Send Orders of Reprints at reprints@benthamscience.org

Current Cancer Drug Targets, **2012**, *12*, **1233-1243**

Anti-tumor Efficacy of a Recombinant Human Arginase in Human Hepatocellular Carcinoma

Ariel K.M. Chow[1,2], Lui Ng[1], Hung Sing Li[1], Chi Wai Cheng[3], Colin S.C. Lam[1], Thomas C.C. Yau[1,2], Paul N.M. Cheng[4], Sheung Tat Fan[1,2], Ronnie T.P. Poon[1,2] and Roberta W.C. Pang[1,2,*]

[1]Department of Surgery, [2]Centre for Cancer Research and [3]Department of Medicine, LKS Faculty of Medicine, The University of Hong Kong, Pokfulam Road, Hong Kong, China; [4]Bio-Cancer Treatment International Limited, Hong Kong

Abstract: Hepatocellular carcinoma (HCC) is considered as auxotrophic for arginine and BCT-100, a new recombinant human arginase, has been synthesized for arginine deprivation to inhibit arginine-dependent tumor growth. The aim of the present study was to evaluate the effects of BCT-100 on the inhibition of *in vitro* cell proliferation of HCC cell lines and *in vivo* tumor growth. The molecular mechanism involved was also studied. The anti-tumor efficacy of BCT-100 on cell proliferation, cell cycle distribution and cellular apoptosis were determined in human hepatoma HepG2 and PLC/PRF/5 cells. Protein expression in the Wnt/β-catenin and Akt signaling pathways were analyzed by western blotting. Tumors were also established subcutaneously and BCT-100, in combination with oxaliplatin, was administrated i.p. to study the anti-tumor growth of the drugs. Treatment with BCT-100 was found to inhibit cell proliferation and enhance caspase-dependent cellular apoptosis. Cell cycle arrest at S phase was observed. Inhibition of Wnt/β-catenin and Akt signaling pathways, with a reduction in survivin and XIAP protein expressions, were also observed. Furthermore, combined treatment of BCT-100 and chemotherapy with oxaliplatin demonstrated synergistic inhibiting effect on tumor growth and the overall survival probability was enhanced as compared with BCT-100 or oxaliplatin treatment alone. These pre-clinical data demonstrate robust anti-tumor activity of BCT100 in HCC, thus providing the basis for its exploitation as a potential therapeutic agent in arginine-driven tumors. The positive effect of testing BCT100 with oxaliplatin in PLC/PRF/5 tumours also supports the rationale of combining BCT-100 and oxaliplatin in the clinical treatment of HCC.

Keywords: Hepatocellular carcinoma, human recombinant arginase, novel therapy, preclinical study, survivin, Wnt/β-catenin signaling pathway, XIAP.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer death worldwide. It is the fourth leading cancer and the third leading cause of cancer-related deaths in Hong Kong [1]. HCC is highly chemoresistant, such that no conventional chemotherapy has been proven to prolong the survival rate of HCC patients [2-4]. Therefore, it is worthwhile to identify more effective therapeutic agents for HCC. Development of research on the molecular biology of carcinogenesis and tumor progression of HCC is leading to a new era of molecular targeting therapy towards several important signaling pathways, such as insulin-like growth factor-1 receptor (IGF-IR), epidermal growth factor receptor (EGFR), hepatocyte growth factor (HGF)/c-MET, mitogen-activated protein kinase (MAPK), Akt and Wnt/β-catenin signaling pathways [5-7]. Most recently, an important molecular target for HCC therapy is arginine deprivation.

Certain cancers such as melanoma and HCC are regarded as auxotrophic for arginine, because they lack the expression of enzymes, argininosuccinate synthetase (ASS-1), argininosuccinate lyase (ASL) and/or ornithine transcarbamylase (OTC) in the urea cycle [8-12]. Arginine is a non-essential amino acid which can be biosynthesized in the gut/kidney axis from citruline [13]. Arginine has long been confirmed to promote tumor growth *in vitro* and *in vivo* [14, 15] and its demand increases in tumor cells. In the urea cycle, OTC is the enzyme that converts ornithine to citrulline, whereas citrulline is converted to argininosuccinate by ASS and then to arginine by ASL. Therefore, without the expression of any one of these enzymes, the urea cycle is terminated and the depletion of arginine by an addition of recombinant arginase cannot be restored, leading to cancer cell death [16, 17].

