genes through the abnormal functioning and expression of DNA methyltransferases (DNMTs) (Aumsuwan et al. 2015). These enzymes catalyze the addition of methyl groups to the CpG sites, and function in the maintenance of genomic stability. There are three different DNMTs that possess this enzymatic activity: DNMT1, DNMT3A, and DNMT3B. DNMT1 is described as the “maintenance” methyltransferase and is the most prevalent DNMT in somatic cells (Robertson et al. 2001). The “maintenance” activity refers to the ability of the methyltransferase to recognize hemimethylated DNA and add the corresponding methyl group to the complementary strand (Chen et al. 2011). For this reason, DNMT1 is generally responsible for maintaining DNA methylation patterns after DNA replication has occurred. DNMT3A and DNMT3B comprise the family of what is known as the de novo methyltransferases. These DNMTs add methyl groups to completely unmethylated DNA, and are in charge of setting the patterns of methylation early on in development and in cell differentiation (Chen et al. 2011). The roles of the maintenance and de novo DNMTs do show some overlap, however, as DNMT3A and DNMT3B may be able to methylate certain CpG sites that were skipped over by DNMT1 (Chen et al. 2011). In addition to these three catalytically conserved enzymes, there are two other DNMTs in the human body that need mentioning. DNMT3L does not possess any inherent enzymatic activity, but it is able
to recruit DNMT3A/3B and stimulate their activity (Chen et al. 2011). A separate methyltransferase, DNMT2, has not been shown to be very active in DNA methylation, but instead has been shown to methylate tRNAs and protect them from cleavage (Ghanbarian et al. 2016). Although these two methyltransferases are a part of the DNMT family, they do not directly catalyze the addition of a methyl group to CpG sites in humans.

**Figure 1:** A: The addition of a C-5 methyl group catalyzed by DNMT. B: The mechanisms of maintenance methylation by DNMT1 vs de novo methylation by DNMT3A/3B. (Chen et al. 2011)
Although the exact mechanisms are still unknown, cancers are able to atypically express these DNMTs and disturb normal DNA methylation patterns (Jin et al. 2013). A recent study was able to demonstrate that DNMT1 is overexpressed in mammary tumors and is essential for both mammary stem cell and cancer stem cell maintenance (Pathania et al. 2015). In the experiment, they introduced a mammary-gland specific DNMT1 gene deletion to mice predisposed to breast cancer, and 85% of the mice were protected. Although the breast development was suppressed, the DNMT1 gene deleted mice had a significantly higher survival rate than those who retained the DNMT1 gene in their mammary gland (Pathania et al. 2015). These results indicate that DNMT1 expression in mammary cells could prove to be an effective target in limiting tumorigenesis in the breast.

Different epigenetic drugs have been created over the years in hopes of limiting DNMT activity, but no exceptional cancer treatment has been found. The epigenetic drug 5-azacytidine (AZA) is a known nucleoside DNMT inhibitor that was approved by the US Food and Drug Administration to treat myelodysplastic syndrome (Amatori et al. 2010). AZA is one of the few known DNMT inhibiting drugs and is currently in a number of clinical trials to test its efficacy for the treatment of haematological cancers. Although AZA was first synthesized over 4 decades ago, the mechanisms behind this epi-drug were not fully understood until quite recently. AZA’s effectiveness and anti-cancer ability stems from its core structure, which is notably similar to the structure of the nucleoside base, cytosine.
Once it has been internalized into the cell, AZA is able to incorporate itself into either DNA or RNA by taking the place of a cytosine base (Amatori et al. 2010). Specifically in DNA incorporation, after DNA replication takes place, DNMT1 enzymes proceed with their maintenance methylation, but problems arise as they encounter AZA bases in the DNA. When a DNMT1 binds to an AZA base, the enzyme is unable to add a methyl group because the C-5 of AZA is replaced with a Nitrogen atom (Carey, 201). The DNMT1 becomes covalently stuck to the AZA base and is later degraded, therefore reducing the amount of DNMT1 available (Carey, 201). This binding and degradation of DNMT1 leads to a passive decrease in methylation and the re-expression of tumor suppressor genes and apoptotic pathway genes that had been previously silenced by hypermethylation (Amatori et al. 2010). However, AZA, like many other potential anti-cancer drugs, has a dangerous toxicity profile and a high level of instability under physiological conditions (Amatori et al. 2010). It
must be taken in very low doses to avoid extreme cytotoxicity. If another DNMT inhibiting compound was found to be similarly effective and have limited adverse effects, it could prove to be a novel cancer therapeutic.

