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ORIGINAL ARTICLE

Mebendazole inhibits growth of human adrenocortical carcinoma cell lines implanted in nude mice

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Abstract Adrenocortical carcinoma is a rare tumor of the adrenal gland which requires new therapeutic approaches as its early diagnosis is difficult and prognosis poor despite therapies used. Recently, mebendazole has been proved to be effective against different cancers. The aim of our study was to evaluate whether mebendazole may result therapeutically useful in the treatment of human adrenocortical carcinoma. We analyzed the effect of mebendazole on human adrenocortical carcinoma cells in vitro and after implantation in nude mice. In order to clarify mechanisms of mebendazole action, metastases formation, apoptosis and angiogenesis were also investigated. Mebendazole significantly inhibited cancer cells growth, both in vitro and in vivo, the effects being due to the induction of apoptosis. Moreover, mebendazole inhibited invasion and migration of cancer cells in vitro, and metastases formation in vivo. Overall, these data suggest that treatment with mebendazole, also in combination with standard therapies, could provide a new protocol for the inhibition of adrenocortical carcinoma growth.

Keywords Adrenocortical carcinoma · Mebendazole · Nude mice · Chemotherapy

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Introduction

Adrenocortical carcinoma (AC) is a rare tumor of the adrenal gland with an incidence of approximately two person every million people.

Generally, at the time of diagnosis only 30% of tumors are limited to the site of development [14], whereas about 50% of them are metastatic stage IV adrenocortical carcinoma [18]. Common affected sites are the peritoneum, lung, liver, and bone. Radical surgical excision of localized tumors allows 5 years survival in 40% of the cases, while for extended cancers, treatment options are only palliative and regard chemotherapy, radiation and surgical removal. In about 60% of the patients, AC induces an excessive hormone secretion and 90% of tumors over-express the IGF-II and the IGF-binding proteins [8].

Xenograft models are useful tools for preclinical screening of anticancer drugs as well as for the evaluation of cellular and molecular biology of cancer.

The human adrenocortical carcinoma cell line H295 was established in 1990 and phenotypic analysis showed that these cells retain the ability to produce most of the major steroids characteristics of the adrenal gland [6, 15]. From this line, cells were selected to grow in a monolayer, thus originating the H295R cell lines which, implanted in nude mice, preserved the ability to produce and secrete steroids [11]. Another cancer cell line developed from the cortex of the human adrenal gland was the SW-13. These cells were tested on animals, showing that they retain the ability to develop tumor when implanted in nude mice [5, 10, 20].

As for other types of cancer, innovative therapies are required for late stage AC treatments and new chemotherapeutics are currently under investigation.

Mebendazole (MZ) (methyl 5-benzoyl-2-benzimidazole-carbamate) is generally used for the treatment of

helmintiasis in humans and animals acting by depolymerizing tubulin and therefore disrupting the functions of microtubules.

Recently, MZ has been proved to inhibit the growth of different cancer cells in vitro and in vivo [12, 16]. In these studies MZ induced depolymerization of tubulin and inhibited normal spindle formation in cancer cells, resulting in mitotic arrest and apoptosis. Finally, antitumoral effects of MZ seemed also due to angiogenesis decrease.

Based upon these findings and the clinically proved safety of MZ [1, 4] this work analyzed the effects of MZ on human adrenocortical carcinoma cell lines in vitro and after implantation in nude mice. Results obtained confirmed the antitumor effects of MZ due to the induction of apoptosis on cancer cells, whereas inhibition of angiogenesis in tumors was not detected.

Materials and methods

Human cell line

H295R human adrenocortical carcinoma cells from ATCC were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 15 mM HEPES, 0.00625 mg/ml insulin, 0.00625 mg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml bovine serum albumin, and 0.00535 mg/ml linoleic acid, 97.5% (ITS+ Premix from BD Biosciences) and Nu-Serum I, 2.5%. SW-13 human adrenal carcinoma cells were maintained in Leibovitz's L-15 medium with 2 mM L-glutamine, and 10%fetal bovine serum.

WI-38 normal human fibroblasts were maintained in Minimum essential medium (Eagle) with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% fetal bovine serum.

Cell cultures were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO_2 in air.

Animals

Athymic 6 weeks old male nude mice Nu/Nu (Harlan, Italy) were used. Mice were kept in laminar-flow cage in a standardized environmental condition. Sterilized food (Harlan, Italy) and water were supplied ad libitum. Experiments were carried out in accordance with the guidelines of the Italian Ministry of Health.

Mebendazole

Mebendazole (MZ) was purchased from Sigma-Aldrich (Italy) and dissolved in dimethylsulphoxide (DMSO).

