

## Potent Inhibition of Tubulin Polymerisation and Proliferation of Paclitaxel-resistant 1A9PTX22 Human Ovarian Cancer Cells by Albendazole

STEPHANIE W.L. CHU, SAMINA BADAR, DAVID L. MORRIS and MOHAMMAD H. POURGHOLAMI

*Cancer Research Laboratories, University of New South Wales Department of Surgery,  
St. George Hospital (SESAHS), Sydney, NSW 2217, Australia*

**Abstract.** *Background: Resistance to paclitaxel (PTX) is a major concern in treating ovarian cancer. Thus, agents effective in taxane-resistant tumours are highly sought. It has recently been shown that albendazole (ABZ) is a potent inhibitor of cell proliferation, angiogenesis and tumour growth. This study was designed to examine the efficacy of ABZ in PTX-resistant human ovarian cancer 1A9PTX22 cells. Materials and Methods: Using both the parent PTX-sensitive 1A9 and PTX-resistant sub-line 1A9PTX22 cells, the effects of both drugs on cell proliferation, tubulin polymerisation and microtubule distribution across the cell was investigated. Results: Comparison of the inhibitory concentration to achieve 50% cell death ( $IC_{50}$ ) revealed that, unlike PTX, ABZ is highly efficacious in inhibiting proliferation of 1A9PTX22 cells. The finding that ABZ but not PTX is highly effective in disrupting tubulin polymerisation in these cells confirmed involvement of the tubulin pathway. Conclusion: Data from this study suggest that ABZ is effective in suppressing growth of PTX-resistant ovarian tumour cells.*

Ovarian cancer is a silent killer that often has no early symptoms. Many women are diagnosed at an advanced stage of the disease. The current treatment for the disease is surgery followed by postoperative chemotherapy. The standard chemotherapy is paclitaxel (PTX) with platinum based drug (1, 2). PTX is an antimitotic drug that causes mitotic arrest by enhancing microtubule stability and thus preventing cells moving from metaphase to anaphase (3).

*Correspondence to:* Mohammad H. Pourgholami, Cancer Research Laboratories, University of New South Wales Department of Surgery, St. George Hospital (SESAHS), Sydney, NSW 2217, Australia. Tel: +61 291132975, Fax: +61 291132456, e-mail: mh.pourgholami@unsw.edu.au

**Key Words:** Albendazole, paclitaxel, ovarian cancer, resistance, tubulin.

The binding site of PTX is unique. It binds to  $\beta$ -tubulin on the inner surface of the microtubules to stabilise the dynamics (4). Although PTX is an effective drug, poor overall outcome in relapsed patients continues to be a major problem (5). The development of a more potent antimitotic drug is becoming increasingly important.

Albendazole (ABZ), a benzimidazole carbamate, is an effective anthelmintic drug with low toxicity in human (6). ABZ exerts its antiproliferative effect in helminths mainly by binding to  $\beta$ -tubulin to inhibit microtubule polymerisation (7). Rolin *et al.* reported that ABZ inhibited cell proliferation by blocking cells at early metaphase (8). Our laboratory demonstrated that ABZ has an inhibitory effect on hepatocellular carcinoma cells both *in vitro* and *in vivo* (9). In addition, we found that PTX-resistant leukaemia cells CEM/dEpoB300 are responsive to ABZ (10). More recently, we have identified ABZ as a potent inhibitor of vascular endothelial growth factor (VEGF) and angiogenesis (11). These findings suggest that ABZ may be an important candidate for anticancer therapy.

The aim of the present study was to look into the antiproliferative effect of ABZ and its effect on microtubules in PTX-sensitive and PTX-resistant ovarian cancer cell lines.

### Materials and Methods

**Chemicals.** ABZ was purchased from ICN Pharmaceuticals, Inc. PTX, colchicine, RIPA lysis buffer, protease inhibitor cocktail, mouse anti- $\alpha$ -tubulin and horseradish peroxidase-conjugated goat anti-mouse IgG were purchased from Sigma Aldrich. Alexa Fluor 488 goat anti-mouse IgG antibody was purchased from Molecular Probes, Invitrogen.