Recent synthesis of a pegylated recombinant human arginase has been developed to deprive arginine and inhibit arginine-dependent tumor growth like that in HCC, melanoma and leukemia [8, 9, 18-20]. BCT-100 is a recombinant human liver arginase produced in an *E.coli* expression system. It is covalently attached, *via* a succinamide propionic acid linker, to polyethylene glycol (PEG) of molecular weight 5000 kDa and has an *in vivo* half life of about 3 days [19, 20]. It inhibits the proliferation of HCC cell lines and xenograft tumor growth [19]. A phase 1 study performed recently has demonstrated that Peg-rhArg1 has preliminary evidence of activity in advanced HCC patients [21]. However, the molecular mechanism through which BCT-100 inhibits cell proliferation and causes apoptosis has not been confirmed yet. The purpose of the

*Address correspondence to this author at the Department of Surgery, LKS Faculty of Medicine, The University of Hong Kong Queen Mary Hospital, 102 Pokfulam Road, Pokfulam, Hong Kong; Tel: 852-22555907; Fax: 852-28175475; E-mail: robertap@hku.hk

1873-5576/12 $58.00+.00

studies reported here was to focus on the mechanism of action of BCT-100 using HCC cells. The effect of such mechanism of action on *in vitro* cell proliferation of PLC/PRF/5 and HepG2 were evaluated. The antitumor efficacy of BCT-100, in combination with oxaliplatin, was also characterized *in vivo* using the PLC/PRF/5 tumor xenograft model.

MATERIALS AND METHODS

Drugs and Reagents

BCT-100 was generously provided by Bio-Cancer Treatment International Limited (Hong Kong). One unit of BCT-100 is defined as the amount of enzyme that can produce 1 μmol urea/min at 30 ºC, pH 8.5. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless specified below.

Expression of ASS-1, ASL and OTC in HCC Specimens

The protocol was approved by the HKU/QMH Ethics Committee and written informed consent was obtained from each patient. Fresh tumor tissues were collected immediately after resection of HCC from 42 patients who underwent hepatectomy at the Department of Surgery, the University of Hong Kong, Queen Mary Hospital. Specimens were collected and frozen with liquid nitrogen. For the analysis of the protein expression of ASS-1, ASL and OTC, specimens were lysed with urea buffer (2 M urea in 100 mM Tris-HCl buffer, pH 7.0). Protein amounts were measured using a Bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein (20 μg) were loaded onto a 10% (w/v) SDS-polyacrylamide gel under reducing condition and transferred to PVDF membrane (Amersham Bioscience, Piscataway, NJ, USA). Blots were probed with the following antibodies: anti-ASS-1 (Novus Biologicals, Littleton, CO, USA), anti-ASL (Abnova Corporation, Taipei City, Taiwan), anti-OTC (Sigma-Aldrich, Saint Louis, MO, USA) and anti-β-actin (Sigma-Aldrich). After probing with horseradish peroxidase-conjugated secondary antibodies, membranes were incubated in Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA). The band intensity of each protein was normalized with that of β-actin band.

Cell Culture

Human hepatoma PLC/PRF/5 (CRL-8024), HepG2 (HB-8065) and Hep3B (HB-8064) cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FBS and 1% Penilicilin/Streptomycin, at 37 ºC and in a humidified incubator with under 5% of CO_2 in the air. Cells were treated with BCT-100 at different concentrations, as indicated below.

Cell Proliferation and Colony Formation

The effect of BCT-100 on cell proliferation was first examined by MTT assay. PLC/PRF/5, HepG2 and Hep3B cells were plated in 96-well culture plates, at 1 x 10^4 cells per well. After 24 h, media were replaced with culture medium (PBS control) or medium with the indicated concentrations (15.6-1000 mU/ml) of BCT-100. After 72 h, cell viability was assessed with the replacement of culture medium with MTT solution (1 mg/ml) (Invitrogen), and incubation at 37 °C for 4 h. After removing the MTT solution, the crystals remaining in the wells were dissolved in 100 μl of dimethyl sulfoxide (DMSO). Absorbance at 570 nm was measured by a 96-well plate reader, with a reference wavelength of 650 nm. The percentage of surviving cells was determined by dividing the absorbance of BCT-100-treated cells by the average absorbance of control cells from 3 replicate samples.

The proliferation rate of PLC/PRF/5 and HepG2 was also analyzed using colony formation assay. 5 x 10^3 cells were plated in 6-well plates. After 24 h, media were replaced with culture medium (PBS control) or medium with BCT-100 (5, 10, 50, 100 mU/ml). Cells were allowed to grow with a change of fresh media every 3-4 days for 2 weeks. Cells were then washed and stained with 0.05% crystal violet. Stained colonies of cells were then solubilized with 1% SDS and the percentage of surviving cells was determined by dividing the average absorbance of BCT-100-treated cells by the average absorbance of control cells from 3 replicate samples.

