Albendazole-Cyclodextrin Complex: Enhanced Cytotoxicity in Ovarian Cancer Cells

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Abstract. *Background: Over recent years, we have identified a potentially new indication for albendazole (ABZ) namely that of an anticancer agent. Our recent data indicate that besides regional use, the drug is quite likely to be useful as a systemic anticancer agent. However, with extremely low solubility, ABZ has to be prepared in a biocompatible solubilized form before any systemic evaluation is possible. The present study aimed at preparing soluble ABZ and evaluating its in vitro antiproliferative efficacy and toxicity. Experimental design: Using β-cyclodextrins (CDs), various formulations of ABZ were prepared and tested in cell culture for antiproliferative efficacy, cell integrity and cell toxicity against human ovarian cancer cell lines 1A9, OVCAR-3 and SKOV-3. Hepatocytes isolated from patients undergoing liver tumor resection were used for toxicity evaluations. Results: Treatment of tumor cells with ABZ–CD + citric acid (CA) solution led to dose-dependent inhibition of cell proliferation. Compared to an ethanolic solution of ABZ, ABZ-CD + CA increased the antiproliferative efficacy of ABZ. Furthermore, in contrast to the ethanolic solution, ABZ-CD-CA complex profoundly ($p<0.001$) reduced the number of OVCAR-3 colonies formed. Fresh human hepatocytes exposed for 3 days to the highest ABZ concentration used in the study (1 μM), revealed no drug toxicity. Conclusion: Complexation of ABZ with β-cyclodextrin leads to the formation of an ABZ solution with potent antiproliferative effects. This solution may find clinical value as an intravenous anticancer agent.*

ABZ is a benzimidazole derivative with a broad spectrum of activity against human and animal helminths. We have previously reported antitumor properties for ABZ in both experimental animals and in a pilot clinical trial (1-3). In addition, more recent results from our laboratory have shown ABZ to be an inhibitor of vascular endothelial growth factor (VEGF) and angiogenesis plus a potent inducer of apoptosis in paclitaxel-epothilone-resistant cells (4, 5). Collectively, these findings are indicative of significant clinical potential for using ABZ in the systemic treatment of cancer. However, due to very low aqueous solubility, ABZ cannot be administered *i.v.* and when used orally, it has been shown to have limited (<5%) and variable absorption (6) with very little (if any) drug reaching the systemic circulation as a result of high hepatic first-pass effect (7). Several strategies have been put forward to enhance ABZ solubility and bioavailability. Incorporating it into 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) is possibly the most convenient and biocompatible approach (8).

Cyclodextrins (CDs) are cyclic oligosaccharides containing covalent linked glucopyranose rings. Alpha (α), beta (β) and gamma (γ) CDs are naturally occurring molecules containing six, seven and eight glucopyranose units respectively. The numbers of these units determine the size of a cone-like cavity, into which many compounds form stable water-soluble complexes (8). Hence, CDs and their derivatives are used in pharmaceutical formulations to enhance solubility and bioavailability of poorly water-soluble compounds (9, 10).

The aim of the present study was therefore to prepare a soluble, biocompatible formulation of ABZ and to test the *in vitro* efficacy and toxicity of the various preparations.

Materials and Methods

Materials. ABZ was purchased from ICN Biomedical Inc. (Australian subsidiary, Sydney). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was obtained from Sigma (Australian subsidiary, Sydney). All materials used in this study were of analytical grade.

ABZ-CD preparation and analysis. Inclusion complex formation between ABZ and CD was prepared by dissolution of ABZ in HP-β-CD. An excess amount of ABZ was dissolved in a 200 mM solution of HP-β-CD and citric acid (50 mM), ascorbic acid (50 mM) or hydrochloric acid (0.02 mM and 50 mM). Suspensions were shaken in

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Key Words: Albendazole, β-cyclodextrin, ovarian cancer, efficacy, toxicity.

