Anti-Proliferative Effects of BZL on MDA-MB-231 Human Breast Cancer Cells

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Abstract Breast cancer is the most commonly diagnosed invasive cancer in women in the United States, and is second only to lung cancer as the cause of deaths due to cancer. In addition to current treatment approaches like surgery, radiation therapy, chemotherapy, and hormone therapy, phytochemicals are being explored for development as potential cancer therapy drugs. BZL, a recent addition to this class of compounds used for alternative or adjuvant therapy, has been shown to cause apoptosis in MDA-MB-T47-D human breast cancer cells in preliminary experiments. An effective dose for BZL has not yet been established, and its mechanism of activity is unknown. In this study, the potential inhibition of cell proliferation induced by BZL was tested on MDA-MB-231 human breast cancer cells. Flow Cytometry was used to determine the inhibition of tumor cell proliferation through cell cycle arrest at the G_1, S, or G_2 phases of the cell cycle. This study finds that BZL induces cell cycle arrest at the G_2-phase, which indicates that BZL has the potential to be used as a drug to target the uncontrolled proliferation of cancer cells. Further work needs to be done to understand the mechanism of action of this drug.

Introduction

In the United States, one of every four deaths is from cancer, making it the second[1] leading cause of death in the country (ACS 2006). Cancer is characterized by unregulated cell growth brought about by several genetic and environmental factors that lead to an accumulation of mutations, which normally occur in the replication or stimulatory pathways of cells. Cancerous cells usually form tumors, which develop in different parts of the body (ACS 2006). Each type of tumor behaves differently and requires various treatment approaches including surgery, radiation therapy, chemotherapy, and hormone therapy, or any of these in combination, to enhance the effectiveness of treatment (ACS 2006; Hemalswarya and Doble 2006; Sun and Liu 2006).

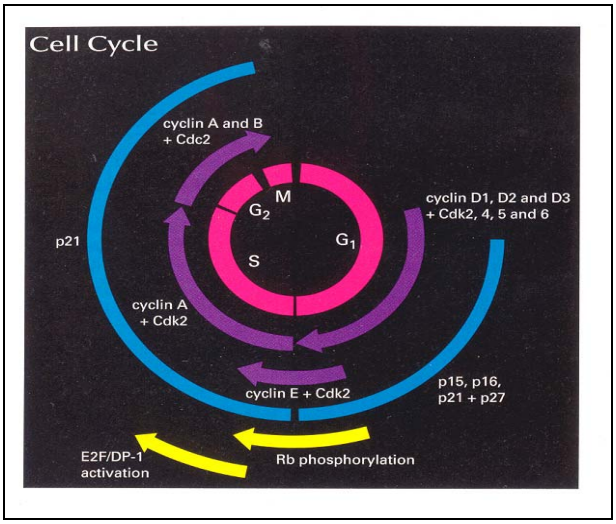
Breast cancer is the most commonly diagnosed invasive cancer in women in the United States, and is the second leading cause of death in women due to cancer (Sun and Liu 2006). Treatment of breast cancer is difficult as there are several distinct classes of breast tumors, and these respond differently to treatment (ACS 2006; Sun and Liu 2006). The anti-estrogen Tamoxifen, one of the most common means of hormonal therapy, is regarded an effective treatment for breast cancer in high-risk women, i.e. women age 40 and older (Katzenellenbogen et al. 2000; ACS 2006), but this is only in one third of breast cancer patients (Sun and Liu 2006). Chemotherapy is another widely used treatment method, and has shown to suppress or prevent carcinogenesis, but it has not increased the cure rates (Hemalswarya and Doble 2006). In addition, chemotherapy drugs can have several side effects, including hair loss, dry skin, nausea, vomiting, and loss of energy (ACS 2006), thus creating a need for treatment strategies that have greater target specificity and minimize the side effects of drug therapy (ACS 2006).