**Cell culture.** 1A9 and 1A9PTX22 were kindly provided by Dr Marianne Poruchynsky of the National Cancer Institute, USA. Cells were grown in RPMI medium supplemented with 10% foetal bovine serum. These cells were grown in 5%  $CO_2$  at 37°C. 1A9PTX22 cells were maintained in 15 ng/ml PTX and 5  $\mu$ g/ml verapamil continuously. Drugs were removed from the medium 4-7 days prior to all experiments (12).

**Cell proliferation assay (MTT assay).** Cells were seeded in 96-well plates at a density of 1,500 and 700 cells (1A9) and 7,000 and 5,000 cells (1A9PTX22) per well for 72 h and 120 h, respectively. Media were changed with RPMI supplemented with 5% FBS containing the appropriate concentration of the drug(s) or the vehicle. Anti-proliferative effects of drugs were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The absorbance was read at 562 nm.

**Tubulin polymerisation assay.** The protocol was provided by Dr. Marianne Poruchynsky (13). Briefly, cells were lysed with hypertonic solution buffer (1 mM  $MgCl_2$ , 2 mM EGTA, 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride, 20 mM Tris HCl pH 6.8) containing protease inhibitors. The samples were centrifuged at 13,000  $xg$  for 10 min at 37°C and supernatant containing depolymerised tubulins was transferred to a new tube. The pellet (polymerised tubulin) was resuspended in an equal amount of hypotonic buffer before analysing 20  $\mu g$  of each sample by Western blot and quantifying by densitometry. The percentage change in polymerised tubulin (%) was determined by dividing the densitometric value of polymerised tubulin by the sum of polymerised and depolymerised tubulin  $\times 100$ .

**Western blot.** Western blot analysis was performed using 4-8% SDS-PAGE. NuPAGE LDS sample buffer (4x) (Invitrogen) was added to the samples and the mixtures were boiled at 100°C for 5 min. Proteins were then loaded on the gel followed by transfer to PVDF overnight at 35 V, 4°C. The membrane was blocked with 5% milk in TBST for 1 h and probed with mouse anti- $\alpha$ -tubulin (0.5  $\mu g/ml$ ) for 1 h at 37°C. The membrane was further probed with goat anti-mouse conjugated with horseradish peroxidase (1:160,000) for 1 h. Bands were detected using chemiluminescence reagent (Perkin Elmer Cetus, Foster City, CA, USA).

**Immunofluorescence.** Cells were grown on sterile coverslips embedded in a 6-well plate. Cells were fixed with ice-cold methanol. Samples were blocked with 5% milk in PBS and probed with mouse anti- $\alpha$ -tubulin followed by alexa fluor 488 goat anti-mouse IgG antibody. Slides were allowed to dry overnight before viewing with confocal microscope. The cells were analysed using an Olympus confocal microscope (Olympus, Tokyo, Japan).

## Results

**Antiproliferative effect of antimitotic drugs against PTX-sensitive and PTX-resistant cells.** The antiproliferative effect of ABZ on 1A9 and 1A9PTX22 were compared to those induced by colchicine and PTX. Results are depicted in Figure 1. Cells were exposed to drugs for 3 days (colchicine) or 5 days (ABZ and PTX). These drugs were chosen as they are classic antimitotic drugs that either depolymerise microtubules (colchicine) or enhance polymerisation of microtubules (PTX). The results demonstrate the inhibitory effect on 1A9 and 1A9PTX22 cells by ABZ ( $IC_{50}=237$  nM  $\pm$  3.65 and 351 nM  $\pm$  90 respectively). While 1A9PTX22 showed very little sensitivity to PTX, 1A9 demonstrated a higher sensitivity ( $IC_{50}=2.01$  nM  $\pm$  0.17). Both cells were sensitive to