Annexin V and Cell Cycle Analysis by Flow Cytometry

The effect of BCT-100 on cellular apoptosis was examined by Annexin V, a PE & 7-AAD apoptosis detection kit (BD Biosciences, San Jose, CA, USA), according to the instructions of the company. Briefly, 1 x 10^5 PLC/PRF/5 and HepG2 cells were grown in 6-well plates. After 24 h, the cells were treated with BCT-100 (50, 100, 200 and 400 mU/ml) for 72 h. They were harvested, washed with PBS, and stained with annexin V/7AAD mixture in binding buffer for 15 min, at room temperature and in the dark. Apoptotic cells were then analyzed by the Cytomics FC 500 flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA).

For the cell cycle distribution analysis, PLC/PRF/5 and HepG2 cells (after treatment) were fixed with ice-cold ethanol (70% v/v) overnight at 4 ºC, washed with PBS, resuspended in PBS containing 5 mg/ml propodium iodide (PI) and 200 μg/ml RNase A and incubated at room temperature for 30 min. Cells with different phases in the cell cycle were determined by flow cytometry and analyzed using FlowJo (version 8.7, Tree Star, Inc. Ashland, OR, USA).

Western Blotting Analysis

After treatment of PLC/PRF/5 cells with BCT-100 (50, 100, 200 and 400 mU/ml) for 72 h, the cells were lysed by ice-cold RIPA containing 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM PMSF, and protease inhibitor cocktail (Roche Diagnostics, Penzberg, Germany) in 50 mM Tris-HCl buffer, pH 7.4. Cells lysates were subjected to western blotting analysis, as mentioned above. Blots were probed with the following antibodies: anti-Wnt3a, anti-Wnt5a/b, anti-low-density-lipoprotein receptor protein (LRP)6, anti-dishevelled (dsh) homolog (Dvl)2, anti-Dvl3, anti-Akt, anti-mammalian target of rapamycin (mTOR) (Cell Signaling Technology Inc., Beverly, MA, USA), anti-β-catenin (BD Biosciences), anti-

survivin (Novus Biologicals Inc., Littleton, Co, USA), anti-x-linked inhibitor of apoptosis protein (XIAP) (Cell Signaling Technology Inc.) and anti- β-actin (Sigma-Aldrich). The band intensity of each protein was normalized with that of the β-actin band.

Caspase 3, 8 and 9 Activities by Flow Cytometry

PLC/PRF/5 cells exposed to BCT100 (50, 100, 200 and 400 mU/ml) for 48 h were analyzed for activities of caspase-3, caspase-8 and caspase-9 by flow cytometry using CaspGLOW^{TM} Red Active Caspase-3, Caspase-8 and Caspase-9 staining kits (BioVision Inc., Mountain View, CA, USA), in accordance with the manufacturer's instructions. Briefly, both floating and attached cells were harvested and washed with PBS. The cells (3×10^5) were then incubated with specific caspase inhibitor conjugated to sulfo-rhodamine (Red-DEVD-FMK, Red-IETD-FMK, and Red-LEHD-FMK for detection of caspase-3, caspase-8, and caspase-9, respectively), in 300 µl of culture medium, at 37 ºC and incubated with 5% CO_2 for 1 h in the dark. After being washed with PBS twice, stained cells were analyzed by flow cytometry.

In Vivo Study

Animal study was approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong. BALB/c-nu/nu (nude) mice were maintained in laminar flow cabinets under pathogen-free conditions. To generate subcutaneous tumors, PLC/PRF/5 cells from mid-log phase cultures were harvested and suspended in a 50% Matrigel (BD Biosciences) in culture medium and 1×10^6 cells in 0.1 ml were subcutaneously injected in the right flank of each mouse. BCT-100 at 400 U/kg body weight and/or oxaliplatin at 10 mg/kg body weight were administered i.p., twice weekly for 16 weeks, starting when all animals in the study had established tumors averaging 200 mm^3 with 10 mice per group. Tumor dimensions and body weights were recorded twice a week starting from the first day of treatment. The mice were sacrificed on week 16 or when tumor sizes exceeded 30% of their body weight. Tumor volume were calculated using the equation $L \times W^2 \times 3.14 / 6$, where L and W refer to the larger and smaller dimensions collected at each measurement. Data were statistically analyzed with one-way ANOVA and were considered statistically significant at $P < 0.05$.