Advances in areas of cell biology and molecular genetics have created new outlets for target specific therapy (ACS 2006; Hemalswarya and Doble 2006) by way of understanding the different mechanisms of cancer, and how the disease advances. This research has not only created avenues for treatment, but also created possibilities for cancer prevention. There is increased interest in alternative treatments, in addition to conventional approaches, including the use of drugs derived from phytochemicals (ACS 2006; Hemalswarya and Doble 2006). Phytochemicals are present in plants as part of their natural defense mechanism (Wargovich 1999) against diseases and environmental stresses, and several types of these compounds are now known to decrease the prevalence of many degenerative diseases such as heart disease in humans. A number of phytochemicals have also shown anti-tumor effects in various experimental systems (Hemalswarya

[1] Heart disease is the leading cause of death for men and women in the United States (ACS 2006)

and Doble 2006; Kummalue et al. 2007). They are now becoming increasingly popular in the prevention of diseases such as cardiovascular disease as well as treatment of cancers (Fahey et al. 1997; Mo and Elson 1999; Grube et al. 2001; Sun and Liu 2006).

Extensive research, involving many different types of phytochemicals such as carotenoids, flavones and flavonoids, isoprenoids, limoniods, and tocopherols, is being conducted. Many of these phytochemicals have been shown to affect different targets within signal transduction pathways leading to gene expression, cell cycle progression and proliferation, and apoptosis (Hemalswarya and Doble 2006), which are important targets as cancerous cells often disable the system's built-in repair mechanisms such as tumor suppressors and other cell cycle checkpoint proteins (Figure1). These proteins are normally activated upon DNA damage, to prevent the division of cells with unrepaired DNA damage. They can act through several signals involved in the cell cycle to induce growth arrest, thus limiting, or stopping, the growth of damaged cells (Fan and Khavari 1999).



(Santa Cruz Biotechnology 2007)
Figure 1: Cartoon showing the G_1, S, and G_2 phases of the cell cycle in the center (pink). The G_1-phase is the growth phase before DNA synthesis, in the S-phase, which is followed by the second growth phase, G_2. Also shown are cell cycle proteins such as the cyclins and cyclin-dependent-kinases (purple), along with tumor suppressors like p21 (blue), that determine the phase transitions and progression of the cell cycle.

An addition to the list of phytochemicals under experimental consideration for cancer therapy is BZL. BZL is a drug mixture of botanical origins. It has uncharacterized chemical components, but has been shown to cause apoptosis in MDA-MB-T47-D human breast cancer cells in preliminary experiments. BZL presents a new research opportunity in that it may inhibit growth of tumors

through decreased proliferation or apoptosis of certain types of cancer cells. An effective dose for BZL has not yet been established, and its mechanism of activity is still unknown. The dose-dependent and time-dependent effects of BZL were tested on MDA-MB-231 human breast cancer cells. The MDA-MB-231 cell line is similar to the MDA-MB-T47-D cells used in preliminary studies, but is a more advanced group of cells that are metastatic. The dose-response and time-course experiments were done as an initial step in elucidating signaling mechanisms that might be involved in tumor cell proliferation or apoptosis.

Methods

The dose-optimization of BZL was carried out on MDA-MB-231 human breast cancer cells, which are among the standard cell lines used for breast cancer research. BZL, obtained from Bionovo, Inc. (Emeryville, CA), comes in a brown freeze-dried powder form, and is dissolved in sterile-filtered nano-water in 100mg/mL aliquots. The control for BZL is sterile-filtered nano-water (nH_2O), the vehicle for the drug. The positive control for BZL is Indole-3-Carbinol (I-3-C), which is used to check for cell cycle arrest, since I-3-C has been shown to cause G_1 cell cycle arrest (Cover et al. 1998). I-3-C is prepared in 99.9% Dimethyl Sulfoxide (DMSO) hence the vehicle control for I-3-C is DMSO.

Cell Culture The MDA-MB-231 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM), supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, and 0.25% Penicillin-Streptomycin. The cells were maintained in a 37°C incubator, and the media was replaced every two days.

Cell Cycle Analysis Flow Cytometry Analysis was used to measure physical properties of individual cells in suspension to establish parameters such as cell cycle arrest or apoptosis. Experiments were set up for 24h, 48h, and 72h time intervals [with three trials for each time point] to determine the most effective duration of exposure to the drug, as well as the optimal dose.

