

**Potent inhibition of cell proliferation and tumour growth by
albendazole: an *in vitro* and *in vivo* study**

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Abbreviations: ABZ = albendazole, VEGF =Vascular Endothelial Growth Factor,
HIF = Hypoxia inducible factor, SRB = Sulforhodamine B, MTAs = Microtubule
Targeting Agents.

ABSTRACT

This study was designed to assess the effect of albendazole (ABZ) on the intraperitoneal tumour growth of ovarian cancer in female nude mice. Initially, *in vitro*, OVCAR-3 cells isolated from the ascites of carrier mice were treated with ABZ (0.01-1.0 micromoles/L) for periods ranging from 2 h to 9 days were examined for cell viability, proliferation, and colony formation. *In vivo*, 21 days after *i.p.* cell inoculation (10 million), one group of mice were euthanased (0 time control), while the other groups were treated *i.p.* thrice weekly for 4 weeks with either the ABZ 150 mg/kg suspension or the vehicle. Blood samples and ascites were collected, just before initiation of treatment and then at the end of the experiment. *In vitro*, ABZ potently inhibited cell proliferation in a dose and time - dependent manner with an IC_{50} value of 0.128 micromoles/L. *In vivo*, tumour weights were 0.44 ± 0.18 g in 0 time control mice, 1.957 ± 0.53 g in the vehicle treated and 0.65 ± 0.10 g in ABZ treated mice ($p = 0.0002$ compared to vehicle treated). Tumour marker levels (CA-125), tumour proliferation index (Ki67), ascites volume, number of tumour floating cells and protein levels in the ascites were all highly suppressed in ABZ treated mice. Plasma and ascitic VEGF concentrations were also dramatically reduced in these mice. These data demonstrate that in this revised experimental model, ABZ arrests tumour growth and has an even greater effect in suppressing ascites formation.

INTRODUCTION

Malignant tumours are characterized by uncontrolled cellular proliferation. Ongoing growth necessitates adequate blood supply to provide oxygen and nutrients and thus making it an angiogenesis dependent event (1). The key mediator of angiogenesis is vascular endothelial growth factor (VEGF), which is induced by many characteristics of tumours, most importantly hypoxia. VEGF has been implicated to play a central role in angiogenesis that is essential for growth and metastasis of tumours and for malignant ascites formation. Through interaction with its tyrosine kinase receptors (VEGFR-1, VEGFR-2 and VEGFR-3) located on vascular endothelial cells, VEGF causes inhibition of apoptosis, induction of cell proliferation, sprouting, migration and tube formation (2-4). It is now generally agreed that VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF (5). *In situ* hybridization studies have demonstrated that

the VEGF mRNA is markedly up-regulated in the vast majority of the tumours so far examined including, lung, breast, prostate, gastrointestinal, renal, bladder and ovary (6). A correlation between VEGF mRNA expression and vascularity of the tumour and degree of malignancy has also been documented (7, 8). Additionally, VEGF expression is correlated to high mitotic activity, FIGO stage (9) and tumour cell proliferation (10). These findings have led to the intriguing conclusion that tumour vessels require constant VEGF stimulation to maintain their proliferative activity and consequently VEGF deprivation of tumours leads to tumour regression.

Albendazole, a widely used broad spectrum benzimidazole carbamate (BZD) anthelmintic drug with an excellent safety record has been shown to bind to β -tubulin and act as a depolymerizing agent (11). In recent years, we have reported on the antiproliferative activity of ABZ(12, 13). Additionally, we recently reported that ABZ down regulates VEGF mRNA leading to suppression of VEGF levels (14). However, despite this, under the conditions employed (late stage disease} intraperitoneal tumour (human OVCAR-3) growth was neither arrested nor retarded (14). Overwhelming evidence however suggest that effective inhibition of VEGF leads to suppression of tumour growth. The availability of VEGF specific monoclonal antibodies capable of inhibiting VEGF-induced angiogenesis *in vivo*, the specific tyrosine kinase receptor inhibitors and the VEGF-Trap have all been shown to decrease tumour burden and provide evidence for a role of VEGF in tumourigenesis (15-18). On this basis, ABZ is expected to inhibit tumour growth. However, in our initial investigations on the effect of ABZ in OVCAR-3 tumour bearing mice, despite suppression of VEGF and ascites formation, no anti-tumour effect was observed (14). In that study drug treatment of mice was initiated at a very late stage of the disease when huge tumour burden was already present. The tumour core is where the drug must reach high enough

concentrations to effectively inhibit tumour growth. It is believed that, in that model, the huge tumour burden present at the time of initiating drug treatment severely restricted ABZ penetration into the highly proliferative layers of the tumour.