In vitro proliferation

The test was performed following the NCI guidelines. H295R, SW-13 and WI-38 cells were plated in serum supplemented growth medium at 5,000 cells/well in 96 well plates. After 24 h, two plates of each cell line were used to measure the cell number for each cell line at the time of drug addition (Tz) by MTT assay. H295R, SW-13 and WI-38 cells in the other plates were treated with MZ dissolved in DMSO at various concentrations: 0 (*C*), 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10 and 100 μM. After 3 days, cell numbers were determined using the MTT assay (*Ti*). Briefly, after incubation with MZ, the solutions were discarded and 20 μl of a solution of 5 mg/ml of MTT (Sigma, Italy) were added and the cultures were incubated for an additional 3 h at 37°C. The supernatant was removed and the precipitate was dissolved in DMSO (100 μl/well). After a few minutes the plate was read on a microtitre plate reader at a wavelength of 570 nm.

Percentage growth inhibition was calculated at each of the drug concentrations levels as:

$$[(\mathrm{Ti} - \mathrm{Tz})/(\mathrm{C} - \mathrm{Tz})] \times 100$$

for concentrations for which $\mathrm{Ti} \geq \mathrm{Tz}$

$$[(\mathrm{Ti} - \mathrm{Tz})/\mathrm{Tz}] \times 100$$

for concentrations for which $\mathrm{Ti} < \mathrm{Tz}$.

Spheroid growth assays 24-well tissue culture dishes were coated with 0.5 ml of 1% agarose (Pharmacia, Biotech, Milano, Italy). Agarose was allowed to solidify at room temperature for at least 30 min. After trypsinizing and washing, 10^4 H295R cells were plated in each well. Plates were briefly swirled to aggregate the cells in the center of each well. After incubating for 24 h, another 0.5 ml of medium was added to each well to reach a final concentration of 0, 1 and 10 μM of MZ dissolved in DMSO. Spheroids were fed every 3 days by carefully removing 0.5 ml medium from each well, and replacing it with fresh medium containing MZ. Spheroids were photographed using 10× magnification on an inverted microscope and the major and minor diameter, d_{max} and d_{min} respectively, were determined and spheroid volume calculated as $V = (1/6)\ \pi\ d_{max}\ d^2_{min}$

Subcutaneous implantation and Tumorigenicity of H295R and SW-13 cells

At near confluence, cells were harvested with trypsin/EDTA solution. Only cell suspensions of >90% viability (trypan blue exclusion) were used. To analyze the tumorigenicity of H295R and SW-13 cells 1, 3 and 6 millions of cells suspended in Hank's salt balanced solution (HBSS)

and 1 millions of cells in matrigel (BD Biosciences, Italy) were injected subcutaneously on the left lateral chest wall close to the axilla of nude mice (40 animals divided in 4 groups for each cell lines). Tumor growth was monitored twice a week using caliper and tumor volume was calculated using the formula: V (mm^3) = $(D \times d^2)/2$, where d (mm) and D (mm) are the smallest and largest perpendicular tumor diameters, respectively.

In vivo metastases formation

Metastases formation was investigated by intravenously and intraperitoneally injection of 6×10^6 H295R or SW-13 cells in nude mice. Metastases count was performed by macroscopic and microscopic examination of visceral organs, lungs and brain after 29 days of oral treatment with 1 mg of MZ, 2 mg of MZ, vehicle or saline.

Therapeutic procedures

Four groups of animals (6 animals each group) were used for subcutaneous inoculation and the in vivo metastases models. Treatments started when tumors were measurable on animals. Based upon the results obtained with the tumorigenicity assay, for H295R cells, treatments started 34 days after tumor cell injection, whereas for SW-13 cell treatments started 21 days after tumor cells injection. For each cell line, the first group of animals was treated orally at 10:00 am with a dose of 1 mg of MZ. The second group of animals was treated orally at 10:00 am with a dose of 2 mg of MZ. The third group of animals received an oral administration of saline solution. The fourth group of animals received an oral administration of DMSO at the concentration used to prepare the 2 mg of MZ dose. The treatment lasted 29 consecutive days.

Autopsy and histology

Mice were sacrificed by CO_2 exposure. Subcutaneous tumors were removed and immediately frozen on powdered dry ice and stored at –80°C.

Matrigel invasion assay

24 BioCoat Matrigel Invasion Chambers (BD Biosciences, Italy) were rehydrated with the addition of 2 ml warm (37°C) serum-free culture medium for 2 h. Following rehydration, the medium was carefully removed. 2,5 ml of medium containing 10% FBS (chemoattractant) were then added to each well of the plate and 2 ml of cell suspension (without serum; 3×10^4 cells) were added in the presence or absence of MZ (0; 0.001; 0.01; 0.1; 1; 10 μM) to the matrigel coated inserts. Plates were incubated for 48 h, then the medium was removed and using a cotton swab, the non-invading cells were removed from the upper surface of the membrane by scrubbing. Cells on the lower surface of the membrane were fixed in 70% ethanol for 30 min and then stained with hematoxylin. The number of invasive cells were then counted in five random fields of the insert and % of control was calculated (number of invasive cells treated with MZ × 100/ number invasive of invasive cells of control group).