Cells were plated onto 6-well tissue culture plates (Table 1) and grown to 30-50% confluency, at which point they were treated with BZL. For each time interval, plates were set up for treatment with the following doses: 30 µl sterile-filtered nano-water/ml media (No Treatment; total volume of 60 µl nH_2O per well); 0.1mg BZL/ml media; 0.3mg BZL/ml media; 0.6mg BZL/ml media; 0.9mg BZL/ml media; and 1µl 200 mM I-3-C/ml media, and 1µl DMSO/ml media were used as positive controls. Treatment of cells involved adding the drug to media in a conical, filtering the drug mixture through a 0.45µm sterile filter, and then applying this mixture to the tissue culture plates for

uniform distribution of the drug. The plates were re-treated and the media was changed every 24 hours.

Table 1: Experimental setup of 6-well plates with four concentrations of BZL, sterile-filtered nano-water negative controls, I-3-C positive controls, and DMSO controls for I-3-C

30 µl/ml nH_2O	30 µl/ml nH_2O	30 µl/ml nH_2O
0.1mg/ml BZL	0.1mg/ml BZL	0.1mg/ml BZL
0.3mg/ml BZL	0.3mg/ml BZL	0.3mg/ml BZL
0.6mg/ml BZL	0.6mg/ml BZL	0.6mg/ml BZL
0.6mg/ml BZL	0.6mg/ml BZL	0.6mg/ml BZL
0.9mg/ml BZL	0.9mg/ml BZL	0.9mg/ml BZL
1µl/ml 200 mM I-3-C	1µl/ml 200 mM I-3-C	1µl/ml 200 mM I-3-C
1µl/ml DMSO	1µl/ml DMSO	1µl/ml DMSO

At the end of each time interval, the cells were harvested—scraped off the tissue culture plates and centrifuged to collect cell pellets. These pellets were resuspended in 400 µl of Propidium Iodide (PI) solution to stain cell nuclei and allow analysis of DNA content. The samples were then run through a Beckman Coulter instrument at the Flow Cytometry Facility at the University of California, Berkeley. Percentages of cells in the G_1, S, and G_2 phase of the cell cycle were determined by analysis with the Multicycle computer program.

Results

Cells treated with BZL experienced arrest in the G_2-phase of the cell cycle. This phase arrest occurs in a dose-dependent manner, with the greatest difference between the control and the 0.9mg/ml dose of BZL, the highest dose tested. The arrest, or increase in the proportion of cells in G_2-phase, is seen at the 24h (Figure 2), 48h (Figure 3), and the 72h (Figure 4) intervals.

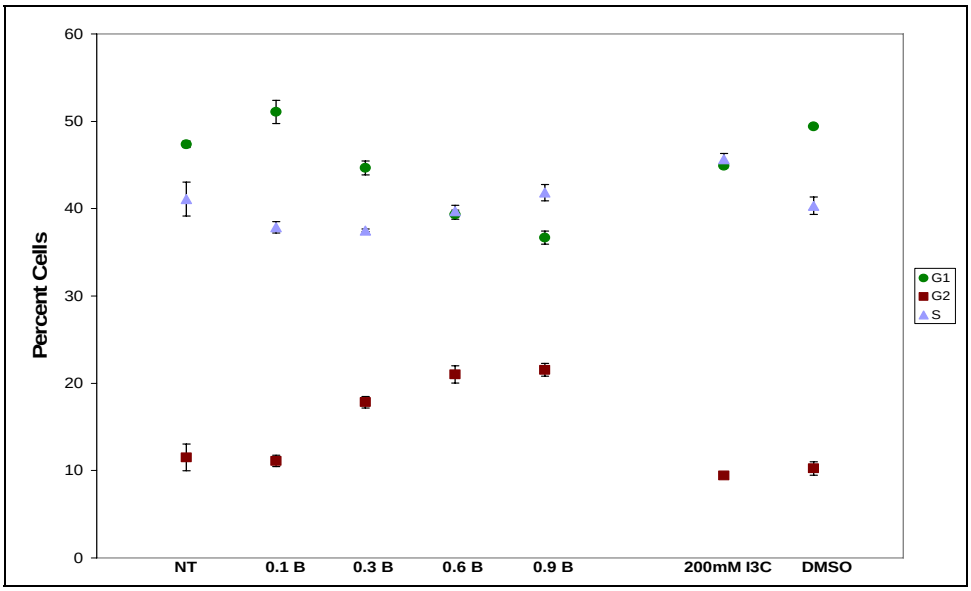


Figure 2: MDA-MB-231 cells for 24h BZL dose-response: four doses of BZL (0.1, 0.3, 0.6, 0.9 mg/ml), with negative control (60 µl nH2O), and positive controls (1 µl/ml I-3-C and DMSO). Points represent cells in G_2-phase (squares), in comparison to the G_1 (circles), and S-phase (triangles); results are ±standard deviation; n=3.

