EFFECT OF THE ANTIPARASITIC DRUG MEBENDAZOLE ON CHOLANGIOCARCINOMA GROWTH

Kanlayanee Sawanyawisuth[1,2,3], Tara Williamson[3], Sopit Wongkham[1,2] and Gregory J Riggins[3]

[1] Department of Biochemistry, [2]Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand; [3]Department of Neurosurgery, School of Medicine, Johns Hopkins University, Baltimore, MD, USA

Abstract. Mebendazole (MBZ) is an anthelmintic drug which inhibits tubulin polymerization and eventually causes apoptosis in target organisms. Antitumor activity of MBZ has been reported in various cancers. The aim of this study was to investigate the effect of MBZ on cholangiocarcinoma (CCA) cells *in vitro* and *in vivo*. MBZ reduced cell proliferation in the KKU-M213 cell line associated with a remarkable enhancement of caspase-3 gene expression and enzyme activity. Oral administration of MBZ slightly reduced the growth rate of subcutaneously xeno-grafted KKU-M213 in nude mice. The TUNEL assay showed an increase of apoptotic cell numbers in the xenograft tumor tissue of MBZ-treated mice. The data obtained in this study suggested that MBZ can suppress CCA cell proliferation via caspase-3 activated apoptosis. Further investigation of the antitumor effects of MBZ might support the use of MBZ as an alternative drug for CCA treatment.

Keywords: cholangiocarcinoma, mebendazole, apoptosis, caspase-3

INTRODUCTION

Liver fluke infection caused by *Opisthorchis viverrini* is a public health problem in Northeast Thailand and is an important risk factor for cholangiocarcinoma (CCA), a cancer of the biliary epithelium (Sripa *et al*, 2007). The poor prognosis of CCA may be, in part, due to its biological aggressiveness and its poor response to conventional chemo-therapeutics (Morise *et al*, 2010). Discovery of alternative anticancer drugs would be helpful for treatment for CCA patients. However, the cancer drug discovery process is time-consuming and extremely costly. One new strategy in the search for such drugs is drug repositioning (or repurposing), in which a new indication for an existing drug is identified.

Mebendazole (MBZ), methyl N-[6-(benzoyl)-1H-benzimidazol-2-yl] carbamate is an anthelmintic drug that has been used extensively for gastrointestinal parasitic infections in humans with the remarkable efficacy and safety (Lacey, 1988). MBZ binds to the tubulin subunits in the gut epithelium and the tegument of helminth parasites, prevents tubulin polymerization and inhibits glucose up-

Correspondence: Kanlayanee Sawanyawisuth, Department of Biochemistry and Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel/Fax: +66 (0) 43 348386
E-mail: kanlayanee@kkumail.com

take (Kohler, 2001; MacDonald *et al*, 2004). Tubulin is important in cell division and is also known as a target for several cancer-chemotherapy drugs, including paclitaxel, colchicine, and vincristine (Jordan and Wilson, 2004).

Anticancer activity of MBZ has been shown in preclinical studies on adrenocortical carcinoma, lung cancer, brain cancer and melanoma (Mukhopadhyay *et al*, 2002; Sasaki *et al*, 2002; Martarelli *et al*, 2008; Bai *et al*, 2011). It is therefore interesting to explore whether MBZ exhibit anticancer activity against CCA. In this study, the effects of MBZ on CCA cell growth *in vitro* and *in vivo* in xenografted mice were examined. The mechanism by which MBZ suppressed proliferation of CCA cell lines was explored. Our results suggest a potential use of MBZ as an anticancer drug for CCA.

MATERIALS AND METHODS

Cell culture and treatment

KKU-M213 cells were grown in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and 1% penicillin/streptomycin under standard conditions. MBZ (Sigma-Aldrich, St Louis, MO) was dissolved in dimethylsulphoxide (DMSO) to a concentration of 100 mM and stored at -20°C as a master stock solution.

Cell proliferation assay

KKU-M213 (3,000 cells) were seeded in 96-well tissue-culture plates (Becton Dickinson, San Jose, CA), allowed to attach overnight and then incubated with various concentration of MBZ for 72 hours. Cell proliferation was measured with a colorimetric assay using a Cell Counting Kit-8 (CKK-8, Dojindo Laboratories) (Kumamoto, Japan) which is a tetrazolium salt-based method. The amount of formazan produced was read at 450 nm on a PerkinElmer VICTOR3 plate reader (Doaners Grove, IL). Measurements were made in triplicate. The absorbance value indicates the number of viable cells.