Apoptosis

DNA fragmentation assay was performed on subcutaneous tumors and tumor cells treated with MZ 1 μM for 24 h. According to the manufacturer's protocol, DNA fragments were extracted using a Genomic cell and tissue kit (Talent, Italy). DNA was then suspended in loading buffer (20% Ficoll 400, 0.1 M Na_2EDTA pH 8.0, 1% SDS, 0.25% bromophenol bleu) (Sigma), heated for 10 min at 65°C and electrophoresed on 1% Agarose gel (Pharmacia, Biotech, Milano, Italy). DNA was visualized by ethidium bromide staining and UV transilluminator.

Western blot analysis

Lysates obtained from H295R and SW-13 cells, both treated with DMSO (control), MZ 0.5 μM and MZ 1 μM for 24 h were resuspended in 0.2 ml of RIPA (0.1% Nonidet-P40, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% sodium azide, 1 mM PMSF (phenylmethylsulfonylfluoride), 0.03 mg/ml aprotinin, 1 mM $NaVO_4$). Proteins were separated on 10 and 14% SDS polyacrylamide gels, transferred overnight at 20 V and incubated for 2 h at room temperature with the primary antibodies followed by the incubation with the secondary HPR-conjugated antibodies. Primary mouse monoclonal and goat polyclonal antibodies included anti-p53 (B-P3), anti-caspase-3, anti-cleaved caspase-3, anti-caspase-9, anti-cleaved caspase-9 and anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To evaluate cytochrome c release, cells were washed in ice-cold PBS and the resulting pellet was resuspended in 0.2 ml of lysis buffer (20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.1 mM PMSF) supplemented with protease inhibitors (5 mg/ml pepstatin A, 10 mg/ml leupeptin and 2 mg/ml aprotinin). After sitting on ice for 15 min, cells were disrupted by 60 times douncing in a mini-potter. Nuclei were pelletted at 1,000 g for 5 min at 41°C and the supernatants were separated and centrifuged for 40 min at 80,000*g*. Then, supernatants loaded onto a 14% SDS-polyacrylamide gel were transferred overnight at 20 V and incubated with anti-cytochrome c mAb (0.5 mg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h, followed by HPR-conjugated anti-mouse antibody.

Immunoreactivity was detected using the Enhanced Chemiluminescence (ECL, Amersham). Densitometric analysis was performed by a Chemidoc using the Quantity One software (BioRad). Each sample was compared to its control (α-tubulin) for the purpose of quantification.

Angiogenesis evaluation

Angiogenesis was evaluated in tumors implanted with matrigel tested for MZ effects and in tumors implanted without matrigel deriving from the tumorigenicity assay. Tumors were placed in OTC compound after autopsy, snap frozen in liquid nitrogen and stored at –80°C. Frozen sections (10–20 μm) were fixed with cold acetone (5 min), acetone/chlorophorm 1/1 (5 min) and cold acetone (5 min). Samples were then rinsed with PBS/triton 1% and treated with 3% hydrogen peroxide in methanol (v/v). Treated slides were incubated in a blocking solution and set overnight at 4°C in a humidified chamber with the endothelial cell marker rat anti-mouse CD31 monoclonal antibody.

Slides were then rinsed with PBS and incubated, first with the blocking solution for 20 min and then with a biotin conjugated goat anti-rat antibody for 1 h. Slides were then rinsed with PBS and incubated for 30 min with the Vector Vectastain ABC Kit (Vinci-Biochem, Vinci, FI).

After 3 washes with PBS, positive reactions were visualized by incubating the slides for about 5 min with stable DAB (Sigma, Italy). Slides were dried and mounted with Universal Mount.

Statistical analysis

The significance of the in vitro and in vivo data was analyzed by one-way analysis of variance (ANOVA), followed by two-tailed *t*-test.

Results

Effect of MZ on H295R cells growth in vitro

We tested the effect of MZ on H295R, SW-13 and WI-38 cells growth in vitro. MZ strongly inhibited the growth of cancer cells at a concentration of 1 μM and higher. On the other hand, MZ had no effect on normal WI-38 fibroblast, even at the higher concentration used. Figure 1 shows data obtained with the MTT assay. H295R: IC50 = 0.23 μM. SW-13: IC50 = 0.27.

Effect of MZ on H295R tumor spheroids growth

To confirm the cytotoxic effect of MZ and to analyze its ability to penetrate the tumor mass, H295R cells were cultured on agarose to form spheroids and then treated with MZ 1 μM. Cells survived more than 20 days without attaching to the agarose-coated well. MZ completely disaggregated tumor spheroids and killed cancer cells in about 20 days (Figs. 2, 3).