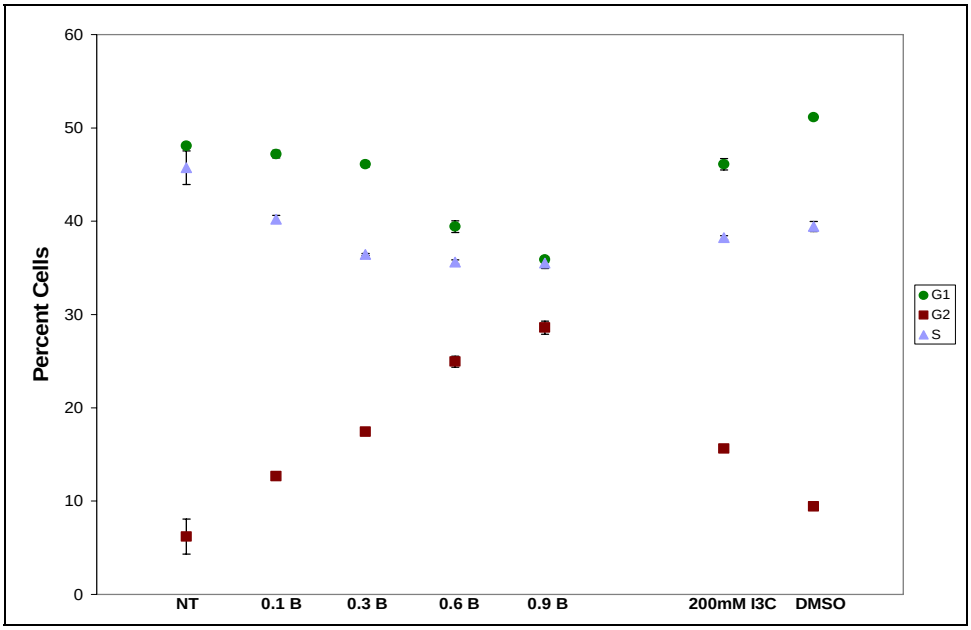


Figure 3: MDA-MB-231 cells for 48h BZL dose-response: four doses of BZL (0.1, 0.3, 0.6, 0.9 mg/ml), with negative control (60 µl nH2O), and positive controls (1 µl/ml I-3-C and DMSO). Points represent cells in G_2-phase (squares), in comparison to the G_1 (circles), and S-phase (triangles); results are ±standard deviation; n=3.

