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Effects of fenbendazole and vitamin E succinate on the growth and survival of prostate cancer cells

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We describe antitumor activities of vitamin E succinate (VES), an anti-oxidant and fenbendazole (FBZ), a commonly used veterinary anthelmintic. We used VES and FBZ, at low concentrations, singly and in combination, to test their inhibitory effects on proliferation of human and mouse prostate cancer cells in vitro. Administered alone, FBZ inhibited proliferation faster than VES in both mouse and human prostate cancer cell lines and a synergistic effect between both was also observed. Apoptosis was the likely mechanism for the observed effect. These drugs may deserve to be tested for their efficacy in the control of prostate cancer using in vivo models.

Key words: Prostate cancer, fenbendazole, vitamin E succinate.

INTRODUCTION

Although various treatments are available for the early stages of prostate cancer (PCa), the options are, however, quite limited for advanced or recurrent disease because of ultimate resistance of these tumors to chemotherapy and endocrine manipulation (Ismail and Gomella, 1997; Sullivan et al., 1998). For this reason, finding novel and alternative methods of treating PCa have received much attention (Agarwal, 2000; Deutsch et al., 2004; Gronberg, 2003; Kline et al., 2004; Vashchenko and Abrahamsson, 2005). Benzimidazoles are a class of drug that are traditionally used for treating parasitic infections in humans and animals and are remarkably safe and efficacious (Lacey, 1988). A primary mechanism of action relates to the binding of benzimidazoles to nematode β-tubulin that inhibits microtubule polymerization and thereby interfering with cell division (Lacey, 1988; Laclette et al., 1980; Sasaki et al., 2002). Several other cancer chemotherapeutics are also known to either stabilize microtubulin (paclitaxel and docetaxel) or destabilize microtubulin (vinblastine and vincristine), but because of high toxicity their therapeutic efficacy is limited (Mukhopadhyay et al., 2002). While parasites are the primary targets of benzimidazoles because of their affinity for non-mammalian microtubulin and rapidly dividing cells (Lacey and Gill, 1994), Mebendazole, a derivative of the Benzimidazoles, has been shown to selectively induce apoptosis in adrenocortical carcinoma in vivo and in vitro (Martarelli et al., 2008), and in melanoma cells in vitro (Doudican N et al., 2008). A Phase I clinical trial reported by Morris et al., 2001 showed Albendazole, another Benzimidazole, had a high maximum tolerated dose when given to 36 patients with malignant tumors, 16% of which showed a decrease in tumor markers (Morris et al., 2001). 

Fenbendazole is a benzimidazole drug commonly used for treating pinworm outbreaks in laboratory rodents (Coghlan et al., 1993). It has a wide margin of safety with an oral LD50 in mice of >10,000 mg/kg (O'Neil, 2001). This drug was shown to have a synergistic inhibition of
lymphoma growth in SCID mice when combined in the
diet with supplemental vitamins (Gao et al., 2008).
However, a specific vitamin was not identified.

There is evidence to suggest that vitamin E (RRR-α-
tocopherol) may be beneficial in preventing or delaying
PCA growth (Busu and Imrhan, 2005; Fleshner, 2002;
Gronberg, 2003; Malafa et al., 2006). Vitamin E succinate
(VES), the most potent derivative of vitamin E for anti-
tumor activity (Busu and Imrhan, 2005), has been shown
to induce apoptosis in PCa in vitro and in vivo through
caspase-4 activation (Malafa et al., 2006). An in vitro
study showed that when human PCa PC-3 cells were
treated in culture with 20 µM of VES, there was a growth
inhibition of 40% after 3 days of treatment (Zu and Ip,
2003). Especially when administered in the diet, VES has
displayed chemopreventive effects (Busu and Imrhan,
2005). In a study performed on the LADY (12T-10)
transgenic mouse strain which harbors expression of
SV40 large T antigen but not small t antigen, a diet with a
combination of antioxidants lycopene, selenium, and VES
was fed from the time of weaning. In these mice, PCa
incidence decreased by 4 times that of controls
Venkateswaran et al., 2004. Supporting data from these
animal models have led to consideration in clinical
medicine. An ongoing human clinical trial, the selenium
and vitamin E cancer prevention trial (SELECT) that is
projected to end in 2013, will likely be informative on
whether vitamin E, in conjunction with selenium, is a
chemopreventive agent (Klein et al., 2003; Lieberman,
2003; Ni and Yeh, 2007).