Additionally at advanced stages of the disease, tumour angiogenesis and growth is fuelled by murine VEGF produced by the host stromal cells and recruited into the tumour (19). Significant VEGF expression also exists in fibroblasts and immune cells that surround and invade the tumour mass (20). Subsequently tumours with greater host stromal invasion and stromal derived VEGF are less responsive to genetic or pharmacological manipulations targeting VEGF production (21). On this basis, we hypothesized that initiation of ABZ therapy at an earlier stage of the disease would provide better grounds for evaluating the anti-tumour effects of the drug. In the present study, using a battery of tests, we demonstrate that ABZ mediates potent *in vitro* antiproliferative responses and profound *in vivo* anti-VEGF and anti-tumour effects. This suggests that, timing of the anti-VEGF therapy has a critical impact on the tumour growth and hence the treatment outcome.

MATERIALS AND METHODS

Cell preparation. Cells were collected from the ascites of carrier mice inoculated with human ovarian carcinoma cell line OVCA-3. These cells were originally obtained from the American Type Culture Collection (ATCC), and prepared for *in vivo* growth and subsequently *in vitro* cytotoxicity assays as previously described (14). To ascertain that the cells collected are epithelial ovarian cancer cells, immunostaining for the detection of CA-125 was performed using the method described by McCormic et al. (22). For *in vitro* antiproliferative assays, cells harvested from ascites of carrier mice were maintained in RPMI 1640 medium with 2

mM l-glutamine, 2 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 0.01 mg/ml bovine insulin, supplemented with 100 units/ml penicillin and 100 units/ml streptomycin and 10% FBS in a humidified atmosphere at 37 °C.

In vitro cytotoxicity assay. Sulforhodamine B (SRB) assay (23) was used to determine the *in vitro* sensitivity of the tumour cells to ABZ. Briefly, cells were plated at a density of 3500 cells/well in 96-well plates and left in the incubator for 24 h. Following attachment, cells were treated with culture medium containing various concentration of ABZ (0.01 to 1.0 micromoles/L). ABZ was initially dissolved in ethanol and then diluted with the medium to provide the final desired ABZ concentration and a final ethanol content of 1%. Control cells were treated with medium containing 1% ethanol. After 72 h of exposure to the drug, cells were fixed in 10% (w/v) TCA for 30 min at 4°C followed by tap water washing ($\times 5$) and stained with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by 5 washes with 1% acetic acid before air-drying. Bound SRB was solubilized with 100 μ l 10mM Tris base (pH-10.5) and the absorbance read at 570 nm. Absorbance readings from the control wells were taken as 100% and absorbance from the ABZ treated wells are presented as % control (mean \pm s.e.m.). Each drug concentration was tested in eight wells and each experiment was repeated at least twice. To test the effect of multiple dosing and longer treatment periods, Trypan blue cell viability assay was used. Briefly, cells (20,000 per well) grown in 6 well plates were treated with various ABZ concentrations (0, 0.1 and 1.0 micromoles/L) for 3, 6 or 9 days. The cell culture medium was changed every other day. At the end of drug treatment period number of viable cells remaining were counted.

Colony formation assay. While the SRB assay measures cell proliferation, colony formation assay measures the productive integrity of the cells following withdrawal of drug treatment. The assays were performed as described by Liebmann *et al* (24) with some modifications. Briefly, exponentially growing cells in Petri dishes were exposed to ABZ for 2 h with the desired drug concentration, then washed with phosphate buffer solution and plated in 0.6% agarose containing Petri dishes. Following 3 weeks of incubation, number of colonies (cluster of cells greater than 50) in each plate was recorded. The experiment was performed twice and the results are presented as % of control.

Animals. For all experiments, 6-8 weeks old female nude athymic Balb C nu/nu mice (Animal Resources Centre, Perth, Western Australia) were used. They were kept under specific pathogen-free conditions and fed autoclaved pellets and sterile water ad libitum. The general health status of the animals were monitored daily. This work had institutional (University of New South Wales) animal ethics committee approval.