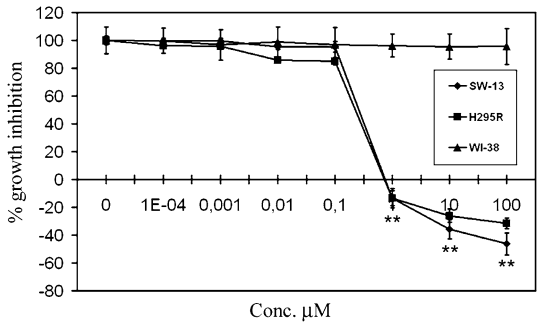

Fig. 1 In vitro cytotoxic effects of MZ on the H295R, SW-13 and WI-38 cell line. H295R: I.C.50 = 0.23 μM. SW-13: I.C.50 = 0.27 Values shown are mean ± SEM (n = 3). ** $P < 0.01$

Tumorigenicity of cancer cells

As shown in Table 1, subcutaneous injection of six millions of H295R cells in HBSS produced tumors in mice at different times and the growth rate was different from animal to animal. The high number of cells needed to implant tumors and their irregular growth (as result from the range of measurable tumors) confirmed the data previously reported by other researcher [11] and indicated that H295R are poorly tumorigenic. SW-13 cells resulted more tumorigenic, but the growth was slow and irregular as for H295R cells. Using matrigel, the growth of both cells lines was regularized and tumors developed rapidly, therefore matrigel was used to implant cells in nude mice.

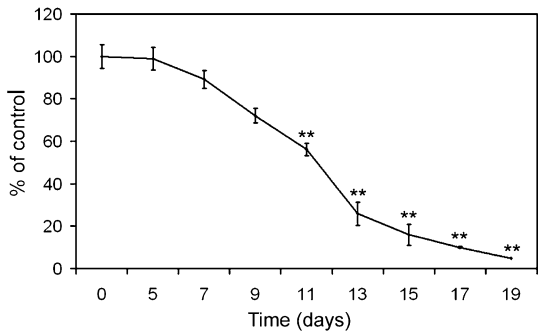

Fig. 2 Effect of MZ 1 μM on H295R spheroids. Spheroids were photographed using ×10 magnification on an inverted microscope and the major and minor diameter, d_{max} and d_{min}, respectively, were determined and spheroid volume calculated as $V = (1/6)\ \pi\ d_{max}\ d^2_{min}$ ** $P < 0.01$

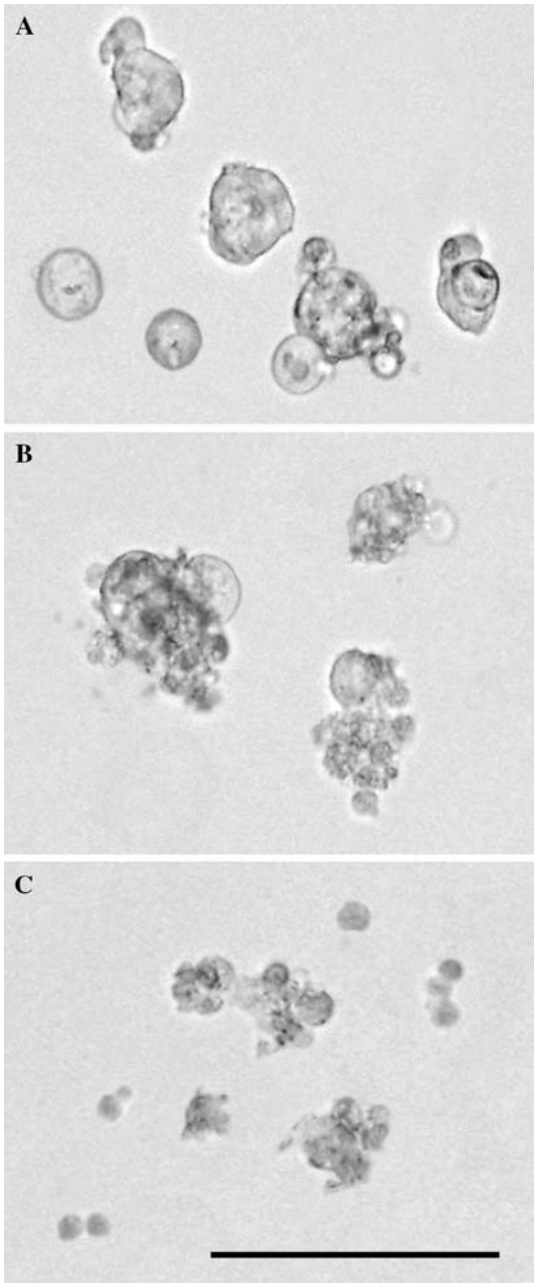

Fig. 3 Disaggregating effect of MZ 1 μM on H295R spheroids. **a** 5 days MZ treatment; **b** 5 days control; **c** 13 days MZ treatment. Bar: 100 μm

