

# In vitro and in vivo suppression of growth of hepatocellular carcinoma cells by albendazole

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## Abstract

Tubulin protein is a major target of drug molecules, and consequently, tubulin inhibitors have attracted great attention as antimitotic antitumor agents for chemotherapeutic use. It has been shown that, the benzimidazole carbamate group of antiparasitics including albendazole act by inhibiting tubulin polymerization. In this study, albendazole was tested in culture against a range of human, rat and mice hepatocellular carcinoma (HCC) cells and in vivo against human SKHEP-1 tumor growth in nude mice. Albendazole induced a dose-dependent inhibition of [<sup>3</sup>H]thymidine incorporation in all cell lines examined and a dramatic decline in cell numbers in SKHEP-1 cells. The inhibitory effect of albendazole was evident at the 100 nM concentration and at 1000 nM, proliferation in all cell lines examined was inhibited by more than 80%, while, proliferation of HepG2, Hep3B and SKHEP-1 were suppressed by more than 90%, compared to control. Cell cycle analysis revealed that, depending on the dose employed, albendazole can arrest SKHEP-1 cells at both G0–G1 (250 nM) and G2–M (1000 nM) phases of the cycle. Albendazole treatment (300 mg/kg per day oral for 20 days) of nude mice inoculated subcutaneously with SKHEP-1, led to profound suppression of tumor growth. Immunohistochemical analysis of these tumors revealed that compared to control, those treated with albendazole have lower growth fractions. These findings demonstrate that albendazole strongly suppresses both in vitro and in vivo proliferation of HCC cells. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

*Keywords:* Albendazole; Cell cycle; Hepatocellular carcinoma; SKHEP-1; Xenografts

## 1. Introduction

Albendazole a benzimidazole carbamate (methyl 5-propylthio-1H-benzimidazole-2-yl carbamate) is a broad spectrum antiparasitic which is used worldwide against a variety of parasites [1,2]. Studies conducted on the mechanism of action of BZs have demonstrated that, by binding to tubulin, these drugs inhibit microtubule polymerization [3–6]. Inhibitors of microtu-

bule polymerization have been shown to exhibit experimentally and clinically, useful antitumor activity [7]. Consequently, the reported activity of BZs against human mammalian tubulin raises the question of whether these agents also possess anticancer properties. In 1985 Lacey and Watson reported the activity of BZs against mouse L1210 leukemia cells [8]. However, except for this and a report investigating the suitability of HCC cells as an in vitro model for the study of albendazole metabolism [9], no other report concerning the possible antitumor effect of BZs has appeared in literature. This coupled with our experience with these drugs [1,10] and the fact

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that to date, there are very limited chemotherapeutic options available for the treatment of HCC [11,12], led to the design of the current study. The study was undertaken to test the effect of albendazole, the most commonly used of the BZs, on cell proliferation rates, cell viability, cell cycle kinetics and the *in vivo* effects of the drug on xenografts in nude mice.

## 2. Materials and methods

### 2.1. Cell culture

HepG2, Hep3-B, Hep1-6, SKHEP-1, PLC/PRF/5, and HTC cells were obtained from European Collection of Cell Cultures (ECACC; U.K.). Novikoff was obtained from Cancer Research Centre (DKFZ) Heidelberg, Germany. Cells were cultured in MEM or DMEM supplemented with 10% FBS, 50 units/ml penicillin, 50 units/ml streptomycin, 25  $\mu\text{g/ml}$  amphotericin B (Gibco, Grand Island, NY) and maintained subconfluent at 37°C in humidified incubators containing 5% CO<sub>2</sub>. Albendazole (Sigma, Australian subsidiary) was dissolved in absolute ethanol at concentrations that were 1000-fold higher than the final medium concentration.

### 2.2. [<sup>3</sup>H]Thymidine incorporation assay

For the study of [<sup>3</sup>H]thymidine incorporation, adherent cells ( $5\text{--}10 \times 10^4$ ) were plated onto 24-well corning tissue culture dishes and were exposed to culture medium (5% FBS) containing the vehicle (0.1% ethanol) or different concentrations of albendazole ( $10^{-8}$  to  $10^{-6}$  M). For Novikoff, a detached rat cell line, 2500 cells were suspended in 2 ml of DMEM (5% FBS) and kept under the same condition as for attached cells. Media were replaced with fresh media on alternate days. At the end of the treatment period (5 days), cell cultures were assayed for thymidine incorporation by the addition of 0.5  $\mu\text{Ci}$  of [<sup>3</sup>H]thymidine (60 Ci/mmol. ICN Biochem, Irvine, CA) to each well for the last 4 h of culture. The amount of radioactivity incorporated into cells was determined using a  $\beta$ -scintillation counter. Results are presented as percentage [<sup>3</sup>H]thymidine incorporation relative to control. For the recovery experiments, SKHEP-1 cells were treated for 1 day with different concentrations of albendazole and then either assayed for [<sup>3</sup>H]thymidine

incorporation or the medium was replaced and the cells treated with fresh medium without the drug for a further 4 days at the end of which, [<sup>3</sup>H] thymidine incorporation assay was performed.