In vivo assessment of tumour growth Mice were inoculated i.p. with 10×10^6 OVCAR-3 cells isolated from the ascites of carrier mice and suspended in 1 ml of medium. Twenty one days later, a cohort was randomly selected and euthanased as day 0 controls. The remaining mice were randomly allocated to 2 groups of vehicle (0.5% CMC, 1mL given i.p.) or drug (ABZ 150 mg/kg) treated groups. Using the leg vein, 0.2 mL of blood was collected from each animal, and then animals were subjected to a peritoneal lavage (2 ml of sterile normal saline injected i.p. and aspirated after kneading). Cell free ascites fluid, ascites cells, plasma and tumours (in case of control group) collected were stored at -80°C for analysis.

Treatment was immediately initiated in the remaining 2 groups and continued thrice weekly for 4 weeks. Abdominal circumference, body weight and general health of each animal were checked each time before drug administration. At the end of treatment period, blood was collected through cardiac puncture, animals were euthanased using lethobarbital i.p. injection (VIRBIC, Sydney, Australia) and immediately subjected to a second peritoneal lavage as described above. Volume of ascites (volume of peritoneal wash aspirated minus 2 ml), total number of viable tumour cells collected from the peritoneal lavage and the weight of tumours dissected from the peritoneal cavity were recorded. All collected samples were appropriately stored at – 80 °C for subsequent analysis.

Maximum proliferation index and tumour marker levels. Immunohistochemical staining of proliferating cells with Ki67 antibodies has widely been substituted for mitotic counting in assessing tumour cell proliferation (25). To do this, the method described by McCormick et al (22) was used with minor modifications. Briefly, paraffin embedded samples (5- μ m thick) were placed on glass slides for Ki67 with staining primary monoclonal mouse human antibodies (Dako, California, 93013, USA). Samples were then deparaffinized in xylene, rehydrated in ascending series of ethanol and washed in Tris-buffered physiological saline. Ki67 positive cells were scored by using Zeiss AxioVision 3.0 image analysis program (Carl Zeiss Vision GMBH, Hallbergmoos, Germany). Ten randomly chosen areas from each sample were examined under microscope at x 200 magnification. The cell was considered Ki67 positive if there was a clearly detectable brown colour in the nucleus. The MPI was expressed as percentage of stained to non-stained cells in these tumour areas.

CA 125 (tumour marker) levels in cell free peritoneal wash were determined by the St. George Hospital Biochemistry laboratories (Sydney, Australia).

VEGF levels in plasma and ascites fluid. VEGF levels in plasma and cell free ascites fluid were determined by means of an enzyme-linked immunosorbent assay (ELISA) according to manufacturers instructions, (Quantikine R&D systems, Minneapolis, USA).

Protein assay. Protein content of the peritoneal wash collected from each mouse prior to initiation of drug treatment and at the end of the drug treatment period were determined using a protein assay kit according to the manufacturer instructions (Bio-Rad Laboratories, Australian subsidiary).

Data analysis and statistics. All data are reported as the mean \pm SE. In vitro cell proliferation data were analysed using student's *t* test followed by Tukeys. Animal data were analysed using Mann-Whitney U test. Effects were considered to be statistically significant at $p < 0.05$.

RESULTS

Inhibition of cell proliferation and colony formation. Immunostaining of cells collected from the peritoneal lavage of carrier mice were confirmed to be epithelial cells highly expressing CA-125 (Fig. 1A). Incubation of these cells with various concentrations of ABZ in culture medium for 72 h resulted in dose-dependent inhibition of cell proliferation (Fig. 1B). ABZ concentrations of over 0.1 micromoles/L inhibited cell proliferation in a dose-dependent manner. Inhibition of cell proliferation was highly significant ($p = 0.001$) in cells treated with 0.25 micromoles/L ABZ concentration. Cell viability in wells treated with the highest ABZ concentration (1.0 micromoles/L) for 3 days, was down to $2.07 \pm 0.72\%$ of the control ($p < 0.001$). Figure 1C

demonstrates that repeated treatment of cells with ABZ concentrations of 0.1 micromoles/L and higher for 3, 6 or 9 days leads to even greater growth inhibition. In contrast to single dose treatment, repeated treatment (3 doses over 5 days) with the 0.1 micromoles/L concentration led to significant ($p < 0.001$) inhibition of cell proliferation. Data from the colony formation assay (Fig. 1D) revealed that, short exposure of cells to ABZ does not affect the integrity of the cell and the cells can recover once the drug has been removed. Collectively these results demonstrate the potent effect of ABZ on inhibiting cell proliferation *in vitro* in OVCAR-3 cells collected from the peritoneal cavity of mice with malignant ascites. It is therefore expected that through interaction with tubulin and also deprivation of the endothelial and the tumour cells of VEGF, the drug would be able to inhibit tumour growth *in vivo*.