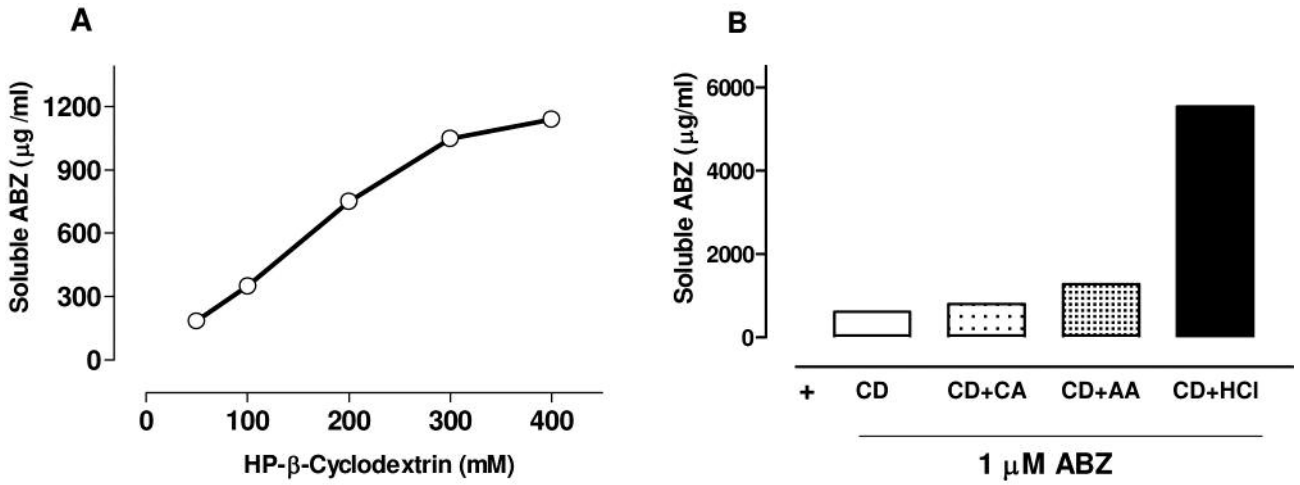

Figure 1. *Amount of ABZ dissolved in increasing concentrations of HP-β-CD (50, 100, 200, 300 and 400 mM). The resultant suspension was shaken in a water-bath (25˚C) for 48 h followed by filtration through a 0.2 µm membrane filter and analyzed for the ABZ content by a validated HPLC technique (A). Solubility of ABZ- HP-β-CD in the presence of various acids. Inclusion complex formation between ABZ and HP-β-CD was prepared by dissolution of ABZ in a solution of HP-β-CD. An excess of ABZ was dissolved in a 200 mM solution of HP-β-CD in citric acid (CA; 50 mM), ascorbic acid (AA; 50 mM) or HCl (0.02 mM - 50 mM). After processing, the resultant solution was assayed for ABZ content (B).*

a water-bath at 25˚C for 48 h, followed by filtration through a 0.2 µm membrane filter. The ABZ content of each preparation was then determined using a validated and fully automated HPLC system as described elsewhere (11). The assay was conducted using a 5 µm C18 column and a mobile phase containing mixture of acetonitrile, phosphate buffer (0.01 M) and tetrabutylammonium hydrogen sulfate (5 mM) at a flow rate of 1 ml/min. Filtered aliquot-fractions containing ABZ were analysed at 291 nm. ABZ retention time was 19.5 min. Linearity of the assay was tested using an ABZ range of 0.01-1 µg/ml, yielding a correlation coefficient of 0.99 with consistent inter- and intra-assay values.

Proliferation assay. Cell lines OVCAR-3 (human ovarian carcinoma cell line) SKOV-3 (human ovarian cystadenocarcinoma cell line) used in this study were originally obtained from the American Type Culture Collection (ATCC) and maintained according to the supplier's instructions. 1A9 (Human ovarian carcinoma) was kindly donated by Marian Poruchynsky of the National Cancer Institute, USA.

Sulforhodamine B (SRB) assay (12) was used to examine the antiproliferative efficacy of ABZ on these cells. Cells plated in 96-well Corning tissue culture dishes at densities of 1,500-5,000 cells/well were left for 24 h at 37˚C under a humidified atmosphere containing 5% CO_2. Following attachment, cells were treated with cell culture medium (RPMI plus 5% fetal calf serum, FCS) containing different concentrations of ABZ (0.1-1 µM). ABZ-CD was diluted with the culture medium to give the final desired drug concentrations. At the end of the treatment period (72 h), cells were fixed in 10% (w/v) trichloroacetic acid for 30 min at 4˚C followed by tap water washing (5×) and stained with 100 µl of 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by five washes with 1% acetic acid before air drying. Bound SRB was solubilized with 100 µl of 10 mM Tris base (pH 10.5) and the absorbance read at 570 nm.

Colony formation assay. The colony formation assay measures the productive integrity of the cells following withdrawal of drug treatment (13). The assays were performed according to the method described by Hamburger and Salmon (14). Briefly, single cell suspensions were treated for 2 h with the desired drug concentration, washed with phosphate buffer solution and plated in Petri dishes containing 0.6% agarose. Following 3 weeks of incubation with non-ABZ-containing medium, the number of colonies (any cluster of cells greater than 50) in each plate was recorded. The experiment was performed twice and the results (mean ± s.e.m.) are presented as % of the control.