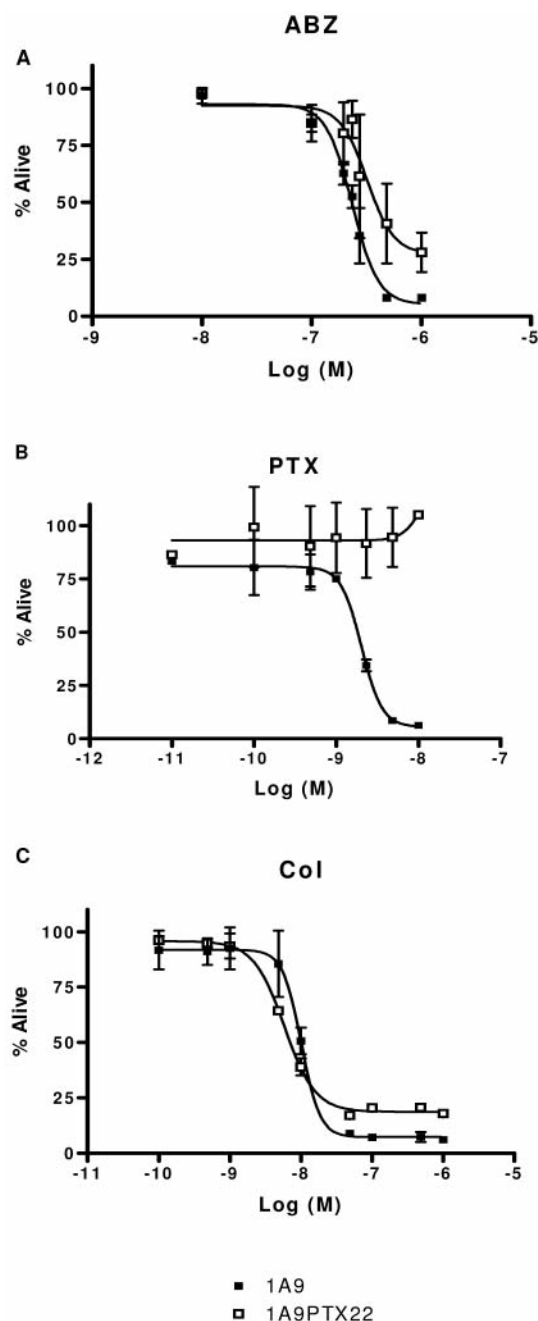


Figure 1. Antiproliferative effect of antimitotic drugs on 1A9 and 1A9PTX22. Cells were treated with drugs for (A) 3 days (colchicine) and (B, C) 5 days (PTX and ABZ) over a range of concentrations. For 5 days' treatment, media were replaced with fresh media containing the appropriate drug every other day. Mean  $\pm$  S.E.M is reported.

colchicine, with 1A9 having an  $IC_{50}$  of 10.3 nM  $\pm$  0.94 and 1A9PTX22 an  $IC_{50}$  of 5.90 nM  $\pm$  0.09.