ABZ inhibits *in vivo* tumour growth. Tumours excised from vehicle and ABZ treated mice are depicted in figure 2A. It is evident from the tumour appearance that ABZ treatment has led to marked suppression of growth. The average tumour weight in 0 control animals (those euthanased at 3 weeks post cell inoculation) was 0.44 ± 0.06 g. For the vehicle and ABZ treated mice, average tumour weights were 1.96 ± 0.2 g ($P = 0.0002$, compared to control mice) and 0.65 ± 0.10 g ($P = 0.068$) respectively (Fig. 2B.) Using the formula “test – control /control x 100”, the tumour growth ratio during the 4 weeks of treatment were found to be 47% compared to 345% for the ABZ and vehicle treated mice respectively. These results demonstrate that, in this experimental nude mice model, initiation of ABZ treatment 3 weeks after cell inoculation leads to arrest of tumour growth.

Reduced Ki67 staining. Immunostaining of proliferating cells with Ki67 antibodies has widely been used for assessing tumour cell proliferation. A reduction of Ki67-defined cell proliferation has been found in patients with good response to chemotherapy for ovarian cancer (26). To gain further insight into the action of ABZ on highly proliferating tumour cells, tumours collected were subjected to Ki67 antibody immunostaining. Percentage of proliferating cells in ABZ treated tumours were 26.1 ± 7.1 % compared to 43.3 ± 5.2 % in vehicle treated tumours ($p = 0.041$).

Inhibition of ascites production. At the end of the treatment period, in contrast to the vehicle treated mice, the ABZ treated animals had no macroscopically visible sign of malignant ascites. In these mice, ABZ treatment led to inhibition of ascites formation resulting in 5/6 mice being completely ascites free. The volume of ascites collected from the vehicle and ABZ treated mice were 1.95 ± 0.12 ml and 0.03 ± 0.03 ml respectively (Fig. 3A; $p = 0.0001$). These results demonstrate the highly potent effect of ABZ in inhibiting ascites production in this experimental model of ovarian carcinoma and peritoneal carcinomatosis.

Suppression of ascitic cell density, tumor marker and protein levels. At euthanasia the number of tumour cells collected from the peritoneal lavage were 0.12 million and 144.8 million cells in ABZ and vehicle treated mice respectively (Fig. 3B). Depicted in figure 3C. are the CA-125 levels in the peritoneal wash of these mice prior to initiation of vehicle or ABZ therapy and at the end of the treatment period. At euthanasia CA-125 levels in the ABZ treated mice were 1680 ± 1163 units/ml compared to 34350 ± 5284 units/ml in the vehicle treated mice ($p < 0.001$). Excessive protein loss into the peritoneal cavity is a feature of malignant ascites brought about by the microvasculature hyperpermeability. Protein concentrations found in the cell free peritoneal wash of vehicle and ABZ treated mice are presented

in figure 3D. As expected, ascitic protein levels in the ABZ mice were extremely low.

Suppression of plasma and ascitic fluid VEGF levels. VEGF plays a central role in both tumour growth and production of ascites. Using a standard ELISA kit, VEGF levels were measured in the plasma and the peritoneal wash collected from animals just before and at the end of drug treatment (Fig. 4A. and 4B. respectively). VEGF levels were highly suppressed in both the plasma and the peritoneal lavage. In the peritoneal wash, VEGF levels were 1563 ± 1163 pg/ml in ABZ treated mice compared to 29420 ± 2771 pg/ml for those treated with the vehicle only ($p = 0.0001$).