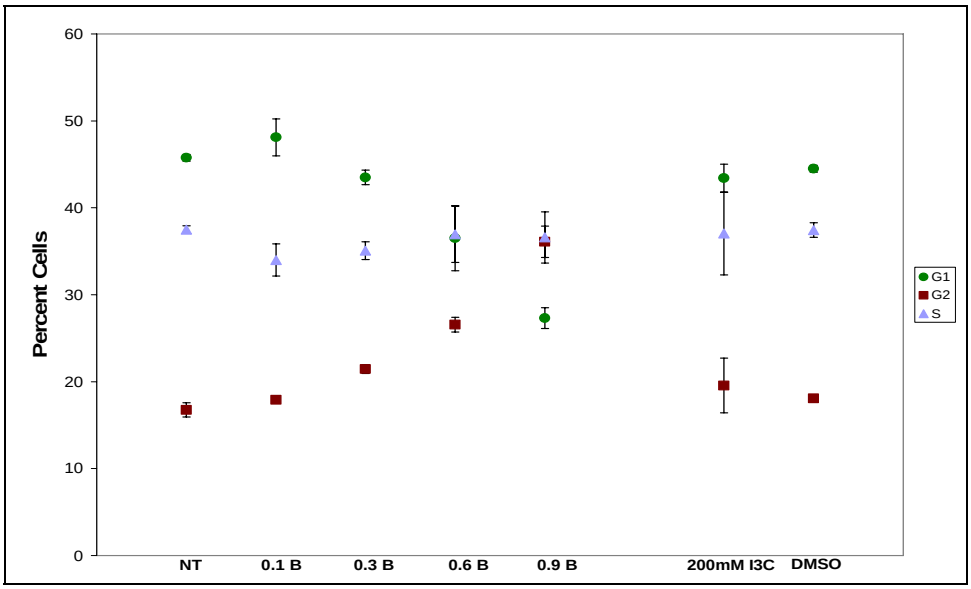


Figure 4: MDA-MB-231 cells for 72h BZL dose-response: four doses of BZL (0.1, 0.3, 0.6, 0.9 mg/ml), with negative control (60 µl nH2O), and positive controls (1 µl/ml I-3-C and DMSO). Points represent cells in G_2-phase (squares), in comparison to the G_1 (circles), and S-phase (triangles); results are ±standard deviation; n=3.

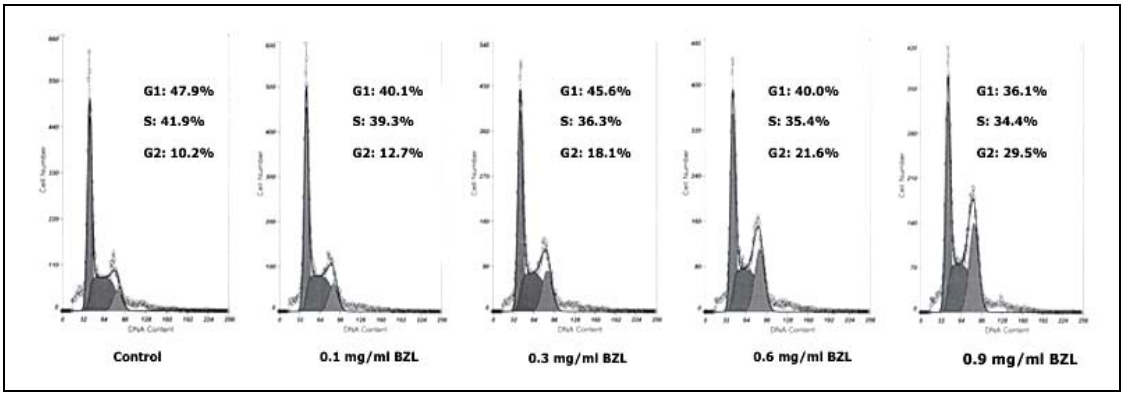


Figure 5: Flow cytometry analysis of MDA-MB-231 cells for 48h BZL dose-response, (0.1, 0.3, 0.6, 0.9 mg/ml BZL), with negative control (60 µl nH2O). Horizontal axis denotes the DNA content, coinciding with the G_1, S, or G_2 phase, of each cell; vertical axis denotes the number of cells in each phase. The G_2-phase is on the right side of each peak (falling on the right of the G_1 and S-phase peaks, respectively). These peaks show a steady increase in the number of cells in the G_2-phase.

There is no clear trend suggesting an S-phase arrest, but there is a decrease in the percent of cells in the G_1-phase, coinciding with an increase, or arrest, in the G_2-phase; this observation holds for all three time intervals tested (Figures 2, 3, and 4). While the G_2 arrest can be seen for all three time intervals, the effect is minimal for the 24h period, where there is only a ten percent difference between the control and the highest tested dose (Figure 2); this small change cannot be fully attributed as an effect of the drug. Compared to the 24h interval, there is a more dramatic G_2 arrest at the 48h interval (Figure 5); BZL seems to have a stronger effect at the 48h and 72h intervals, with

a change of about twenty percent between the 0.9 mg/ml BZL dose and the control (Figures 3 and 4). While the G_2 arrest for the 48h and 72h intervals is comparable (Figure 6), the results were more consistent for the 48h interval, suggesting that BZL may not work in a time-dependent manner; i.e. the effect of the drug is not a related to time.