Quantification of caspase-3 activity

Caspase-3 activity was measured using the Caspase-Glo 3/7 Assay (Promega, Madision, WI) according to the manufacturer's protocol. Cells were seeded in black clear 96-well plates (Becton Dickinson), incubated overnight, and treated with MBZ for 24 hours. Then, 50 μl of Caspase-Glo 3/7 reagent was added to each well and the luminescent signal was measured using a PerkinElmer VICTOR3 plate reader. The assay was carried out in triplicate. The luminescence value indicates degree of caspase activity.

Apoptosis-related protein expression

Profiles of apoptosis-related proteins were analyzed using a human apoptosis array kit (#ARY-009, R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Vehicle- and MBZ-treated KKU-M213 cells lysates were diluted and incubated overnight with the array membrane. The array was washed to remove unbound protein, incubated with the antibody cocktail, and then developed using streptavidin-horseradish peroxidase and chemiluminescence detection reagents. The pixel intensity was quantified using Image J software. Relative fold change of protein expression induced by MBZ treatment was compared to the vehicle control sample.

Apoptosis analysis by TUNEL (TdT-mediated dUTP biotin nick end-labeling) method

Cell apoptosis in CCA tumor tissues transplanted into mice was determined using the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI) according to the manufacturer's protocol. In brief, paraffin-embedded tissue sections

(5 μm thickness) were deparaffinized in xylene, rehydrated in a descending series of ethanol concentrations and digested using proteinase K. After incubation with the equilibration buffer, samples were incubated in 50 μl of TUNEL reaction mixture for 1 hour. The labeled ends were detected after incubation in the 3,3′-diaminobenzidine (DAB) chromogen and the slides were counterstained with hematoxylin. We counted TUNEL positive cells from five randomly selected microscopic fields at 200x magnification.

***In vivo* tumor growth in the xenograft model**

All the animal studies were approved by the Johns Hopkins University Animal Care and Use Committee (MO13M21). Female athymic nude mice (4-6 weeks old; $n = 5$ in each group) were purchased from the National Cancer Institute (Frederick, MD). KKU-M213 cells ($3x10^6$) were resuspended in 100 μl PBS and then mixed with an equal volume of growth factor–reduced Matrigel (BD Biosciences, Sandiago, CA). The mixture was subcutaneously (s.c.) injected into the right dorsal flank. At day five after implantation of the tumor cells, MBZ tablets (Aurochem Laboratories, Mumbai, India) were resuspended in phosphate-buffered saline (PBS) and mixed with 50% sesame oil (Sigma). An MBZ dose of 50 mg/kg/day was administered by oral gavage for five days with two days off, each week. Control animals were fed with PBS mixed with 50% sesame oil (Bai *et al*, 2011). Tumors were measured three times a week with calipers, and tumor volumes were calculated by the formula 0.5 ($L \times W^2$) where L is the length and W is the width of the tumor. At the time of death, tumors were collected from mice for further analysis.

Statistical analysis

All determinations were performed at least in triplicate. The results are presented as a mean value ± standard deviation (SD). Differences of mean values of all groups were compared using one-way ANOVA. A $p \leq 0.05$ was considered a significant result. If statistical significance was inferred from one-way ANOVA, all pair-wise differences were calculated using the Scheffe method. Stata version 10.1 (College Station, TX) was used to perform the statistical analyses.

Table 1
Absorbance values of the CKK-8 cell-viability assay in MBZ-treated KKU-M213 cells at 72 hours.

MBZ concentration (μM)	Absorbance value (mean ± SD)	*p*-value
0	1.114 ± 0.068	Reference
0.001	1.164 ± 0.025	0.64
0.1	1.076 ± 0.010	0.999
1	0.615 ± 0.018	<0.001
10	0.613 ± 0.021	<0.001
50	0.491 ± 0.010	<0.001

RESULTS

MBZ suppresses *in vitro* proliferation of KKU-M213 cells

Proliferation of KKU-M213 cells was analyzed using the CKK-8 assay. Cells were treated with various concentrations of MBZ (1 nM - 50 μM in DMSO) for 72 hours. DMSO-treated cells were used as a vehicle control. MBZ concentrations more than 1 μM significantly suppressed the growth of KKU-M213 cells ($p < 0.001$, Table 1) but the response was not strictly dose-dependent.