DISCUSSION

Malignant neoplasms are characterized by uncontrolled cellular proliferation. Adequate blood supply and nutrients are required to sustain their growth. Tumour growth and metastasis are angiogenesis-dependent events (27, 28). VEGFs are widely regarded as the most important proteins involved in the development of vasculogenesis, angiogenesis and lymphangiogenesis (3). Extensive research with various anti-VEGF agents have led to the intriguing conclusion that tumour vessels require constant stimulation with VEGF to maintain not only their proliferative properties but also some key morphological features (1, 29, 30). While, overwhelming experimental evidence suggest that inhibition of angiogenesis can lead to suppression of tumour growth (21), the degree of tumour suppression has been shown to be highly dependent on the pharmacological manipulation, tumour type and its VEGF dependency, stromal VEGF contribution and importantly stage of disease at initiation of drug therapy (21). In advanced disease, other pathways including growth

factors, inflammatory cytokines and hormones also up-regulate VEGFmRNA expression. Furthermore, as tumours grow, they begin to produce a wider array of angiogenic molecules both VEGF related such as VEGF-C, PlGF and VEGF-independent molecules such as bFGF or IL-8 (20, 30). Moreover, antiangiogenic agents in clinical studies have shown little regression of existing tumours, but have been effective in preventing the recurrence of disease when used in an adjuvant setting. These findings suggest that the greatest role of antiangiogenic agents may be in the prevention of new blood vessel formation rather than resorption of existing tumour vessels (31). Our findings are consistent with these reports. Here, we found that ABZ treated mice had significantly smaller tumours than the vehicle treated group ($p = 0.0002$) but not significantly different ($p = 0.068$) from the 0 time control group ($p = 0.068$). Hence demonstrating the arrest of tumour growth in ABZ treated mice. This correlates well with the *in vitro* results showing that ABZ potently inhibits proliferation of the very aggressive ascitic tumour cells in a dose and time-dependant manner. In addition, the *in vivo* results revealed that, tumour cell proliferation index, tumour marker levels, ascites formation, ascitic tumour cell number, ascitic protein concentration, plasma and ascitic fluid VEGF levels were all considerably suppressed in ABZ treated mice.

VEGF expression is highly regulated by hypoxia, which induces binding of hypoxia-inducible factor-1 α to the hypoxia response element in the VEGF gene promoter region (32). Recent reports by Majeesh et al. and Escuin et al. (33, 34) have revealed that, regardless of chemical structure, binding site, or net effect on microtubule polymer mass, all microtubule-targeting agents (MTAs) down-regulate HIF-1 α . A proangiogenic transcription factor critically involved in tumour survival, progression, and metastasis, HIF-1 α highly regulates VEGF production (35-37). Prior

to them, in 1992, Ettenson and Gotlieb (38) had reported antiangiogenic properties for MTAs *in vitro* which was later confirmed *in vivo* by Belotti et al. in 1996 (39). It has also been reported that endothelial cells are extremely sensitive to MTAs (40). In experimental animal models, both anti-VEGF agents (monoclonal antibodies, VEGF trap, TKR inhibitors) and MTAs have been shown to inhibit tumour growth. At present it is unknown to what extent the anti-tumour effect of the later group of drugs can be attributed to their indirect anti-VEGF properties. Interestingly, the primary mode of action of ABZ in susceptible parasites has been attributed to its binding to β -tubulin leading to inhibition of microtubule polymerisation. Additionally, ABZ is known to bind to β -tubulin and to act as a depolymerizing agent (41, 42). Over the last few years we have progressively reported on the potential anticancer properties of ABZ and most recently on its anti-VEGF properties and with these latest developments, we are currently investigating the binding of ABZ to human tumour tubulin and the contribution of such an event to the anti-VEGF and anti-tumour effects of the drug.

In conclusion, a salient feature of the results presented here is that albendazole potently inhibits tumour growth, an effect that may at least partly be mediated through affective suppression of tumour VEGF and hence suggesting potential therapeutic value for the drug in the treatment of highly VEGF dependent cancers.

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Figure legends:

Figure 1. OVCAR-3 cells isolated from the ascitic fluid of carrier mice were used for all *in vitro* tests. Initially these cells were immunostained and examined for CA 125 expression (magnification $\times 200$). The brown staining confirms the epithelial nature of these tumour cells (A). Dose-response inhibition of proliferation of tumour cells *in vitro* where cells were treated with a single dose of ABZ (0.01 –1.0 micromoles/L) for 72 h (B). Cell viability as assessed by Trypan Blue, after 3, 6 or 9 days of treatment with ABZ (C). Cell integrity following ABZ treatment was assessed using the colony formation assay, where after 2 h of drug exposure, cells in agar plates were incubated for 3 weeks (medium without drug) and the number of colonies formed were counted (D). Columns, mean; bars, SE.