### 2.3. Cell counts

SKHEP-1 cells ( $2.5 \times 10^4$ ) were plated in six well plates. The cell treatment procedure was as described for the thymidine assay. At the end of the treatment period (1, 3 or 5 days), cells were trypsinized and counted with a hemocytometer using the trypan blue exclusion method. In all experiments, cells treated with the medium containing 0.1% ethanol were taken as the control for albendazole treated cells. All counts were obtained in quadruplicate and each experiment was repeated at least twice.

### 2.4. Cell cycle analysis

SKHEP-1 cells ( $5 \times 10^4$ ) were plated onto six-well tissue culture plates. Triplicate samples were treated with the indicated concentrations of albendazole (100, 250 and 1000 nM). The medium was changed everyday. After 72 h the relevant group of cells were collected, washed twice with phosphate buffer and treated with ribonuclease, Triton X-100 and propidium iodide (Sigma) based on the method described by Taylor [13]. The percentage of cells within the G1, S, and G2-M phases of the cell cycle were determined using a FACScan flow cytometer (Becton Dickinson FACSsort) and Multifit LT cell cycle analysis software (Verity Software INC.)

### 2.5. Tumor formation in nude mice

Six to 10 weeks old male BALB/c Nu/Nu mice (Animal Resources Center, Perth, Australia) were inoculated subcutaneously with  $10^6$  SKHEP-1 cells into the right flank. Twenty-four hours after inoculation animals were randomly assigned to one of the treatment groups ( $n = 10$  per group), receiving 25, 50 or 150 mg/kg twice daily oral albendazole suspended in sesame oil for 20 days.

The control group was treated with the vehicle (sesame oil). Using vernier calipers, tumor diameter (mm) was measured on day 8 and then every 3 days up to day 20-post tumor cell inoculation. Tumor volumes were calculated using the formula:  $ab^2/2$ , where  $a$  and

Table 1  
Effect of albendazole on [<sup>3</sup>H] thymidine incorporation in HCC cell lines<sup>a</sup>

Cell line	[albendazole] nM			
	10	100	500	1000
HepG2	93.1 ± 5.6	72.9 ± 6.3	12.2 ± 2.9	3.5 ± 0.4
Hep3-B	105.7 ± 8.6	68.5 ± 5.7	22.6 ± 1.8	9.3 ± 0.7
SKHEP-1	89.6 ± 6.1	63.7 ± 3.1	4.5 ± 1.1	0.6 ± 0.1
PLC/PRF/5	92.7 ± 4.7	69.4 ± 5.2	26.9 ± 2.7	18.4 ± 2.1
Novikoff	96.5 ± 8.8	71.6 ± 5.9	29.2 ± 3.3	10.3 ± 1.8
HTC	98.4 ± 7.5	86.0 ± 6.9	28.5 ± 2.2	11.4 ± 1.3
Hep1-6	97.7 ± 4.3	79.5 ± 3.2	28.1 ± 2.5	5.6 ± 0.6

<sup>a</sup> Cells were treated with different concentrations of albendazole (10–1000 nM) for 5 days at the end of which [<sup>3</sup>H]thymidine incorporation was measured. Values (% control) represent mean ± SEM of several determinations.

*b* are the smaller diameters in millimeters, respectively [14] and a piece of the tumor was preserved in paraffin for immunohistochemical determination of maximum proliferation index (MPI). Here, after fixation, the specimen was processed for the detection of Ki-67 antigen with the monoclonal antibody MIB1 according to the method described by McCormick [15].

The animal model was chosen on the basis of SKHEP-1 being the most tumorigenic human liver cancer cell line in nude mice [16] and our previous experience with the model [17].

## 2.6. Statistical analysis

Differences between different treatment groups were analyzed using ANOVA followed by Tukey's test. *P* values of less than 0.05 were considered to represent a significant difference.