Natural products are an encouraging area of research because many natural compounds have exhibited chemopreventive characteristics while maintaining a low toxicity profile. There are numerous herbal remedies that have shown promise in the treatment of cancer, and some molecules derived from natural products have already been reported as having DNMT inhibiting abilities. The marine sponge *Pseudoceratina purpurea* contains several derivatives that exhibit DNMT1 inhibitory abilities, as does a type of red alga by the name of *Peyssonnelia caulifera* (Medina-Franco *et al.* 2010). The National Center for Natural Products Research here at the University of Mississippi has taken a special interest in the roots and rhizomes of the plant *Dioscorea villosa*, also known as the wild yam. Found throughout the Eastern United States, *Dioscorea villosa* is commonly used as a dietary supplement to treat menstrual cramps and menopausal symptoms, but has recently shown promise as an anti-cancer agent (Aumsuwan *et al.* 2015). The roots and rhizomes of *Dioscorea villosa* are rich in steroidal saponins; some of these saponins show promise as anti-cancer agents (Aumsuwan *et al.* 2016). *Dioscorea villosa* root extract was recently found to decrease the cell viability in several breast cancer cell lines, and was identified as a possible demethylating agent (Aumsuwan *et al.* 2015). Further examination into the components of the root extract revealed that the bioactive compound dioscin was a major factor responsible for this epigenetic activity.
Dioscin (DS) is a steroidal saponin that has been reported to possess anti-inflammatory, hepatoprotective, and antiviral effects (Wu et al. 2015; Zhang et al. 2015; Liu et al. 2012). DS has also been shown to possess anti-proliferative and apoptotic abilities in a variety of cancer cells (Aumsuwan et al. 2016). In a previous study (Aumsuwan et al. 2016), DS was found to reduce cell proliferation in two separate breast cancer cell lines: MCF-7 (estrogen receptor positive; noninvasive) and MDA-MB-231 (estrogen receptor negative; invasive). A RT-qPCR was also utilized to determine the effect of DS and AZA (a known DNMT inhibitor) on the mRNA content of two commonly hypermethylated tumor suppressor genes in breast cancer: GATA3 and CDH1. DS and AZA were ineffective in altering GATA3 and CDH1 mRNA expression in the MCF-7 cell line, but both DS and AZA were able to significantly increase GATA3 and CDH1 mRNA content in the MDA-MB-231 cells. These results first tell us that the cellular effects of DS and AZA treatments are cell line specific. In addition, these results indicate that DS may be able to alter the

**Figure 3**: The chemical structure of Dioscin. (Aumsuwan et al. 2016)
methylation status on the GATA3 and CDH1 gene promoters in invasive breast cancer cells. An additional RT-qPCR was run to examine the mRNA content of DNMT1 in MCF-7 and MDA-MB-231 cells treated with DS, and no significant change was found. From these results, it was hypothesized that DS may have anti-cancer potential similar to AZA because it is able to affect gene methylation and re-express tumor suppressor genes that were previously silenced.

The research I conducted is essentially an extension of what was found in the paragraph above. Because DS and AZA had similar effects in the breast cancer cell lines, I wanted to determine if DS is able to work through a similar mechanism as AZA, and if so, if DS could be a novel and safe breast cancer therapeutic. DNMT1 was the apparent starting point because of its critical role in cell maintenance, and because it is known to be overexpressed in mammary tumors. Therefore, the goal of my experiment was to determine if DS is able to inhibit DNMT1 protein expression in breast cancer cells.
Materials and Methods

Plant Material

Dioscin (3β, 25R)-spirost-5-en-3-yl O-α-L-rhamnopyranosyl-(1 → 2)-O-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside) was isolated at the National Center for Natural Products Research (NCNPR), University of Mississippi, Mississippi, USA; the identity and purity (91.2%) were confirmed by chromatographic and spectral analysis.

Cell Culture

Human breast cancer adenocarcinoma, MCF-7 (ER+) and MDA-MB-231 (ER-) cell lines were purchased from American Type Culture Collection (ATCC). Both cell lines were maintained in phenol red free DMEM-F12 (1:1) medium supplemented with 10% dextran charcoal treated fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 50 U/mL penicillin and 50 μg/mL streptomycin as Pen-Strep (Life Technology, Grand Island, NY), and 2 mM of L-glutamine (Life Technology) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. To maintain both cell lines in identical culture conditions, human recombinant insulin (0.01 mg/mL) was omitted for MCF-7 cells. The cells (~400 x 10³ cells/mL) were allowed to attach in the
culture flasks, and after 24 hours were treated with various concentrations of DS (3,
6 μM) or AZA (1μM)(Sigma-Aldrich, St. Louis, MO) as a known inhibitor of DNMT enzyme activities for three days. The media of the cells treated with AZA were replaced every 24 hours. After total removal of the media at 72 hours of treatment, the cells were trypsinized, resuspended in the medium, washed twice with PBS, and were used for biochemical analysis. (Aumsuwann et al. 2016)

**Cell Lysis**

Cells were put on ice and kept at a temperature of 4°C. The medium was removed by aspiration and cells were washed twice with PBS. Cells were treated with 150 μL lysis buffer (1 mL 10x protease inhibitor, 9 mL lysis buffer) and allowed to incubate for 1 hour at 4°C. Cells were mechanically removed via scraping and centrifuged at 12,000 rpm for 20 minutes. Supernatant was gathered and protein concentration was measured via Bradford protocol.