Figure 2. Depicted in section A are tumors excised from peritoneal cavity of mice treated i.p. (1 mL) 3 times weekly for 4 weeks with the vehicle or ABZ (150 mg/kg). Arrest of tumour growth in ABZ treated mice is demonstrated in section B. C_0 is 0 time control representing tumour weight (g) in the group of mice euthanased 3 weeks after cell inoculation. Values represent mean \pm SE and the median for each group.

Figure 3. Ascites volume (A), number of floating tumour cells (B), tumour marker level (C), protein concentration (D), in vehicle (VEH or ABZ treated mice). 1 and 2 refer to pre and post treatment values in the same group of animals respectively. Mice treated with ABZ barely produced any ascites during the treatment period. Columns, mean; bars, SE.

Figure 4. VEGF levels in plasma (A) and ascites fluid (B) of vehicle (VEH) and ABZ treated mice showing huge difference between the 2 group at the end of the treatment period (ABZ-2 versus VEH-2; $p < 0.0001$). Columns, mean; bars, SE.

Figure 1A

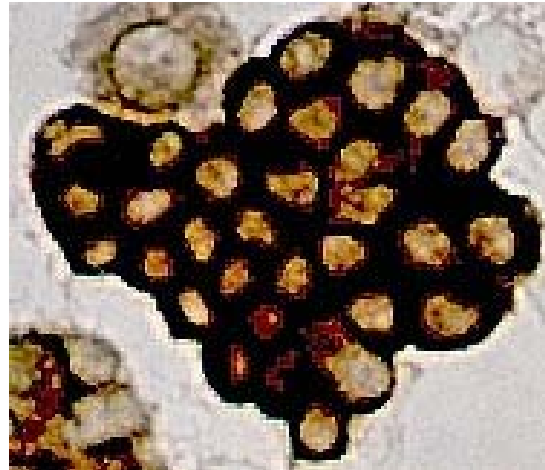