**ABZ induces depolymerisation in 1A9 and 1A9PTX22 cells.** To study whether ABZ interacts with human ovarian cancer

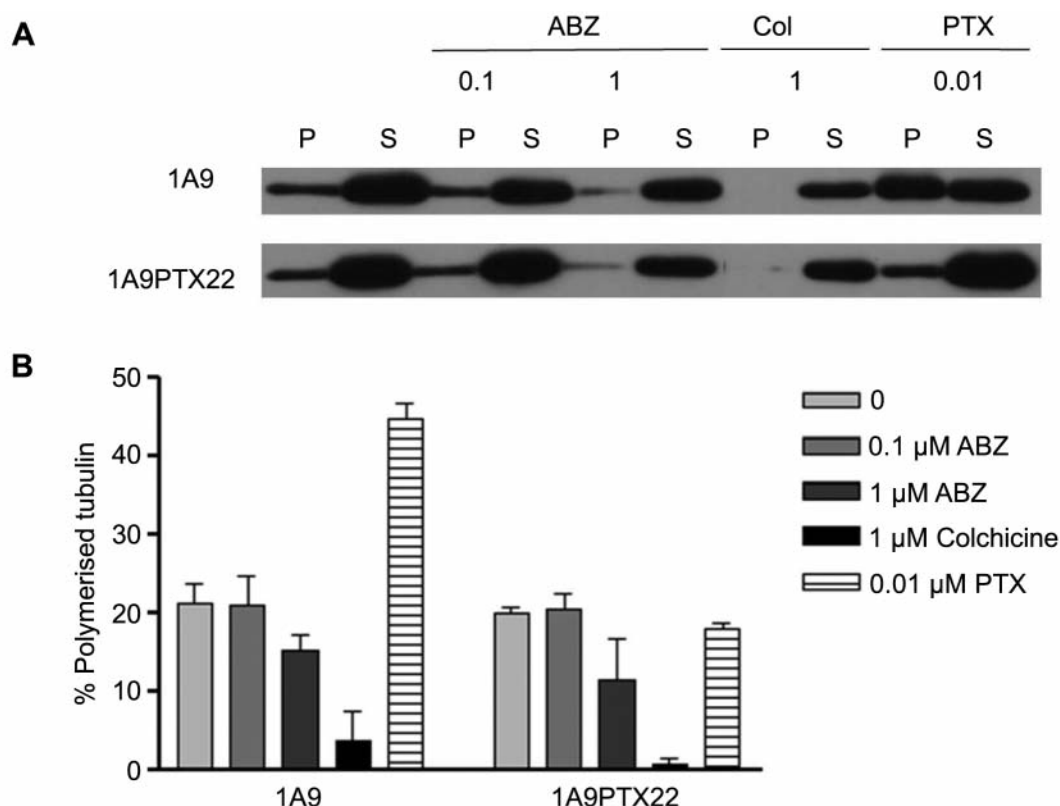
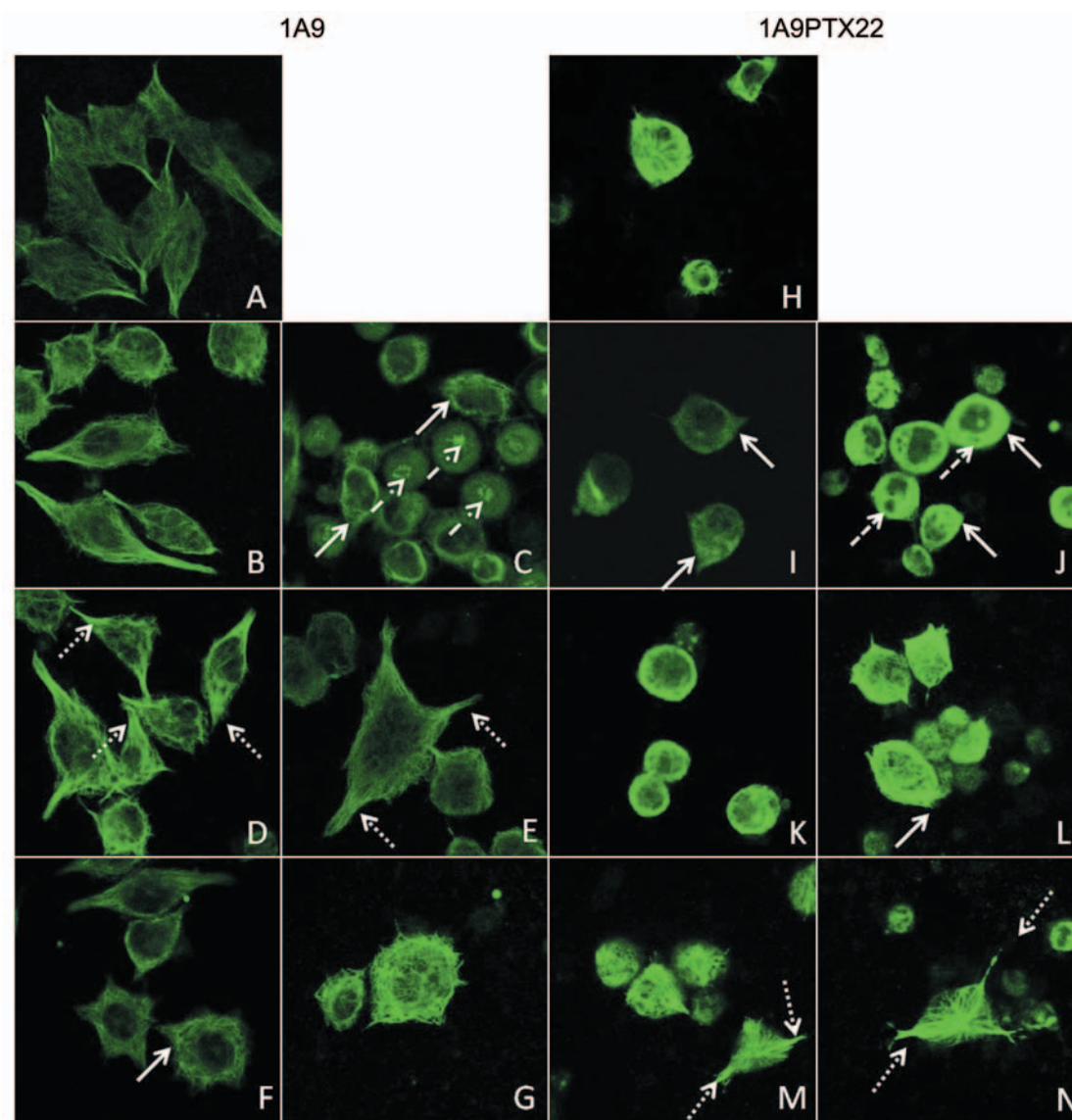