Toxicity assay. ABZ-CD toxicity was tested using fresh human hepatocytes isolated from undiseased portions of liver of cancer patients under going surgical liver resection. These cells were isolated utilizing a protocol described elsewhere (15). Digested liver tissue suspended in ice-cold suspension medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotic] was filtered through a series of 800, 400 and finally 100 µm Teflon meshes. The resultant suspension was washed by low speed centrifugation (×3) at 50 ×g for 3 min and a 20% Percoll wash was performed on the re-suspended pellet. Cells were then plated in uncoated 6-well plates at a density of 0.875×10^6 cells/well. Culture medium (William E) enriched with 10% FCS, endothelial growth factor (EGF; 5 ng/ml), insulin (5 µg/ml), antibiotics and hydrocortisone (0.5 µg/ml) was used to grow the cells. Four days later, cells were treated with the various preparations of ABZ (0.1-1 µM) in ethanol, HP-β-CD or HP-β-CD + citric acid for 3 days, following which cell viability was assessed using the MTT assay as described elsewhere (5).

Statistical analysis. All calculations, graph preparations and statistical analysis were performed using the statistical package GraphPad Software Prism Version 3 (La Jolla, CA 92037, USA). To compare the sensitivity of each cell line to the different ABZ

preparations, IC_{50} values (concentration of the drug that leads to 50% inhibition in cell proliferation) were calculated. Differences in cell response to drug preparations were tested for statistical significance using one way analysis of variance (ANOVA) and Student's *t*-test. *P*-values of less than 0.05 were considered to present a statistically significant difference.

Results

Solubility of ABZ preparations. Results obtained show a strong linear relationship between ABZ solubility and HP-β-CD concentration used (Figure 1A). Addition of citric acid, ascorbic acid or hydrochloric acid increased the solubility of ABZ in the HP-β-CD by 20% , 90% and 820% respectively (Figure 1B). However, based on pH and biocompatibility issues, the HP-β-CD-citric acid complex was chosen for further studies.

Antiproliferative activity of ABZ preparation. Effect of ABZ dissolved in ethanol, HP-β-CD (200 mM) or HP-β-CD plus citric acid on the proliferation of 1-A-9, OVCAR-3 and SKOV-3 cells is depicted in Figure 2. The results clearly demonstrate the profound dose-dependent inhibition of all 3 human ovarian cancer cells by ABZ. While in 1A9 cells the IC_{50} values were similar irrespective of the solvent used, OVCAR-3 and especially SKOV-3 demonstrated higher sensitivity to ABZ HP-β-CD + citric acid preparation ($p<0.001$ when compared to the ethanol preparation). These results reveal for the first time that dissolving ABZ in HP β-CD plus citric acid profoundly improves the antiproliferative activity of ABZ.

Colony formation assay. To evaluate cell integrity and the ability to remain viable and grow into colonies following drug exposure, colony formation assay was performed utilizing OVCAR-3 cells. As depicted in Figure 3A, while, the number of colonies formed in ABZ-ethanol-treated plates were identical to vehicle-only-treated plates, exposure of cells to ABZ-CD and ABZ-CD+citric acid led to the formation of significantly lower numbers of colonies ($p<0.01$ and $p<0.001$, respectively).

Toxicity assay. The ABZ-CD complex toxicity was examined using fresh human hepatocytes as described. Cells were incubated for 3 days with 1 μM of various ABZ preparations (ethanolic, ABZ-CD and ABZ-CD + citric acid) and then tested using the MTT assay. Results obtained show no reduction in cell viability following treatment with ABZ irrespective of the solvent used (Figure 3B).

Discussion

Agents with anti-VEGF properties are in clinical use for the treatment of colorectal cancer and are in clinical trials for a wide range of other VEGF-related cancers (16). We recently showed ABZ to be a VEGF inhibitor and also a potent