## 3. Results

### 3.1. Inhibition of [<sup>3</sup>H]thymidine incorporation by albendazole

[<sup>3</sup>H]Thymidine incorporation assay was used to determine the effect of albendazole on cell proliferation in a number of human (HepG2, Hep3-B, PLC/PRF/5, SKHEP-1), rat (HTC and Novikoff) and mice (Hep1-6) HCC cell lines. Results obtained show that,

in all cell lines examined, albendazole effectively reduces thymidine incorporation (Table 1). When treated with the 100 nM concentration of albendazole, compared to other cell lines, SKHEP-1 demonstrated the highest level of sensitivity to albendazole ( $P < 0.01$  compared to control), while the rat cell line HTC was the least responsive of all. Treatment with the 1000 nM concentration of albendazole reduced thymidine incorporation to less than 20% of control values ( $P < 0.001$ ) in all cell lines and to less than 5% in SKHEP-1 and HepG2. Here again SKHEP-1 cells displayed the highest level of sensitivity to albendazole. In these cells, thymidine incorporation was reduced to  $0.6 \pm 0.1\%$  of the control values corresponding to 99.4% inhibition. For this reason, SKHEP-1 was employed for all further investigations. Exposure of SKHEP-1 cells to different concentrations of albendazole for 1 day, revealed that, concentrations of 250 nM and over of albendazole still produce profound inhibition of thymidine incorporation (Fig. 1a). Removal of the drug and treat-

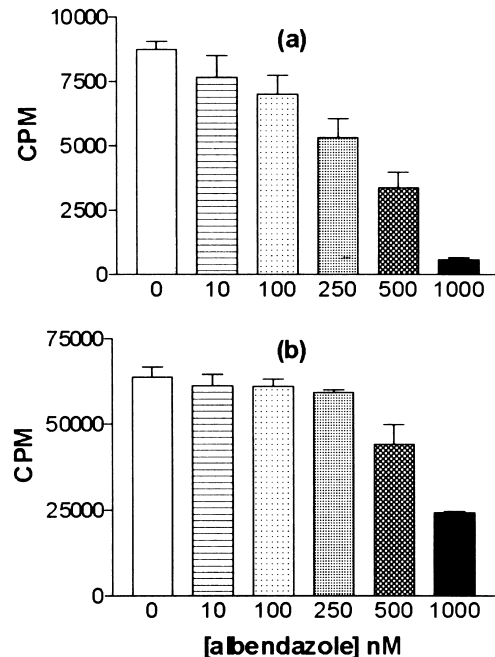


Fig. 1. [<sup>3</sup>H]Thymidine incorporation [expressed as counts/min (CPM)] in SKHEP-1 cells was measured either (a) immediately after 1 day treatment with albendazole or (b) after 1 day treatment with albendazole followed by 4 days treatment with the medium alone (not containing the drug). Data points are the mean ± SEM.

ment of cells with the normal medium for a further 4 days led to the recovery of thymidine incorporation by the cells (Fig. 1b). Except for the 500 and the 1000 nM concentrations, cells exposed to all other concentrations of albendazole were able to recover from the inhibitory effect of the drug.

### 3.2. Albendazole inhibits proliferation of cells leading to a decline in cell number

Counting of viable cells treated with different concentrations of albendazole for 1, 3 or 5 days produced a dose dependent decline in the number of cells, showing the profound inhibition of proliferation of SKHEP-1 cells by the drug (Fig. 2).

This was evident from day 3 at the 500 and the 1000 nM concentrations. Compared to control, cells exposed to the 1000 nM concentration of the drug, were significantly reduced in number ( $P < 0.001$ ).

### 3.3. Dose-dependent effect of albendazole on the cell cycle kinetics

Flow cytometric analysis of albendazole-treated cells, revealed that, the drug induces a dose-dependant effect on the cell cycle kinetics of SKHEP-1 HCC cells. Fig. 3 demonstrates the changes induced on the distribution of cells in the different phases of the cell cycle following 3 days treatment with different concentrations of albendazole. From this, it is clearly evident that exposure of cells to the 250 nM concentration causes accumulation of cells in the G0–G1 phase and associated with this was a reduction in the percentage of cells in both S and G2–M phases

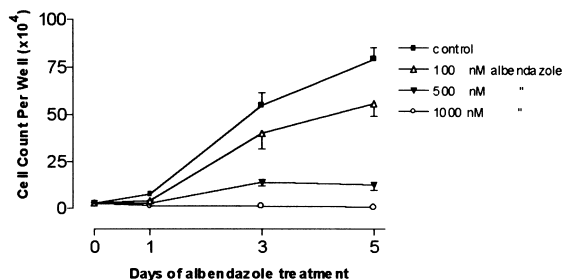


Fig. 2. Time-course effects of albendazole on SKHEP-1 cell number. Cells growing in 6 well plates were treated for 1, 3, or 5 days with albendazole (0, 100, 500 or 1000 nM) and number of viable cells were counted using the Trypan blue exclusion method. Data points are the mean  $\pm$  SEM.