**Western Blot**

Lysate samples were boiled in sample buffer and loaded onto SDS-PAGE gel (BioRad) with a concentration of 25 μg of protein in each well. The gel was run at 70 V, 400 mA for 2 hours, and was then transferred onto a blotting membrane and placed in a blocking solution (Invitrogen; BSA based) overnight at 4°C. The membrane was rinsed and allowed to incubate with the polyclonal rabbit DNMT1
IgG antibody (1:100; Santa Cruz Biotechnology; sc-20701) for 24 hours at 4°C. WesternDot™ 625 Western Blot Kit from Invitrogen was then utilized for immunodetection of proteins. With washing in between each step, the membrane was incubated with the goat anti-rabbit IgG secondary antibody (5:12000) for 1 hour at 4°C and the Qdot® 625 streptavidin conjugate (5:12000) for 1 hour at 4°C. Pictures were taken of the membrane and analyzed. Anti-beta Actin antibody (Cell Signaling) was utilized as a loading control.
Results and Observations

Two separate Western blots were run to give an accurate representation of dioscin’s effects on the DNMT1 protein. For each Western blot, both cell lines received the following treatments: Dimethyl sulfoxide (DMSO) only, 1μM AZA, 3μM DS, and 6μM DS. Both DS and AZA had been resuspended in DMSO in their stock solutions, so the DMSO only treatment served as our control. This made sure that the observed results were due to DS and AZA, and not due to the DMSO.

The image of Western blot 1 (Figure 4) was taken September 28, 2016. The MDA-MB-231 cells proved to be much more fragile in culture than the MCF-7 cells. The treatment of 6μM DS was found to be toxic to the MDA-MB-231 cells, and we were not able to collect enough lysate for western blot analysis. Therefore, the treatment of 6μM DS was omitted for the MDA-MB-231 cells in Western blot 1 (Figure 4). Two separate isoforms of DNMT1 protein were detected on the membrane, which is consistent with the background information given by Santa Cruz Biotechnology and expected because of the polyclonal nature of the antibody. The first isoform (A) was detected at roughly 145 kDa, while the second isoform (B) was detected at 120 kDa. The loading control was confirmed to be accurate, as the beta Actin bands (C) were located at the expected weight of 45 kDa.
**Figure 4:** Western blot 1 analysis of the cell lines MCF-7 (lanes 2-6) and MDA-MB-231 (lanes 7-10) with selected treatments. Lane 1: molecular weight markers; Lane 2: MCF-7, DMSO; Lane 3: MCF-7, 1μM AZA; Lane 4: MCF-7, 6μM DS; Lane 5: MCF-7, 3μM DS; Lane 6: MCF-7, untreated; Lane 7: MDA-MB-231, DMSO; Lane 8: MDA-MB-231, 1μM AZA; Lane 9: MDA-MB-231, 3μM DS; Lane 10: MDA-MB-231, untreated.

The image of Western blot 2 (**Figure 5**) was taken November 11, 2016. The MDA-MB-231 cells responded more favorably to the 6μM DS treatment, and we were able to collect enough lysate for western blot analysis. The 6μM DS lane was therefore added for the MDA-MB-231 cell line in Western blot 2 (**Figure 5**, lane 8). The two DNMT1 isoforms (A,B) were detected at 145 kDa and 120 kDa, and the beta Actin bands were again located at 45 kDa. While there is a significant amount of non-specific binding and background noise in between the beta Actin bands and the
DNMT1 bands, this can be chiefly attributed to the polyclonal nature of the DNMT1 antibody. If a monoclonal antibody were used, the binding would be much more specific.

**Figure 5:** Western blot 2 analysis of cell lines MCF-7 (lanes 2-5) and MDA-MB-231 (Lanes 6-9) with selected treatments. Lane 1: molecular weight markers; Lane 2: MCF-7, DMSO; Lane 3: MCF-7, 1μM AZA; Lane 4: MCF-7, 6μM DS; Lane 5: MCF-7, 3μM DS; Lane 6: MDA-MB-231, DMSO; Lane 7: MDA-MB-231, 1μM AZA; Lane 8: MDA-MB-231, 6μM DS; Lane 9: MDA-MB-231 3μM DS.