MBZ induces caspase-3 activation in KKU-M213 cells

To determine whether the decrease

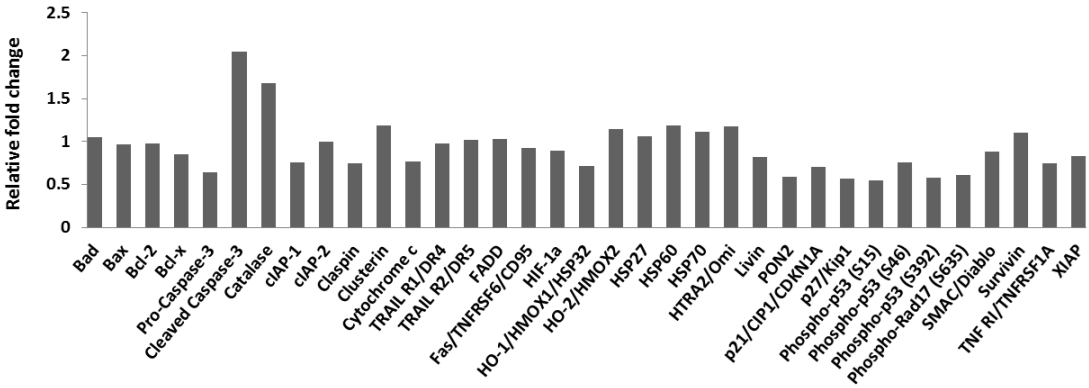

Fig 1–Relative fold-changes in expression of apoptosis-related proteins in KKU-M213 cells. Lysates of KKU-M213 cells were treated with DMSO as a vehicle control, or with 0.5 μM MBZ for 24 hours. These were subjected to apoptosis arrays.

Table 2
Caspase-3 activity in MBZ-treated KKU-M213 cells at 24 and 48 hours.

MBZ concentration (μM)	Caspase-3 activity (mean±SD)			
	At 24 hr	*p*-value	At 48 hr	*p*-value
0	770 ± 68	Reference	1,638 ± 44	Reference
0.5	1,397 ± 95	0.009	3,217 ± 418	0.025
1	1,705 ± 251	0.001	4,079 ± 769	0.003

of cell viability after MBZ treatment was due to the activation of apoptotic process, the activity of the executioner, caspase-3, was measured. KKU-M213 cells were treated with 0.5 and 1 μM MBZ for 24 and 48 hours. Table 2 demonstrates that MBZ treatment induced a significant increase in caspase-3 activity in a dose- and time-dependent manner.

The effect of MBZ on expression of apoptosis-related proteins was further determined using an apoptosis array screen kit. The results showed that KKU-M213 cells treated with 0.5 μM MBZ for 24 hours did not show remarkable changes of most apoptosis-related proteins compared to the vehicle control (expressed as relative fold-change). The most substantial change, however, was the increase in the expression of cleaved caspase-3 (2.05-fold) and the concomitant decrease in the expression of procaspase-3 (Fig 1).

Effect of MBZ on CCA tumor growth in athymic nude mice

The effect of MBZ on *in vivo* tumor growth was examined using a xenograft nude mouse model with subcutaneously implanted KKU-M213 cells. From day 29 post-implantation, tumors of MBZ-treated mice began to grow slower than those in the control group. This suppression of growth rate continued until the end of the experiment on day 45. Mean tumor volume of MBZ-treated mice was 977.83 ±