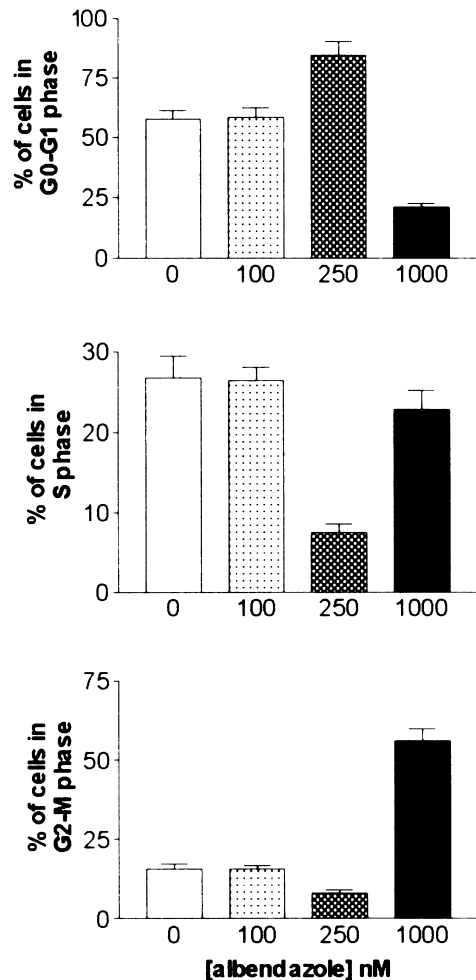


Fig. 3. Effect of albendazole on cell cycle stage of SKHEP-1 cells. Cells were treated with different concentrations of albendazole (0, 100, 250, and 1000 nM) for 3 days, stained with propidium iodide and analyzed for DNA content by flow cytometry. A total of 10 000 nuclei were analyzed from each sample. Data points are the mean  $\pm$  SEM of the percentage of cells within G0–G1, S and G2–M phases of the cell cycle.

of the cell cycle. Changes induced by the 500 nM concentration of the drug were identical to those of the 250 nM concentration (data not shown). However, as depicted in the same figure, treatment of the cells with the 1000 nM concentration of albendazole leads to a totally different pattern of changes. Here, arrest and accumulation of cells in the G2–M phase of the cell cycle was accompanied by a dramatic reduction in percentage of cells in the G0–G1 phase, while

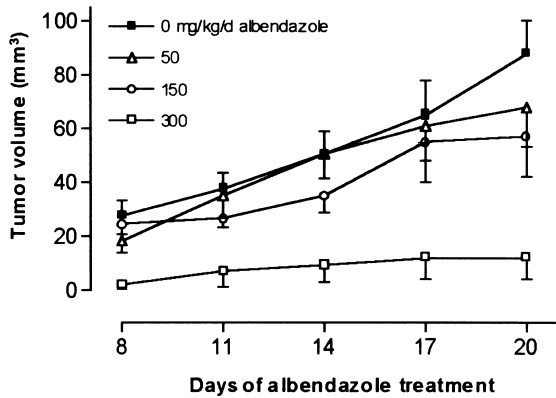


Fig. 4. Effect of different doses of albendazole (0, 50, 150 & 300 mg/kg per day in two divided dose given orally in sesame oil) on SKHEP-1 subcutaneous tumor formation and growth in nude mice. Changes in tumor volumes were measured every 3 days. Each value represents mean  $\pm$  SEM of ten animals.

percentage of cells in the S phase remained unchanged.

#### 3.4. Effect of albendazole on tumor growth in vivo

In control animals, SKHEP-1 tumors grew to a mean volume of  $87.9 \pm 12.3 \text{ mm}^3$  at 20 days post inoculation. In animals receiving 50 and 150 mg/kg per day, tumor growth was slightly but not significantly retarded. However, tumor growth was profoundly suppressed in animals receiving the 300 mg/kg dose of albendazole (Fig. 4) with a mean tumor volume of  $12.0 \pm 7.8$  ( $P < 0.001$ ). Results from the immunohistochemical analysis of tumors revealed that, tumors from animals receiving the 50 and 150 mg/kg dose of albendazole had reduced MPIs of  $22.54 \pm 1.53$  (mean  $\pm$  SEM) and  $13.36 \pm 3.04$  respectively compared to  $34.2 \pm 3.13$  for the control. There was not enough tissue for the analysis of MPI in tumors of mice receiving the 300 mg/kg per day dose.