Several spontaneous mouse models of prostate cancer
have been developed based on genetic alterations that
are prominent in human prostate cancer to capture the
natural course of initiation and progression of this cancer
(Mimeault and Batra, 2011; Roy-Burman et al., 2004).
One such model, the conditional Pt en deletion mouse
model with a Luciferase reporter (cPt en−/− L) (Wang et al.,
2003; Liao et al, 2007), leads to development of prostatic
adenocarcinoma. After castration, tumor burden begins to
decline rapidly due to the dependence of the primary
tumor on androgen presence. However, the residual
tumors undergo recurrent growth to give rise to castration
resistant prostate cancer (CRPC).

The E8 cell line is a neoplastic epithelial cell line
derived from the primary prostate tumor of the cPten+/−
mouse which maintains sensitivity to androgen for growth
(our unpublished data). The previously described cE1
carcinoma cell line (Liao et al., 2010) was derived from a
CRPC tumor of the cPten−/− model. This cell line can
grow in the absence of androgen but still remains
sensitive to androgen stimulation. The PC-3 cell line, a
human prostate cancer cell line of bone metastatic origin,
was obtained from ATCC (Manassas, VA).

In this study we used malignant epithelial cell lines
(Liao et al, 2010) derived from both primary and CRPC
tumors of this model along with a human prostate cancer
 cell line to determine the effect of FBZ and VES, singly
and in combination, on their in vitro growth potential.

MATERIALS AND METHODS

E8 and cE1 cells were cultured in DMEM media supplemented with
10% FBS, 1% penicillin/streptomycin, 25 µg/ml bovine pituitary
epithelium, 5 µg/ml insulin, and 6 ng/ml recombinant human
epithelial growth factor. PC-3 cells were cultured in DMEM media
supplemented with 1% Penicillin and Streptomycin, 10% FBS, 1x
Non-essential amino acid, and 1x essential amino acid. All cells
were grown until 70% confluent.

To evaluate the inhibitory effect of FBZ (Santa Cruz
Biotechnology, Santa Cruz, CA) and VES (supelco analytical,
bellfonte, PA) on the growth of E8, cE1, and PC-3, the cells (2 x
10⁴) were seeded in 12-well plates in the maintenance media for 12
h. A dose response curve was performed on E8 cells to determine
the activity of each agent. FBZ was diluted serially from 2.1, 1.1,
1.0, and 1.00, from a starting concentration of 450 ng/ml to 2.25
ng/ml. VES was serially diluted in the same manner but with a
starting concentration of 50 µg/ml diluted down to 0.025µg/ml.
Intermediate concentrations were also tested that included 56, 140,
168, and 200 ng/ml for FBZ. For VES, intermediate concentrations
included 30 and 40 µg/ml. The vehicle control media contained
ethanol in equal volume. The cultures were monitored for up to 4
days. Once it was determined that FBZ had a stronger effect than
VES on proliferation, an experiment was performed on the PC-3
cell line to see whether these drugs had a synergistic effect on
proliferation. Synergy was measured by cell counts that were
smaller in number when the two drugs were used together at the
optimal dosage than when used alone. The VES concentration of
25 µg/ml was chosen since it was within the IC₅₀ of the drug and
was the highest dose with which growth inhibition was not observed
in the E8 cells for the first four days. A 12 well plate was treated
with dosage combinations of VES+FBZ with a corresponding
vehicle control well. The dosage of 25 µg/ml VES and 14 ng/ml of
FBZ were determined to be the optimal dosages for which growth
inhibition occurred without acute detachment and cell floatation,
and these dosages were used on the other cell lines.

Each cell line was seeded onto six-well plates where three wells
were treated with the vehicle control and three were treated with 25
µg/ml of VES and 14 ng/ml FBZ. The culture medium was changed
every 2 days and the cell proliferation rate was determined at time
points 1,3,5, and 7 days by cell counting (Beckman Coulter Cell
Counter, Brea, CA).This growth analysis was repeated in
independent triplicates on all 3 cell lines.

Cellular apoptosis in E8, cE1, and PC-3 cells was measured using
APO-BRDUTM Terminal deoxynucleotidyl transferase
mediated dUTP Nick End Labeling (TUNEL) kit (Phoenix Flow Systems,
San Diego, CA). After signs of cell death were observed
(4 days for E8 and cE1, 3 days for PC-3), cells were collected, and
TUNEL was performed and quantified by flow cytometric analysis
on an LSRII machine (SORP, BD biosciences) following the
instructions of the manufacturer.