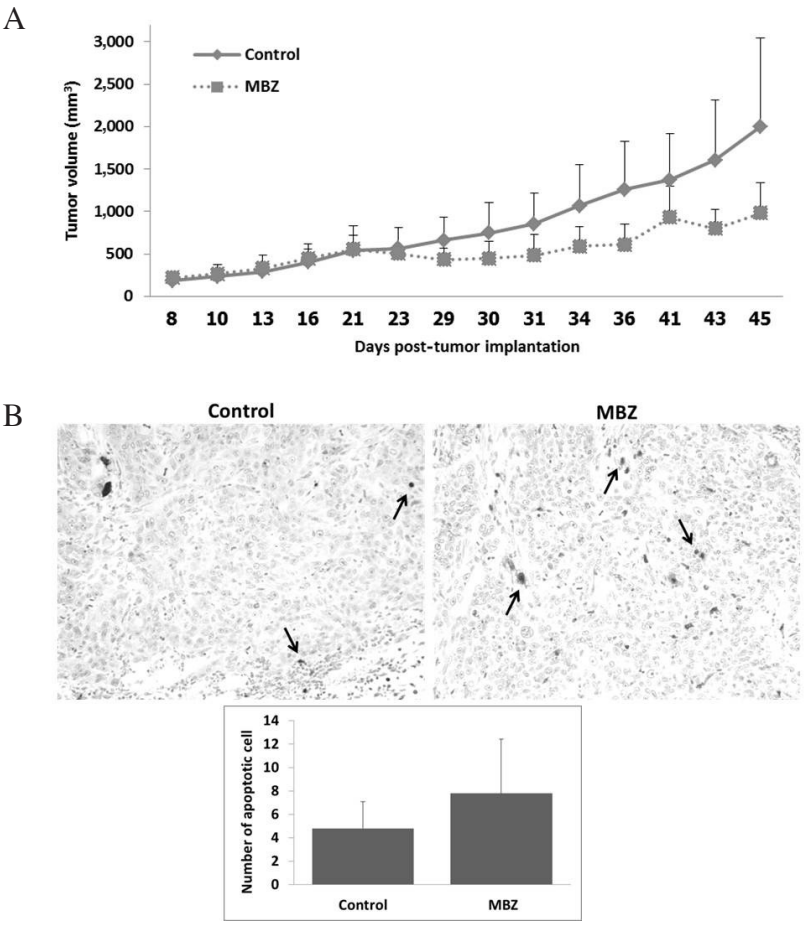

Fig 2–Effect of MBZ on CCA tumor growth *in vivo*. (A) KKU-M213 CCA cells were implanted in athymic nude mice. MBZ was given orally beginning 5 days after tumor implantation at a daily dose of 50 mg/kg for 5 days, with 2 days off, each week. Tumor volume was measured 2-3 times a week during the course of treatment. (B) Representative tumor sections from mice were analyzed using TUNEL assay for apoptotic cell death. The number of TUNEL positive cells (indicated arrows) in tumors was counted from five random microscopic fields. Magnification, x200.

362.81 mm^3, which was smaller than that of the control mice (2,003.00 ± 1,038.60 mm^3, $p = 0.22$, Fig 2A).

Then, xenograft tumor sections were analyzed using the TUNEL assay to determine proportions of apoptotic cells. Tumor tissues from mice treated with MBZ displayed 1.63-fold more positively stained apoptotic cells, than did those from the control mice ($p = 0.24$, Fig 2B).

DISCUSSION

MBZ, a broad-spectrum antiparasitic drug with a well-understood record of side effects and safety (Lacey, 1988; Sajid *et al*, 2006; Vuitton, 2009), is a microtubule-disrupting agent and shows antitumor activity in preclinical models (Mukhopadhyay *et al*, 2002; Sasaki *et al*, 2002; Martarelli *et al*, 2008; Bai *et al*, 2011; Doudican *et al*, 2013). Our work is to examine the antitumor effects of MBZ in CCA. We demonstrated here that MBZ inhibited the proliferation of KKU-M213 cells *in vitro* and significantly induced caspase-3 enzyme activity. Caspase-3 is the crucial executioner caspase, cleaving critical cellular substrates that induce the morphological and biochemical features of apoptosis. Thus, activation of this death protease can indicate the apoptosis event in MBZ-treated cells. MBZ inhibited the growth of CCA cells in a dose-independent manner, suggesting that MBZ may have other molecular targets besides tubulin. At least, our preliminary findings reported here are consistent with

other studies on lung cancer, melanoma and adrenocortical carcinoma (Sasaki *et al*, 2002; Doudican *et al*, 2008; Martarelli *et al*, 2008). This indicated that MBZ can induce caspase-3-dependent apoptosis in several cancers including CCA. Since the response of CCA cells to MBZ might be variable, evaluation of anti-CCA effects of MBZ must be tested using more cell lines of various phenotypes.