Effect of MZ on cancer cells growth in vivo

The MZ antitumor activity was also tested in vivo. Tumors were established by subcutaneous injection of H295R cells in matrigel in nude mice. A 1 mg dose of MZ significantly inhibited the growth of H295R cells. The growth reduction obtained at a dose of 2 mg of MZ was not very different from those obtained with the lower dose. The experiment was then repeated with the SW-13 human cell line. MZ inhibited tumor development with a kinetic similar to that of H295R cells. These results are shown on Fig. 4.

Table 1 Tumorigenicity of H295R and SW-13 cells after subcutaneous implantation in nude mice

No of cells injected	Tumorigenicity	Range of measurable tumors (days)
1×10^6 H295R in HBSS	0/10	0
3×10^6 H295R in HBSS	0/10	0
6×10^6 H295R in HBSS	9/10	34–68
1×10^6 H295R in matrigel	10/10	25–34
1×10^6 SW-13 in HBSS	0/10	0
3×10^6 SW-13 in HBSS	8/10	22–46
6×10^6 SW-13 in HBSS	7/10	20–42
1×10^6 SW-13 in matrigel	10/10	18–28

Different number of H295R or SW-13 cells in HBSS or matrigel were injected subcutaneously in nude mice. Tumor growth was monitored daily

In vivo metastases formation

To analyze if MZ could inhibit metastases formations, six millions of H295R or SW-13 cells were injected intravenously and intraperitoneally in nude mice. Twenty days later, mice injected with H295R cells received a daily oral administration of 1 mg of MZ, 2 mg of MZ, vehicle or saline. Treatment of mice injected with SW-13 started 10 days after cell implantation. In both cases, analyses were performed after 29 days of oral treatment. SW-13 was unable to form metastases when injected intravenously (data not shown) and H295R cells did not form metastases also when injected intraperitoneally, confirming that these cells are poorly aggressive and cannot grow in nude mice unless implanted with matrigel or at very high concentration (data not shown). SW-13 formed metastases only in the intestines and the daily oral administration of 1 mg of MZ reduced the mean metastases number to about 50%. Differently to the subcutaneous models, the effects obtained at a dose of 2 mg of MZ were more evident and the reduction in the number of metastases was approximately 75% (Table 2).

Matrigel invasion assay

As it was impossible to determine if MZ could inhibit H295R cell metastases formation and since MZ disrupt the microtubules network, we analyzed its effects on H295R cells migration and invasion. Cells were treated with increasing doses of MZ for 48 h in the matrigel invasion chamber and cells on the lower surface of the membrane were fixed and then stained with hematoxylin and then counted. Data represented on Fig. 5 indicated that MZ

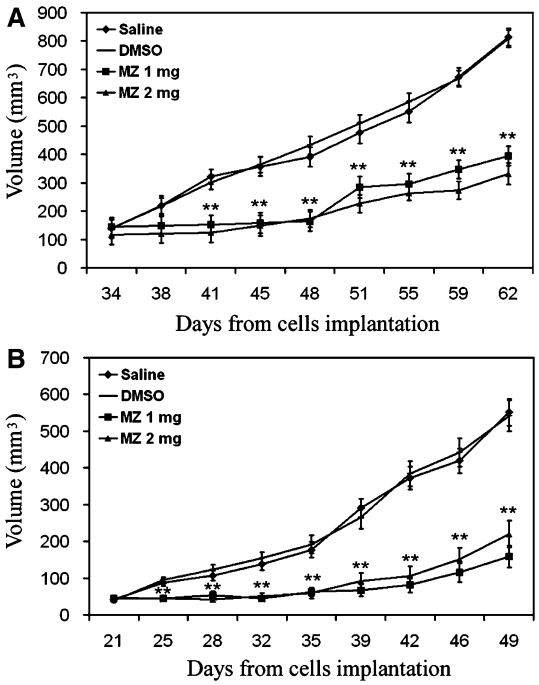

Fig. 4 **a** Inhibitory effects of MZ on the growth of H295R cells subcutaneously implanted in athymic nude mice. **b** Inhibitory effects of MZ on the growth of SW-13 cells subcutaneously implanted in athymic nude mice. Values shown are mean ± SEM ($n = 6$). ** $P < 0.01$

Table 2 In vivo metastases formation 6×10^6 SW-13 cells were injected intraperitoneally in nude mice

Experimental groups	SW-13	
	Mean metastases count	Control (%)
Vehicle	24	100
MZ 1 μM	12.8	53
MZ 2 μM	6.2	26