For the pilot in-vivo experiment, normal mice were euthanized
with an overdose of isoflurane anesthesia followed by cervical
dislocation at 11 months of age. Their prostates were dissected out
into the separate lobes, paraffinized, and cut into sections using a
microtome, (Mikron Instruments Inc, Germany). Sections were
stained with hematoxylin and eosin. Immunohistochemical analysis
was performed on slides of parallel paraffin sections of
paraformaldehyde-fixed tissue using a modified Avidin – Biotin
Complex technique, as described previously (Zhong et al., 2006).
Antigen retrieval was accomplished by boiling the slides in 10
mmol/L of citric acid buffer (pH 6.0) for 15 min. Antibodies to
cytokerein 8 (CK8; 1:100 TROMA-1 antibody; Developmental
Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa),
androgen receptor (ARsc-815 1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), and vimentin (R28 1:50; cell signaling technology, davers, MA) were incubated overnight at 4°C. Sections were incubated with biotinylated secondary antibody for 30 min at room temperature. Apoptosis was detected with TUNEL assay using the In Situ death detection kit from Roche according to manufacturer’s directions. All sections were then detected with the ABC elite kit (Vector Laboratories Inc, Germany) and 3, 3-diaminobenzidine (DAB Sigma, Dako North America, Carpinteria, CA) as substrate. All slides were dehydrated through graded alcohols to xylenes and mounted with coverslips. Animal studies were approved by the University of Southern California Institution Animal Care and use committee and housed according to federal guidelines.

**Statistical analysis**

Statistical analysis was performed with excel 2007 (microsoft, Redmond, MA). The results of the cell proliferation assay were evaluated as the mean ± SD of at least three different experiments performed in triplicate. For both the cell proliferation assay and the TUNEL assay, differences between control cell numbers were compared with that of treated cells and were analyzed by independent t-test, P values of < 0.01 were considered statistically significant. Flow cytometric data was collected on BD FACS Diva software version 6.0 (San Jose, CA) and analyzed on Flow Jo software version 9.3 (Treestar, Ashland, OR).

**RESULTS AND DISCUSSION**

Growth assays for all three cell lines were performed (Figure 1A to D). After testing E8 cell line singly with FBZ then VES, we determined that neither FBZ at 22.5 ng/ml nor VES at 25 µg/ml had any significant inhibitory effect on proliferation, at least during the four days of exposure (Figure 1A). However, when we used a lower concentration of FBZ (14 ng/ml) together with VES (25 µg/ml), beginning at the third day, a synergistic inhibitory effect on proliferation was observed that became robust in the subsequent days (Figure 1B). A similar pattern of inhibition was seen with the cE1 (Figure 1C) and PC-3 cells (Figure 1D). All cells were seeded at 2 × 10⁴ cells/ml and proliferation reached to 3.5 × 10^6 for control E8 cells, 3.91 × 10^5 for treated E8 cells, 2.37 × 10^5 for control cE1 cells, 1.2 × 10^5 for treated cE1 cells, and 3.7 × 10^5 for the control PC-3 cells, 733 for treated PC-3 cells by day 7. The treatment of each cell line yielded a highly significant (P<0.01) level of cell inhibition at 5 to 7 days.

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In the subsequent days (Figure 1B). A similar pattern of inhibition was seen with the cE1 (Figure 1C) and PC-3 cells by day 7. The treatment of each cell line yielded a highly significant (P<0.01) level of cell inhibition at 5 to 7 days.

An average of 22.4 ± 9.42% treated cE1 cells underwent apoptosis by day 4 compared to 2.25 ± 1.71% of vehicle control cells. For PC-3, apoptosis occurred in 93 ± 2.9% of treated cells in contrast to 27.2 ± 9.89% labeled control cells. These results of increased apoptosis induced by the combination of FBZ and VES were all statistically significant (P<0.01), indicating that the observed inhibitory effect on proliferation might be related to induction of apoptosis by the agents at the concentrations used.

FBZ+VES significantly inhibited growth of PCa cells and induced apoptosis in vitro. To our knowledge, this is the first time these drugs have been tested together in PCa cell lines. In a previous study, a concentration of 50 µg/ml of VES was shown to be effective on PC-3 cells (Malafa et al., 2006). For FBZ, we tested a range of concentrations based on a dosage used for Mebendazole treatment of melanoma cells in culture (Doudican et al., 2008). A dosage of 25 µg/ml of VES and 14 ng/ml of FBZ was optimal for the treatment of prostate cancer cell lines we tested. Higher concentrations of FBZ produced a rapid rate of cell death during the initial 48 h period and lower concentrations did not cause any change in cell growth compared to controls. The agents were combined at the specified dosages because alone neither produced results that were as striking as when they were together. This synergistic ability of inhibition is very interesting, although the mechanisms involved is unclear. One hypothesis is that vitamin E activates multiple pro-apoptotic pathways such as targeting NF-kB (Ni and Yeh, 2007), and caspase-4 (Malafa et al., 2006), and if used with an agent that inhibits cell division such as one of the benzimidazoles, can be antagonistic to tumor growth. These agents are worth further exploration since they are both relatively safe and easy to administer.