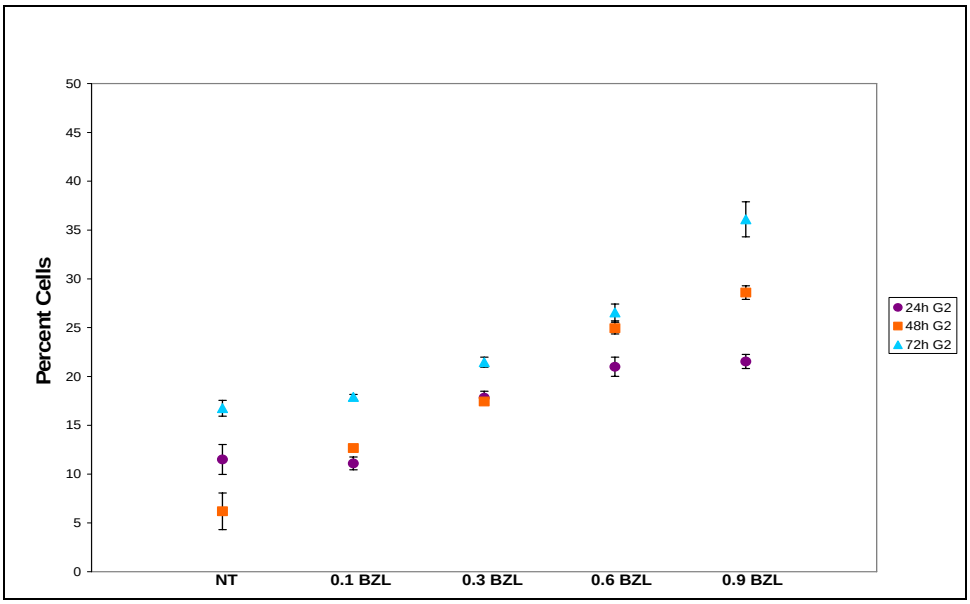


Figure 6: MDA-MB-231 cells in G_2-phase for 24, 48, and 72h BZL dose-response: four doses of BZL (0.1, 0.3, 0.6, 0.9 mg/ml), with negative control (60 µl nH2O). Points represent cells in G_2 for the 24h (circles), 48h (squares), and 72h (triangles); results are ±standard deviation; n=3.

Discussion

While preliminary studies using BZL show induction of apoptosis in MDA-MB-T47-D cells, this study features the effect of BZL on cell proliferation in MDA-MB-231 cells. Flow Cytometry analysis shows an arrest at the G_2-phase of the cell cycle. This response occurs in a dose-dependent manner, with the most noticeable effects at the 48h time interval compared to the 24h and 72h intervals also tested. A G_2/M-phase arrest implies that the cells do not proceed to mitosis, thus inhibiting tumor cell division (Fan and Khavari 1999). However, the cells were fully confluent at the time of harvest (for all three time intervals), implying that proliferation (cell division) continued despite the G_2-phase arrest induced by BZL.

This study finds that BZL acts in a dose-dependent manner, but the G_2-phase arrest is not affected by time, as the effects were the strongest for the 48h, and not the 72h, interval. This effect is difficult to explain, but could be due to variations in the cell line tested, along with the growth conditions of these cells (Kummalue et al. 2007). In addition, the MDA-MB-231 cells are an

aggressive line of breast cancer cells (Sundar et al. 2006), and have a high rate of proliferation and survival, which could explain why the drug does not stop proliferation despite the G_2-arrest.

The mechanism of the G_2-arrest, suggested by flow cytometry, still needs to be investigated. An initial step could be to perform cell count and viability experiments (Kummalue et al. 2007; Lim et al. 2007; Shyam Sundar, personal communication), in order to determine whether the cells that continue to proliferate are actually intact and functional, or have become fragmented (approaching death via necrosis or apoptosis). Additional experiments can be done to monitor the cells' DNA content, through fluorescence or radioactive tag, as it undergoes the G_2-phase arrest (Shyam Sundar, personal communication; Weber et al. 2007), where continued increase in DNA content means that the G_2-arrest is not sufficient to stop the growth of the cancer cells. These experiments may provide further insight into the continued proliferation of these cells. Once a potential mechanism has been determined, Western blot analysis can be used to probe for proteins involved in the G_2 arrest. Another approach is to measure transcriptional up-regulation or down-regulation of signaling components involved in the cell cycle using RNA levels.

This study only explored the effects of BZL on one cell line, and its mechanism of action remains unknown. Further studies should continue, probably using less metastatic breast cancer cells, using higher doses, and different time points. And BZL should also be tested in combination with other plant derived drugs (Kummalue et al. 2007; Gary Firestone, personal communication).

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