Figure 2. ABZ induced microtubule depolymerisation in both 1A9 and 1A9PTX22 cells. Exponentially dividing cells were treated with the vehicle (control), colchicine (1  $\mu$ M), PTX (0.01  $\mu$ M) or ABZ (0.1 and 1  $\mu$ M) for 24 h and then lysed by hypotonic buffer. The 2 fractions (polymerised and depolymerised) were separated by centrifugation and the pellets were then suspended in lysis buffer. Equal aliquot of polymerised (P) and depolymerised (S) fractions were loaded on SDS-PAGE and evaluated by Western blotting using an antibody to  $\alpha$ -tubulin. The change in percentage of polymerised tubulin (% P) induced by each treatment was determined using the formula:  $\% P = P / (P + S) \times 100$ . Mean  $\pm$  S.E.M is reported.

cells, we tested its ability to promote tubulin polymerisation. The cells were treated with antimetabolic drugs at selected concentrations for 24 h. Figure 2A shows the Western blot results demonstrating the depolymerising effect of colchicine in 1A9 (% P=3.7%) and 1A9PTX22 (% P=0.7%) cells. These results show that both cell lines are sensitive to colchicine. PTX, on the other hand, increased tubulin polymerisation of 1A9 by 1-fold (% P=44.6%). Since 1A9PTX22 is resistant to PTX, only a very low percentage of polymerised tubulin was observed (0.1-fold). We found that at 0.1  $\mu$ M ABZ did not have an effect on either of the cell lines (Figure 2B). However, at a higher concentration (1  $\mu$ M), the level of polymerised tubulin decreased in 1A9 and 1A9PTX22 cells (0.28- and 0.42-fold, respectively). It appears that ABZ exerts a stronger inhibition of tubulin polymerisation in PTX-resistant cells.

*ABZ causes bundling of microtubules in 1A9 and 1A9PTX22.* Cells were exposed to colchicine, PTX and ABZ at various concentrations for 24 h (Figure 3). Normal,

untreated 1A9 cells displayed long filamentous microtubule distribution across the cell (Figure 3A). It was observed that colchicine caused a disorganised microtubule network in 1A9PTX22 cells (Figure 3I, J) and that there were blebbing cells (Figure 3J). Figure 3C demonstrates mitotic arrest caused by colchicine in 1A9 cells. PTX is a polymerising agent (3, 14). Results show that bundling of microtubules occurred in 1A9 after 24 h treatment with PTX (Figure 3D, E). The microtubules were long and thick along the periphery of the cells and microtubules no longer covered the entire cell. In the resistant 1A9PTX22 cells, a stronger dose of PTX (100 nM) was used to promote disruption of the cell cytoskeleton. The intensity of the microtubules was greatly increased compared to the control (Figure 3H, L). ABZ caused a formation of bundles of short microtubules around the edges of the cells in both cell lines (Figure 3F-G, M-N). At higher concentration (1  $\mu$ M), there was a total reorganization of the microtubule network. In 1A9PTX22 cells, the bundles of microtubules appeared to be longer compared to those of 1A9 cells (Figure 3G, N).



1A9 (A-G) and 1A9PTX22 (H-N) cells were treated with vehicle (A+H), 0.1 nM colchicine (B+I), 10 nM colchicine (C+J), 0.01 nM PTX (D+K), 1 nM PTX (E), 100 nM PTX (L), 0.25  $\mu$ M ABZ (F+M) and 1  $\mu$ M ABZ (G+N) for 24 h. Disorganised microtubules:  $\leftarrow$  mitotic arrest,  $\leftarrow$  - bleb,  $\leftarrow$  - mitotic bundles,  $\leftarrow$  ..... Representative of 3 separate experiments.