We determined that apoptosis occurred in all three cell lines as a result of the combination treatment. PC-3 had the shortest treatment time and the most cell death when compared to E8 and cE1. This may be as a result of human cells being more sensitive to this combination of drugs versus mice having a higher tolerance. Between the two mouse cells lines tested, the more androgen independent cE1 cells derived from CRPC appears to be more resistant to the effects of FBZ+VES than the primary tumor E8 cells from the same model. Further studies will be needed to understand which mediators may be responsible for drug resistance specific to these cell types. Because all three cell lines had a generally similar response to the treatment in combination, it can be concluded that both mouse androgen dependent as well as human and mouse CRPC cells are susceptible to FBZ +VES. Thus, these drugs may potentially have efficacy independent of the hormonal environment.

As a preliminary study, a small group of normal mice were fed with either FBZ, VES, or a VES+FBZ combination administered in the feed for 206 days at which point they were humanely euthanized. Mice fed a normal rodent diet that was not supplemented were used...
Figure 1. Effect of VES+FBZ on the proliferation and apoptosis of human and mouse prostate cancer cell lines. (A) Growth inhibitory properties of dosages of VES or FBZ on the E8 mouse primary prostate cancer cell line. (B) Inhibitory effect of combination of VES and FBZ at low concentrations on the proliferation of E8 cells. Similar treatment of a mouse prostate cancer cell line, cE1 derived from a recurrent cancer (C), and a human prostate cancer cell line, PC-3 derived from a bone metastasis (D). Wells were treated with 25 µg/mL VES and 14 ng/mL FBZ and an equal volume of Ethanol as the vehicle control. Representative TUNEL assays conducted for E8 cells treated for 4 days (E), cE1 cells treated for 4 days (F), and PC-3 cells treated for 3 days (G) with the same concentrations of VES+FBZ. These graphs are merged to show the vehicle control cells in relation to that of the experimental groups of cells. The white peaks represent the population of live cells (vehicle control) and the gray peaks represent the cell fraction undergoing apoptosis (FBZ+VES treated). Asterisk (*) denotes statistical significance at a P value <0.01.

as age-matched controls. While serum markers of organ failure would have been ideal indicators of toxicity, we focused on how these drugs affect the function and anatomy of the prostate on histopathology. General physical changes such as weight loss, hair coat, behavior, ability to urinate and defecate normally, respiration, and activity were monitored by a veterinarian throughout the study for signs of toxicity and illness but
no abnormalities were observed. Body weight was measured monthly and significantly increased (P<0.001) by the end of the study indicating the diet was palatable, non-toxic, and the mice maintained a positive energy balance. Upon necropsy, grossly, the dorsolateral, anterior, and ventral prostatic lobes were morphologically normal. Histopathology analysis was performed on their prostates and normal glandular structures were observed in all groups (Figures 2 and 3). No significant observable differences in architecture were seen between the groups and controls except there was more secretory material present in the lumen of the glands from the FBZ+VES group. We do not believe this is due to the drugs since the single treatments did not produce the same result. This is more likely to be a physiological process that is occurring such as an increase in prostatic fluid at the time of death. Expression of androgen receptor and cytokeratin 8 was present and did not differ between cells. Stromal proliferation as marked by Vimentin staining was minimal and also did not differ between groups. The amount of cells in each group had a minimal population of cells undergoing apoptosis and also did not differ. The similarities between groups may indicate these drugs are non-toxic even when given at 150 ppm of FBZ and 2000 IU of VES orally for prolonged time periods. While the preliminary data showed these drugs are relatively benign further in vivo studies will be necessary to establish the validity of these observations at higher concentrations. Additionally, a large study in which animal models that develop spontaneous tumors are fed

Figure 2. Illustration of comparative histological analysis of the prostate tissue sections of normal mice kept on the FBZ + VES diet (first row) and control diet not containing FBZ or VES (second row). Representative staining for Hematoxylin and Eosin (H and E), Cytokeratin 8 (CK8), androgen receptor (AR), terminal deoxynucleotidyl transferase mediated dUTP Nick end labeling (TUNEL), and vimentin on serial sections of the dorsolateral prostatic lobe are shown. Arrows, areas enlarged in the insets (400X). Bar, 100 µm.
these agents in the diet at the highest non-toxic concentrations would be worthwhile for exploration of these drugs’ efficacy in vivo.

In summary, combination therapy with VES and FBZ deserves further investigation as a possible treatment modality for prostate cancer.

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