Treatments started 10 days from cells implantation and last 29 consecutive days. Metastases count was performed by macroscopic and microscopic examination of visceral organs, lungs and brain

significantly inhibited cell invasion in a dose dependent manner. I.C.50 was 0.085 μM a non cytotoxic dose as demonstrated by the in vitro proliferation test (Fig. 1.). The total inhibitory effects attained at 1 and 10 μM are attributable also to MZ cytotoxicity. *MZ induced apoptosis in H295R and SW-13 cells in vitro and in vivo*

To evaluate if the inhibitory effect of MZ on the in vitro and in vivo growth of H295R and SW-13 cells was due to apoptosis, analysis of DNA fragmentation was performed by agarose gel electrophoresis. As shown in Fig. 6, specific DNA cleavage, evident in electrophoretically analysis as a typical ladder pattern due to multiple DNA fragments, was

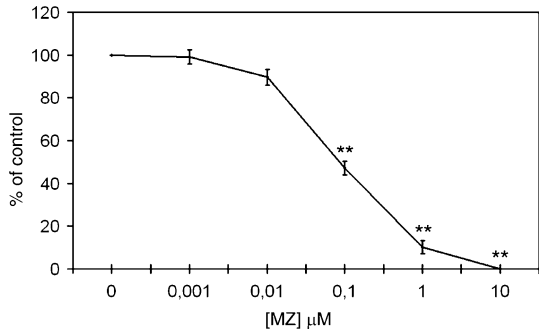

Fig. 5 Cells were treated with increasing doses of MZ for 48 h in the matrigel invasion chamber and cells on the lower surface of the membrane were fixed and then stained with hematoxylin and then counted. MZ inhibited invasion of H295R cells at non cytotoxic concentration; I.C.50: 0.085 μM. ** $P < 0.01$

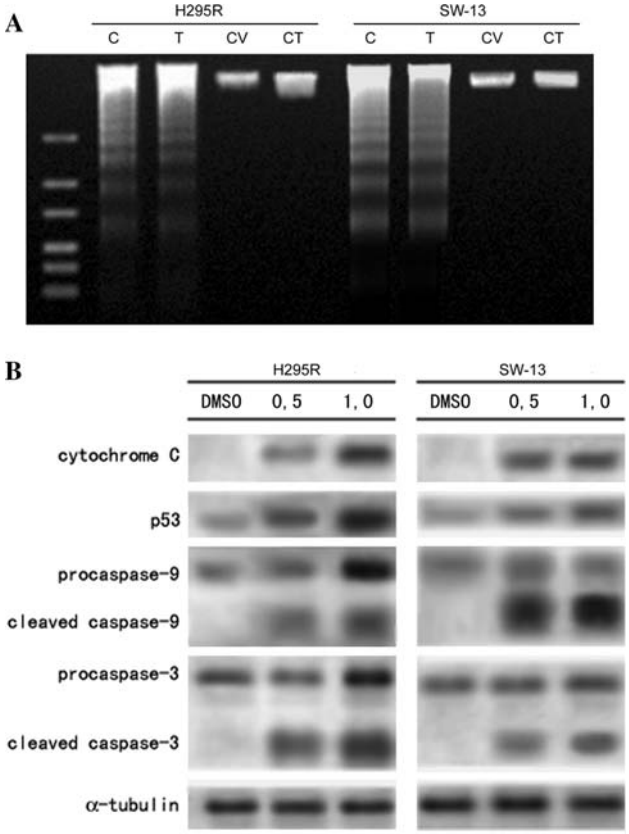

Fig. 6 **a** Apoptosis induction by MZ. *CV* cells treated with vehicle, *C* cells treated with 1 μM of MZ, *CT* tumors treated with vehicle, *T* tumors treated with 1 mg of MZ. Results represent one out of three separate experiments. **b** Lysates from untreated and treated cells were separate on SDS-PAGE and probed with the appropriate antibodies. MZ induced procaspase-9 and procaspase-3 expression and activation and cytochrome *c* release from mitochondria in H295R and SW-13 cells after a 24 h treatment. Also p53 expression was induced by the MZ treatment

observed in tumors and cells treated with MZ 1 μM, but not in vehicle-treated cells and tumors. Also a number of apoptotic gene family proteins were measured using western blot analysis. p53 protein was elevated in both the cell lines treated with MZ, suggesting its involvement in the apoptosis induction of the cells. Moreover, MZ significantly released cytochrome *c* in a dose-dependent manner. Indeed, using a monoclonal antibody, a single immunoreactive band was observed in H295R and SW-13 cells 24 h after treatment. This result suggests that MZ-induced apoptosis requires cytochrome *c* release from mitochondria. To analyze whether caspase-9 and -3 were activated during MZ-induced apoptosis, the proteolysis of procaspase-9 and procaspase-3 was measured by western blot analysis. A 24 h MZ treatment induced the cleavage of procaspase-9 and -3 in a dose-dependent manner as resulted by the presence of caspase-9 and caspase-3 fragments.