Figure 3. Effects of anti-mitotic drugs on the cytoskeleton of 1A9 and 1A9PTX22 cells.

## Discussion

Resistance to PTX remains a major obstacle to successful chemotherapy. Therefore, there is an increasing demand for effective alternative treatments to increase survival rates of cancer patients. A few mechanisms have been suggested for the development of resistance, including alterations in the tubulin,

increase in multidrug resistance (MDR), and dysfunctional apoptosis machinery (15-17). However, the exact mechanism is not yet fully understood. In the present study, we used human ovarian carcinoma PTX-sensitive 1A9 and PTX-resistance 1A9PTX22 cells. 1A9PTX22 has a point mutation on  $\beta$ -tubulin and is 24-fold more resistant to PTX compared to 1A9. It is not PTX dependent in growth (12).



Benzimidazoles selectively bind to the (+) end of the microtubule to form the “capping” effect, inhibiting further addition of tubulin subunits (18-22). A recent study using NMR spectroscopy has reported that benomyl, an antifungal benzimidazole derivative, binds to a novel site on  $\beta$ -tubulin (23). ABZ is a safe and effective anthelmintic used against a variety of nematode infections (6). However, its effect(s) on cancer cell tubulin-binding has not to date been described. We have previously reported that ABZ induces apoptosis in hepatocellular carcinoma cells by arresting cells at the G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phases of the cell cycle (9). Additionally, we found that in PTX-resistant leukaemic CEM/dEpoB300 cells, the response to ABZ is linked to caspase-3 and Mcl-1 expression levels following ABZ treatment (10). Here, we demonstrate that through disrupting tubulin polymerisation, ABZ also inhibits the cell growth in PTX-resistant human ovarian carcinoma 1A9PTX22 cells. These findings suggest that ABZ may be a potential substitute for PTX in PTX-resistant patients.

Microtubules are highly dynamic structures and are made up of tubulin subunits. They are involved in various activities of the cells, including one of the most complex and most important cellular functions, mitosis. As reported, in the absence of drugs, the microtubule network of the cells is well organised with long and filamentous microtubules covering the entire cytoplasm. On treatment of cells with colchicine, a classic depolymerising agent, a dose-dependent effect was obtained, where higher drug concentrations induced substantial changes to the microtubule network in both cell lines. The microtubules appeared to be broken down into smaller fragments. In 1A9PTX22, there were formations of blebs, which is an indication of apoptosis. Conversely, treatment with polymerising agent PTX (3, 14, 24, 25) led to disorganisation in PTX-treated 1A9 cells but not in 1A9PTX22 cells. When treated with ABZ, formation of microtubule bundles in both 1A9 and 1A9PTX22 cells was quite evident. The effect was more notable in 1A9PTX22 cells where there was total rearrangement in the microtubule network. At a lower concentration (0.25  $\mu$ M), the microtubule bundles were thick and short. On the other hand, at 1  $\mu$ M, the microtubules were longer. In 1A9 cells, although the microtubule bundles were shorter than in PTX-treated 1A9 cells, the intensity of the cellular microtubules was greater. Since all three antimetabolic drugs shift the dynamic equilibrium that normally exists in cells, tubulin polymerisation assay was carried out on each drug to determine their influences on the polymerisation of the microtubules. Interestingly, ABZ reduced the percentage of polymerised tubulin in a dose-dependent manner. The effect was more evident in 1A9PTX22 cells where there was 0.42-fold decrease in the polymerisation. Comparable results were obtained in OVCAR-3 nude mice cells (data not shown). Collectively, these observations indicate that ABZ not only depolymerises parasite microtubule but is also a depolymerising agent in human cancer cells.

In conclusion, this report shows for the first time the effect of ABZ on tubulin depolymerisation in cancerous cells. The microtubule-disrupting effect of ABZ and PTX correlated well with their antiproliferative activity *in vitro*. More interestingly, in contrast to PTX, ABZ was highly effective in disrupting microtubules and inducing cell death in 1A9PTX22 cells. Further studies on these potentially useful therapeutic effects of ABZ are warranted.

## Acknowledgements

This study was supported by Rodney Horan – Great Australian Stroller for Liver Cancer. The authors would like to thank Dr. Marianne Poruchynsky for providing the cells and tubulin-binding assay protocol.

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Received June 18, 2009

Accepted July 9, 2009