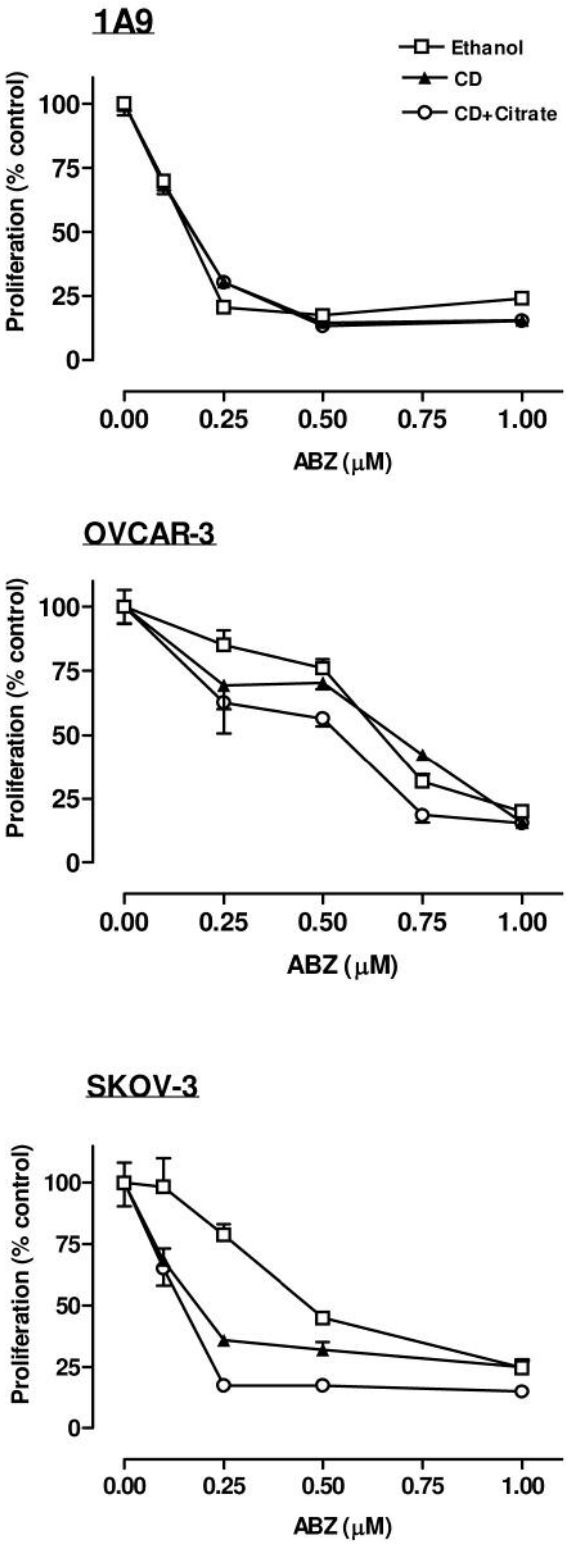

Figure 2. *Proliferation of 1A9, OVCAR-3 and SKOV-3 cells in culture medium containing different concentrations of ABZ (0.01-1.0 μM) dissolved in ethanol, HP-β-CD or HP-β-CD + citric acid. Following 3 days of treatment, cell proliferation was measured by SRB assay and results (mean ± s.e.m.) are expressed as % of control.*