We have shown here in a subcutaneous CCA xenograft model that MBZ slightly slowed the tumor growth rate in association with a slight increase of the number of apoptotic cells. These minor anti-proliferative effects are probably due to the inability of MBZ to overcome the rapid tumor mass formation of KKU-M213 cells in nude mice, since we administered the drug on day five after implantation. In agreement with the study on adrenocortical carcinoma, MBZ was shown to be more effective during the initial phase of the treatment, when the severity of the tumors was minimum (Martarelli *et al*, 2008). These data suggest that MBZ may be suitable for cancer treatment at the early stage when tumor masses are small. However, most CCA patients are diagnosed at an advanced stage. In order to halt the aggressively proliferating CCA cells before developing the disease, the use of MBZ as a preventive drug might be considered, since it is inexpensive and commercially available, well tolerated and safe. Further assessment of the molecular mechanism and chemopreventive effect of MBZ in the animal model of CCA carcinogenesis is a prerequisite before embarking on human studies.

In summary, our results reported here provide the evidence for the first time that MBZ reduces CCA cell proliferation with marked increase of caspase-3-dependent apoptosis. Activation of this major executioner caspase suggests that it is one of the pathways by which MBZ induces cell death in CCA. Additional investigation of antitumor effect of MBZ might support the use of MBZ as an alternative drug in CCA.

ACKNOWLEDGEMENTS

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Health cluster (SHeP-GMS) and Khon Kaen University to SW. KS is supported by the Faculty of Medicine, Khon Kaen University, Thailand. GJR is supported by the Irving J Sherman Research Professorship in Neurosurgery and the Virginia and DK Ludwig Fund for Cancer Research. We are grateful to Dr Banchob Sripa, Faculty of Medicine, Khon Kaen University for providing CCA cell lines in this study. We would like to thank Prof Yukifumi Nawa for the critical reading of this manuscript via the Faculty of Medicine Publication Clinic, Khon Kaen University, Thailand. Conflicts of interest: None declared.

REFERENCES

Bai RY, Staedtke V, Aprhys CM, Gallia GL, Riggins GJ. Antiparasitic mebendazole shows survival benefit in 2 preclinical models of glioblastoma multiforme. *Neuro Oncol* 2011; 13: 974-82.

Doudican N, Rodriguez A, Osman I, Orlow SJ. Mebendazole induces apoptosis via Bcl-2 inactivation in chemoresistant melanoma cells. *Mol Cancer Res* 2008; 6: 1308-15.

Doudican NA, Byron SA, Pollock PM, Orlow SJ. XIAP downregulation accompanies mebendazole growth inhibition in melanoma xenografts. *Anticancer Drugs* 2013; 24: 181-8.

Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. *Nat Rev Cancer* 2004; 4: 253-65.

Kohler P. The biochemical basis of anthelmintic action and resistance. *Int J Parasitol* 2001; 31: 336-45.

Lacey E. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *Int J Parasitol* 1988; 18: 885-936.

MacDonald LM, Armson A, Thompson AR, Reynoldson JA. Characterisation of benzimidazole binding with recombinant tubulin from *Giardia duodenalis*, *Encephalitozoon intestinalis*, and *Cryptosporidium parvum*. *Mol Biochem Parasitol* 2004; 138: 89-96.

Martarelli D, Pompei P, Baldi C, Mazzoni G. Mebendazole inhibits growth of human adrenocortical carcinoma cell lines implanted in nude mice. *Cancer Chemother Pharmacol* 2008; 61: 809-17.

Morise Z, Sugioka A, Tokoro T, *et al*. Surgery and chemotherapy for intrahepatic cholangiocarcinoma. *World J Hepatol* 2010; 2: 58-64.

Mukhopadhyay T, Sasaki J, Ramesh R, Roth JA. Mebendazole elicits a potent antitumor effect on human cancer cell lines both *in vitro* and *in vivo*. *Clin Cancer Res* 2002; 8: 2963-9.

Sajid MS, Iqbal Z, Muhammad G, Iqbal MU. Immunomodulatory effect of various anti-parasitics: a review. *Parasitology* 2006; 132: 301-13.

Sasaki J, Ramesh R, Chada S, Gomyo Y, Roth JA, Mukhopadhyay T. The anthelmintic drug mebendazole induces mitotic arrest and apoptosis by depolymerizing tubulin in non-small cell lung cancer cells. *Mol Cancer Ther* 2002; 1: 1201-9.

Sripa B, Kaewkes S, Sithithaworn P, *et al*. Liver fluke induces cholangiocarcinoma. *PLoS Med* 2007; 4: e201.

Vuitton DA. Benzimidazoles for the treatment of cystic and alveolar echinococcosis: what is the consensus? *Expert Rev Anti Infect Ther* 2009; 7: 145-9.