## 4. Discussion

Albendazole is a drug that has been in use in the clinic for nearly two decades as the treatment of choice in a number of parasitic diseases. However, the mechanisms of action described for the drug suggest that, it may hold greater potential than it is currently employed for. To explore this possibility,

we set out testing the drug against a range of HCC cell lines under both in vitro and in vivo conditions.

Results from the cell proliferation studies clearly demonstrated that all human, rat and mice liver cell lines examined are profoundly inhibited by albendazole. This was manifested by the significant reduction of thymidine incorporation following treatment with albendazole doses of 100 nM and over. Similarly treatment of SKHEP-1 cells with albendazole led to a dose and time dependent reduction of cell number. The reason behind the higher sensitivity of SKHEP-1 to albendazole is not clear at this stage. However, lacking the enzymes needed for the conversion of the drug to less active or inactive metabolites, may partly account for this observation [9]. Flow cytometric analysis of the cell cycle revealed that, albendazole causes differential dose-dependent effect on the cell cycle kinetics of SKHEP-1. Accumulation of cells in the G<sub>0</sub>-G<sub>1</sub> phase following treatment with albendazole concentrations of up to 500 nM with an associated decline in percentage of cells in S and G<sub>2</sub>-M phases, indicates that progression out of the G<sub>1</sub> phase was blocked. Many natural triggers for programmed cell death, including glucocorticoid hormones act at G<sub>1</sub>-G<sub>0</sub> transition and the cells die in a process described as 'premature aging' [18]. However, following treatment with the 1000 nM concentration of albendazole, the pattern of cell distribution was reversed, leading to the accumulation of cells in the G<sub>2</sub>-M phase of the cycle. This indicates that the primary effect of albendazole at this concentration may be mediated by a transition delay through G<sub>2</sub>-M or mitosis. Rolin et al. while working on the drug metabolizing capacity of HepG2 cells and using albendazole as a model substrate, observed that, by interfering with mitosis, albendazole (40 000 nM) blocks cell division at metaphase [9]. It is well documented that, several of the cell cycle specific drugs commonly used in the chemotherapy of cancer including the vinca alkaloids [19,20] and the taxanes [20–22] act by arresting cells at the G<sub>2</sub>-M phase of the cell cycle. In general, BZs produce many biochemical changes, however, the primary mode of action of these drugs in susceptible nematodes has been described as the inhibition of microtubule polymerization by binding to  $\beta$ -tubulin [23,24]. In addition to their involvement in a number of cellular functions, microtubules also play a key role in the formation of

the mitotic spindles, disruption of which would consequently lead to cell death [25].

However, beside the tubulin, other mechanisms of action have been described for the BZs including disruption of the energy metabolism of the host. In fact initial studies of the mode of action of BZs focused on their role in carbohydrate metabolism as these compounds have been shown to inhibit glucose uptake both in vitro and in vivo in many helminth species. Albendazole has been shown to block glucose uptake by larval and adult stages of susceptible parasites, depleting their glycogen stores and decreasing formation of ATP leading to the death of the parasite [23]. Differences in energy metabolism between normal and malignant cells has also been described, and it is well known that in many malignant cell lines, glucose utilization is several fold higher than in normal cells [26]. In the rat HCC cell line HTC, the rate of glucose uptake is 40-fold greater than that of the normal liver cells [27]. Moreover, it has been shown that 2-Deoxyglucose (2-DG), a glucose analogue that competitively inhibits cellular uptake and utilization of glucose causes death of malignant cells.

Data from our work in nude mice suggests that, at the higher dose of the 300 mg/kg per day, albendazole presumably reaches the necessary concentrations required to suppress tumor formation. The very high rate of metabolism of albendazole in mice and the poor blood supply to the subcutaneous tumor, are amongst a number of factors that could account for the high dose of the drug required to suppress tumor growth in these animals.

The MPI data also confirm the ability of albendazole to reduce tumor proliferation rate. The Ki-67 antigen used in this assay is tightly linked to proliferation and has been used in a large number of studies to estimate the growth fraction of tumors [15].

In conclusion, the present report reveals for the first time that, albendazole a benzimidazole carbamate with extensive clinical use as an anthelmintic drug, can also inhibit HCC cell proliferation under both in vitro and in vivo experimental conditions. These results led us to examine the drug in patients with liver cancer refractory to all other available treatments. Preliminary results obtained here, have basically confirmed our experimental data, in suggesting the possibility of potential usefulness of albendazole in the treatment of liver cancers (unpublished data).

This is a novel use for albendazole and the potential efficacy of the drug in other forms of cancer is also currently under investigation in our laboratories. However, further studies in larger population of patients would be needed before assuming a role for albendazole in the treatment of liver cancer.

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