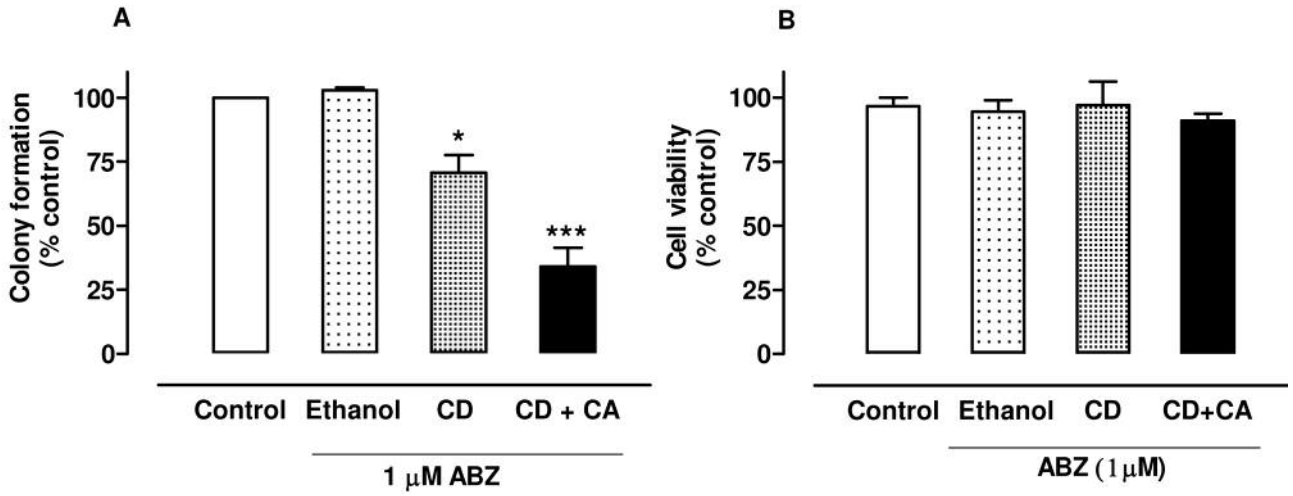

Figure 3. *Effect of different ABZ preparations on colony formation in agar Petri dishes. OVCAR-3 cells treated for 2 h with 1 μM ABZ preparations were incubated for 3 weeks, following which plates were examined for the presence of colonies (cluster of cells greater than 50). Control plates (no treatment) were taken to represent 100% growth and the drug-treated plates are expressed as % of control. * , *** Represent p-values of less than 0.05 and 0.001 respectively as compared to untreated control values (A). Viability of human hepatocytes incubated in culture with different preparations of 1 μM ABZ for 3 days. CD represents β-cyclodextrin while CD + CA represents β-cyclodextrin plus citric acid. Cell viability was measures by MTT assay and results (mean ± s.e.m.) are expressed as % of control (B).*

inducer of apoptosis in paclitaxel-resistant leukemic cells, thus substantially increasing its potential as an anticancer agent (4, 5). However, for use as a systemic agent, the drug must be solubilized first in a biocompatible agent.

HB-β-Cyclodextrin seems to be an appropriate candidate due to its high water solubility and low toxicity in humans, with very high complex forming potential (9, 17). Using a saturated solution of ABZ, the solubility of ABZ in HP-β-CD was found to be linear. Addition of citric acid (50 mM) enhanced ABZ solubility in HP-β-CD. The result is a highly soluble multicomponent inclusion complex containing ABZ, citric acid and HP-β-CD. It has been suggested that these multicomponent complexes with HP-β-CD could be tools of the future to develop intravenous drug delivery of such water-insoluble molecules (18).

This is the first report to show the effect of ABZ or ABZ-CD complex against a range of human ovarian cancer cells *in vitro*. Compared to the ethanolic preparation, the CD complex led to a profoundly higher degree of cell inhibition. Moreover, in OVCAR-3 and SKOV-3 cells, the inhibition was triggered at a lower ABZ concentration (0.1 μM). 1A9 Cells were generally more responsive to all 3 ABZ formulations, with 30% cell inhibition at 0.1 μM and over 70% inhibition at 0.25 μM ABZ concentrations. The difference in cell responsiveness to the various formulations was most evident in SKOV-3 cells with profoundly higher inhibition in the presence of HP-β-CD + citric acid complex (IC_{50} value of 1.09 μM), as compared to HP-β-CD alone (IC_{50} value of 1.94 μM) or ethanol (IC_{50} value of 3.88 μM). These results demonstrate that solubilization of ABZ in HP-β-CD + citric acid significantly ($p<0.001$) improves the ABZ potency in inhibiting cell proliferation.

In the colony formation assays, no difference was observed between control and ABZ delivered in ethanol, suggesting that after drug removal, the ABZ effect does not persist. In contrast, exposure of cells to either HP-β-CD or HP-β-CD + citric acid led to significant ($p<0.001$) reductions in the colony numbers formed. This indicates that exposure of cells to the CD complexes leads to a longer lasting inhibitory effect.

The difference observed in the cell proliferation and the colony formation assays may be explained on the basis of the CD properties. Studies conducted on skin cells have revealed that CDs determine the drug delivery to the cell by acting on the cell membrane through inclusion of phospholipids and cholesterol (19), extraction of proteins (20) and by removal and disorganization of the lipid matrix resulting from complexation (21-23). How the presence of HP-β-CD + citric acid leads to increased drug effect is still not well understood. However, there are suggestions that HP-β-CD complex forms a labile complex to release the free drug at the cell site and the presence of citric acid enhances the multicomponent complex system resulting in better bioavailability of the drug (24, 25). The use of HP-β-CD as a possible vehicle for water insoluble drugs has been previously described (26-28). The use has specially been advocated for intravenous drug administration, as CDs are less lipophilic, hemolytic and toxic than other drug vehicles (29).

In conclusion, results from the present study demonstrate that solubilization of ABZ with HP-β-CD leads to an enhanced antiproliferative activity. Further work to investigate the systemic antitumor efficacy of the ABZ-HP-β-CD complex in experimental animals bearing tumors is warranted.

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Received March 11, 2008
Revised June 18, 2008
Accepted July 3, 2008