Angiogenesis evaluation

H295R and SW-13 cells were implanted subcutaneously in nude mice which then received a daily oral administration of MZ. Tumor samples were collected after 29 days of treatment for immunohistochemical analysis. As shown in Fig. 7, the anti-mouse CD31 antibody staining revealed an intricate network of blood vessel both in control and treated tumors demonstrating that the antitumoral effect of MZ was not due to angiogenesis inhibition (Sections a, b). The intense vascularization as well as the presence of vessels with regular lumen (Sections c, d) are attributable to the use of matrigel for cells implantation. Tumors implanted without matrigel resulted less vascularized and rich of immature vessels (Sections e, f).

Discussion

This study confirms the antitumoral effects of MZ previously reported by other researchers. MZ significantly inhibited H295R and SW-13 human adrenocortical carcinoma cells growth in vitro and in vivo and analysis revealed that these effects were due to apoptosis induction. Mechanisms responsible for the antitumoral properties of mebendazole are not totally known yet. Classical

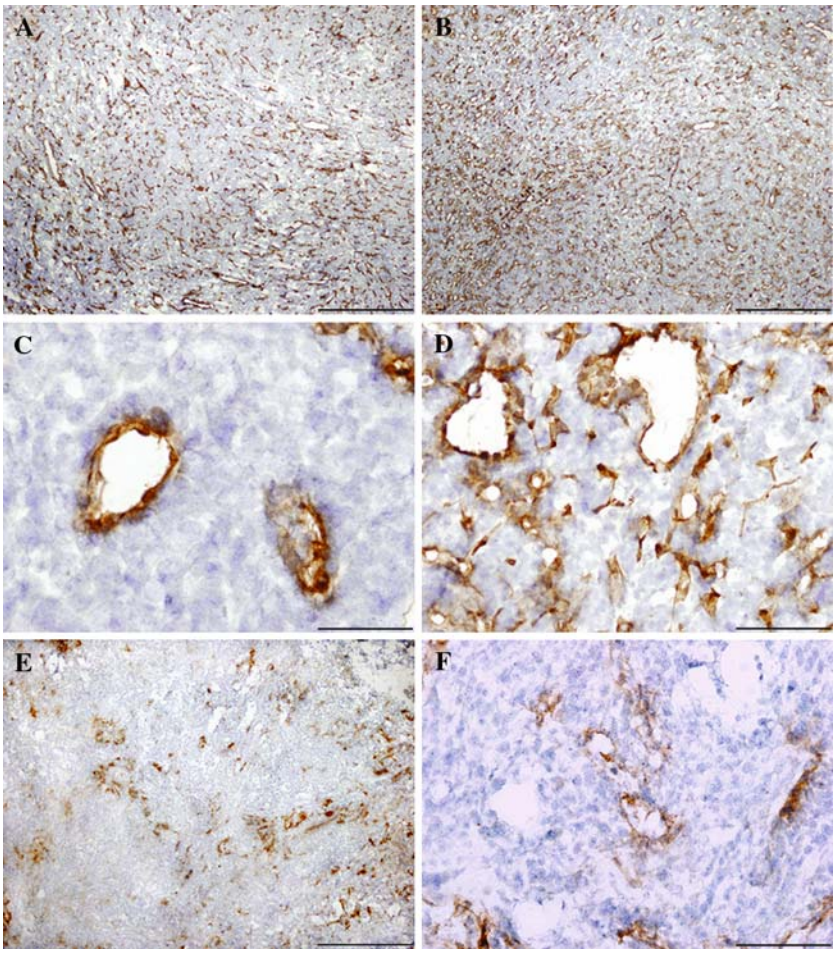

Fig. 7 CD31 staining. Control tumors implanted with matrigel (**a**, **c**). MZ treated tumors implanted using matrigel (**b**, **d**). Untreated tumors implanted without matrix component (**e**, **f**). **a**, **b** and **e** bar: 500 μm. **c**, **d** and **f** bar: 100 μm

chemotherapeutics, such as Paclitaxel or Vinca alkaloids, act preventing the formation of spindle microtubules [9]. However, as many studies demonstrated, interfering with the mechanism of cell division is not sufficient to block cancer growth. Recently, researchers found that MZ induced depolymerization of tubulin and inhibited normal spindle formation in Non-small cell lung cancer cells, thus resulting in cancer cells apoptosis [12]. The ratio of soluble/pellets tubulin in cancer cells treated with MZ was significantly higher compared to control group, indicating depolymerization of microtubules. On the contrary, cells treated with paclitaxel had a lower ratio than control group, confirming its effects on preventing microtubules breakdown [16]. Depolymerising tubulin of assembled microtubules, MZ could be more effective than classical chemotherapeutics, inhibiting cancer cell growth by different ways. Indeed, in the cited study, MZ resulted more efficient in reducing the number of lung colonies if compared to Paclitaxel. It has been suggested that MZ could be considered a microtubule inhibitor with pleiotropic effects and further studies will elucidate the mechanisms of MZ action.