The intensities of the DNMT1 protein bands were quantified using the Bio-Rad ChemiDoc MP Imaging System. The intensities for each of the treatments were normalized in terms of the control (DMSO only), and relative intensity ratios were developed to analyze the DNMT1 protein concentrations.
The results in Table 1 and Table 2 indicate that DS and AZA were able to decrease the protein concentration of the first isoform of DNMT1 in both the MCF-7 cells and the MDA-MB-231 cells. However, only the 1μM AZA and the 6μM DS treatments were able to inhibit the protein expression of the second DNMT1 isoform. These relationships are more clearly depicted by Figure 6.
**Figure 6:** Mean expression of DNMT1 protein (± average deviation) for Western blots 1 and 2.

**Figure 6** demonstrates that DS and AZA were much more effective in decreasing the protein expression of the first DNMT1 isoform in both the MCF-7 and MDA-MB-231 cell lines. Protein expression for both DNMT1 isoforms exhibited a dose dependent response to DS in the MCF-7 cells. AZA was also able to decrease the protein expression of the first isoform of DNMT1 by 70% and the second isoform of DNMT1 by 30%. Protein expression in the MDA-MB-231 cells did not exhibit a dose dependent response to DS. DS and AZA were able to decrease protein expression of the first isoform of DNMT1 in the MDA-MB-231 cells, but they actually increased protein expression of the second isoform of DNMT1.
Table 3: The percent increase/decrease of DNMT1 protein concentration in breast cancer cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCF7</th>
<th>MDAMB231</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZA 1uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70% decrease</td>
<td>30% decrease</td>
<td>34% decrease</td>
</tr>
<tr>
<td>DS 3uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21% decrease</td>
<td>no decrease</td>
<td>30% decrease</td>
</tr>
<tr>
<td>DS 6uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36% decrease</td>
<td>19% decrease</td>
<td>42% decrease</td>
</tr>
</tbody>
</table>
Discussion

The present study was focused on the steroidal saponin, dioscin, and its efficacy as a DNMT1 inhibitor. The results obtained from the two Western blot experiments are not perfect, but they are promising. DS was able to inhibit the protein expression of both isoforms of DNMT1 in MCF-7 cells and the protein expression of the first isoform (145 kDa) of DNMT1 in MDA-MB-231 cells. DS and AZA exhibited similar effects on the DNMT1 protein concentration, as we hoped they would. These complementary results reinforce our hypothesis that DS is a potential DNMT1 inhibitor. It can also be concluded that DS is acting post-transcriptionally to inhibit DNMT1 protein expression. We know this because the mRNA content of DNMT1 in MCF-7 and MDA-MB-231 cells remained unaltered when treated with DS (Aumsuwan et al. 2016). Therefore, DS may be acting on the DNMT1 mRNA and inhibiting some step of translation, or DS may be directly degrading the DNMT1 protein with a mechanism similar to AZA’s.

While these results support our hypothesis that DS is a DNMT1 inhibitor, there are still some questions that remain. DS was able to inhibit DNMT1 protein expression in the MCF-7 cells; this was not expected considering that in a previously conducted experiment, DS increased GATA3 and CDH1 mRNA expression in the MDA-MB-231 cells but not in the MCF-7 cells (Aumsuwan et al. 2016). For this reason, DS’s inhibition of DNMT1 may not be the only contributing factor in the re-
expression of GATA3 and CDH1. Analysis of DS’s effect on other epigenetic proteins may be needed in order to explain the exact mechanism by which DS is able to re-express these tumor suppressor genes.

DS is a proven anti-breast cancer agent in vitro. It is able to reduce cell proliferation and increase expression of crucial tumor suppressor genes. Whether or not DS’s anti-breast cancer potential stems solely from its DNMT1 inhibiting activity remains to be seen, but there is a clear path to follow in order to determine this. The effect of DS on other epigenetic proteins involved in methylation and demethylation must be determined. DS’s impact on DNMT3A and DNMT3B protein expression should be examined, as well as its impact on other DNMT binding proteins and the recently discovered TET enzymes. As our knowledge in the field of epigenetics increases, so will our knowledge on epigenetic mechanisms and drug treatments. DS may one day be used as an anti-invasive therapeutic in the treatment of breast cancer, but we have yet to elucidate the exact mechanism of action or how it will perform in an animal model. For now, DS is a promising and natural anti-cancer agent with the potential to limit DNMT1 protein expression, and therefore methylation, in breast cancer cells in vitro.
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