Figure 1B

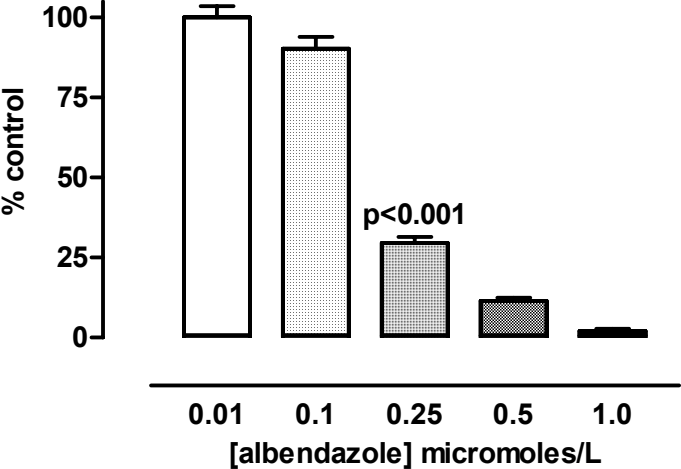


Figure 1C

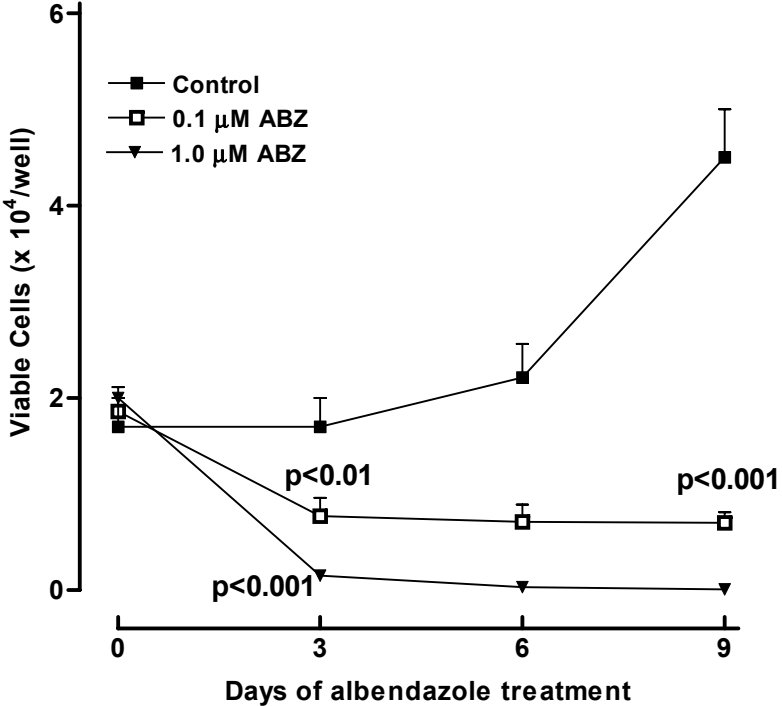


Figure 1D

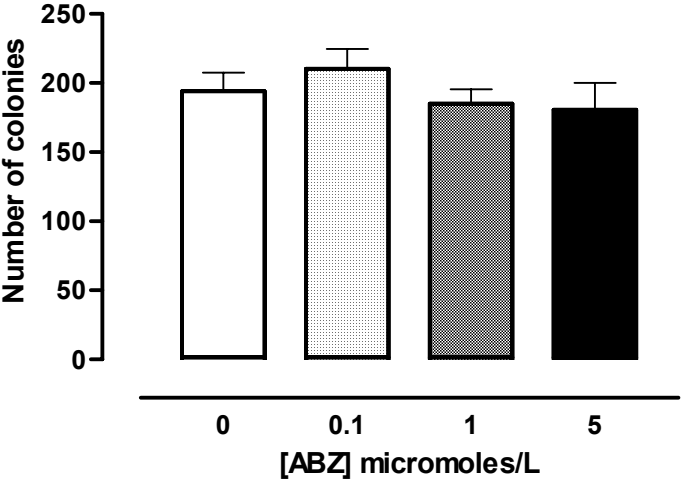


Figure 2A



Figure 2B

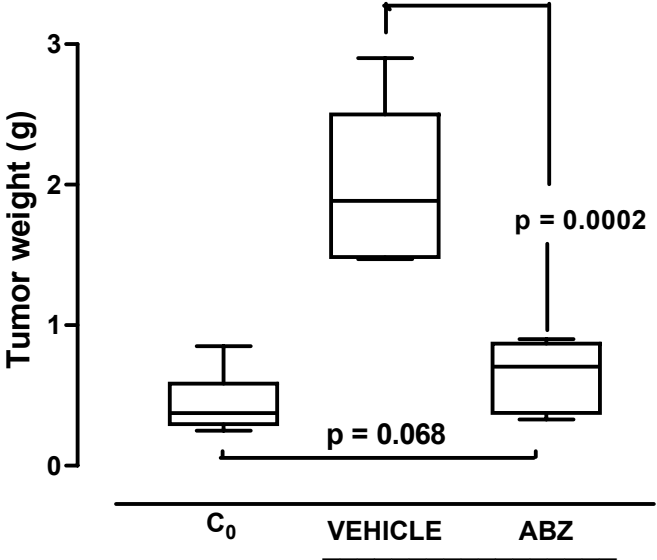


Figure 3A

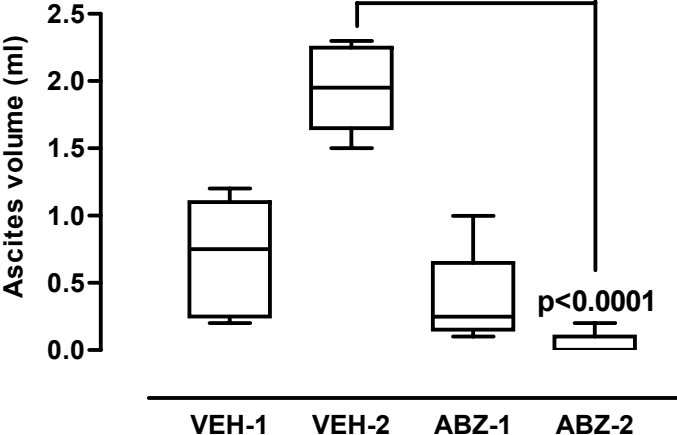


Figure 3B

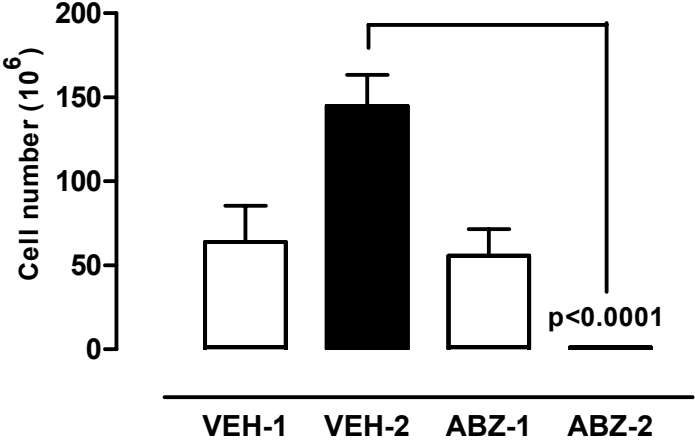


Figure 3C

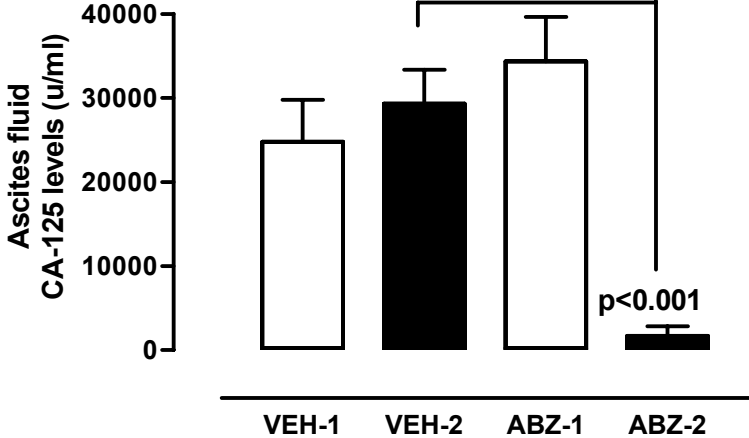


Figure 3D

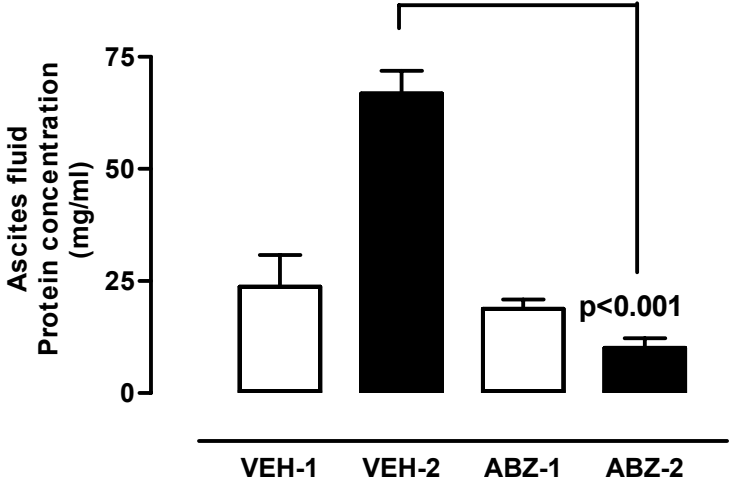


Figure 4A

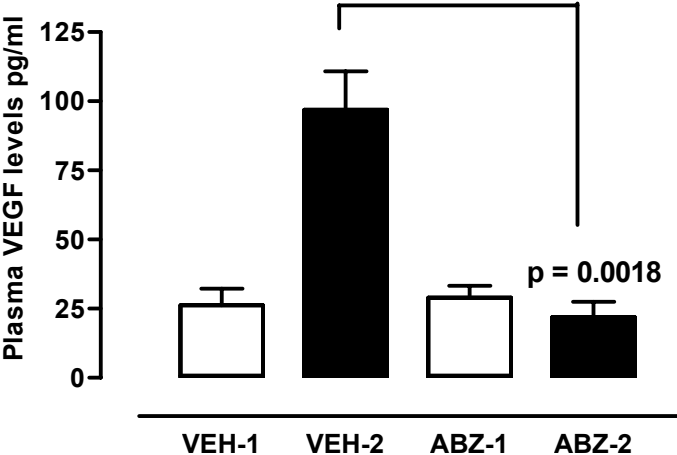


Figure 4B