As shown in Fig. 1, in the in vitro tests the I.C. 50 for MZ was approximately 0.25 μM. The decrease between 0.1 and 1 μM of MZ of the Fig. 1 curve totally matches the results previously obtained by other authors using different cell lines. It is known that the cytotoxicity of drugs is due to a number of damaging effects over a certain threshold which depends on the nature of the cells. Therefore, a dose below the cell specific threshold is not able to induce cell death or significantly inhibit cell proliferation. This assumption seems also to be valid for apoptosis where the concentration of death stimulating agents, in order to be effective, need to exceed certain levels. Since MZ was cytotoxic against the H295R and SW-13 cells (Fig. 1) by inducing apoptosis (Fig. 6), one explanation for the differences of MZ effects at 0.1 and 1 μM could be that the threshold for the cytotoxic effects of this drug is set between this two concentrations. Analysis of growth rate of H295R cells subcutaneously implanted in nude mice, evidenced that MZ was shown to be more effective during the initial phase of the treatment, where tumors increment was minimum. It is known that since cancer cells grow rapidly and adherently, cytotoxic drugs affect mainly the external layers of tumors, thus being insufficient to have an effect on the internal cells. Results obtained with the tridimensional in vitro culture (Figs. 2, 3) support this hypothesis showing that MZ required several days to completely dissolve the tumor spheroids. On the contrary, in the monolayer culture, MZ rapidly killed the cancer cells. Nevertheless, the effects of MZ were highly significant as it strongly reduced tumor increment. As conventional cancer therapy is relying on combination therapies, MZ efficacy can be increased combining MZ with other cytotoxic or anti-angiogenic drugs which can mutually improve their diffusion in the tumor mass.

Differently from other works, in this study MZ did not inhibit angiogenesis in tumors. The use of matrigel, required for the H295R and SW-13 cells implantation, can be tricky for angiogenesis analysis. Matrix components improve microvessel formation and therefore the inhibitory effects of drugs on microvessel formation can be emphasized. Indeed, antiangiogenetic drugs are commonly tested in vivo in matrigel plugs implanted subcutaneously in C57BL or nude mice. Analysis of the CD31 staining revealed a consistent blood vessels network in both, control and treated tumors, demonstrating that the antitumoral effects of MZ on the in vivo cells growth were not due to angiogenesis inhibition. In all tumors implanted with matrigel, vessels with regular lumen were present, whereas tumors implanted with HBSS were characterized by immature vessels with a poor contact between endothelial cell and a discontinuous lining (Fig. 7). However, since immature tumor microvessels are more susceptible to the effects of drugs [3, 7, 19], this can explain the discrepancies between our and previous works. Matrigel increased the tumorigenicity and stabilized the growth of H295R and SW-13 cells as only 10^6 of them were sufficient to generate subcutaneous tumors in nude mice (Table 1). The necessity of stromal components, such as extracellular matrix, to grow in mice is a representative feature of poorly aggressive human cancer cells. It has been suggested that cancer associated stroma can promote the growth of tumors by stimulating early angiogenesis [17] and data here presented support this hypothesis as matrigel increased tumorigenesis of H295R and SW-13 cells as well as blood vessels formation.

MZ did not inhibit normal fibroblast growth in vitro and, at the doses used to suppress adrenocortical carcinoma, no side effects were noted. Considering the clinical data deriving from its use as antihelmintic [1, 13], MZ toxicity should not represent a central concern in cancer therapy.

MZ inhibited the metastases formation of SW-13 cells confirming the result obtained by previous studies which demonstrated the in vivo inhibitory effects of MZ on metastases formation [12, 16]. H295R cells were unable to form metastases in mice when injected intraperitoneally or intravenously. However, anti-metastatic properties of MZ can be assumed considering its effects in the invasion test, which showed that non cytotoxic doses of MZ significantly reduced the number of H295R cells invading the extracellular matrix and migrating through the membrane pores.

Metastases are the main cause of cancer death. Whereas standard therapies are essentially sufficient for primary tumors, no effective treatment is available once cancer cells colonize local limphnodes or distant sites [2] Based

upon the data here presented, MZ appears to be appropriate for metastases prevention and treatment, when tumor masses are small and single circulating cancer cells are present. Overall, these data suggest that a mebendazole treatment, also in combination with standard therapies, could provide a new protocol for the inhibition of adrenocortical carcinoma growth.

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