RESULTS

Expression of ASS-1, ASL and OTC in HCC Specimens

As the expression of ASS-1, ASL and OTC may affect the sensitivity of HCC cells towards BCT-100 treatment, the protein expression of these enzymes from 42 HCC samples were studied (Fig. **1)**. Expression of all 3 enzymes was found in 24% (10/42) of the tumor samples (Table **1**). Therefore, 76% of the HCC patients, with any one of these three enzymes' expression missing, are predicted to be sensitive towards BCT-100 treatment.

Table 1. The expression of ASS-1, ASL and OTC in 42 pairs of HCC samples.

Protein Expression of Enzymes			No. of Case among the Tumor Samples
ASS-1	ASL	OTC	
+	+	+	10
+	+	-	2
+	-	-	4
+	-	+	0
-	+	-	10
-	-	+	3
-	+	+	6
-	-	-	7

'+' and '-' represents positive and undetectable protein expression of the indicated enzymes by western blotting analysis.

BCT-100 Inhibits Cell Proliferation, Induces Apoptosis and Arrests Cells at S Phase of the Cell Cycle in HCC Cells

Fig. **2A** demonstrates the expression of ASS-1, ASL and OTC in PLC/PRF/5, HepG2 and Hep3B cells. ASS-1

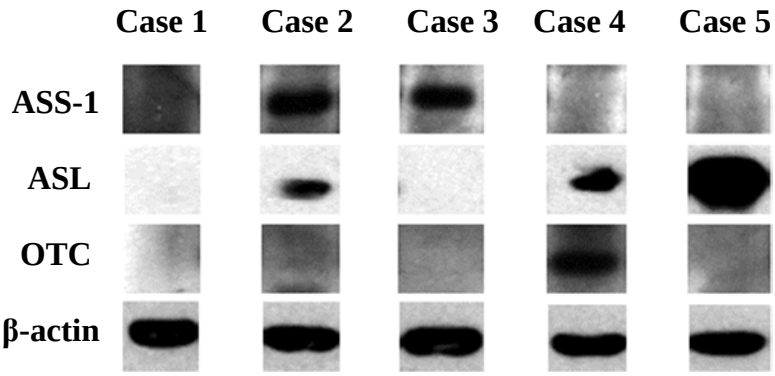

Fig. (1). Expression of ASS-1, ASL and OTC in 5 representative cases from 42 HCC specimens was evaluated by Western Blot analysis. Those with clear bands are regarded as positive expression whereas those with no bands were regarded as negative/undetectable protein expression.

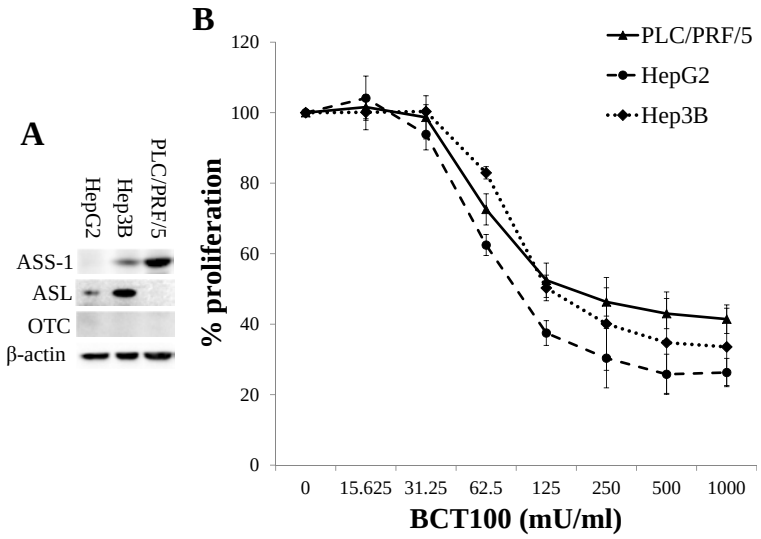

Fig. (2). Expression of ASS-1, ASL and OTC negatively correlates with sensitivity to BCT-100 treatment in HCC cell lines. **A.** Expression of the three proteins in PLC/PRF/5, HepG2 and Hep3B cells was evaluated by Western Blot analysis. ASS-1 expression was undetectable in HepG2. ASL expression was undetectable in PLC/PRF/5. OTC expression was undetectable in the three cell lines. One representative experiment out of three is shown. **B.** BCT-100 inhibited the proliferation of the HCC cells in a dose-dependent manner. MTT assay was performed by treating PLC/PRF/5, HepG2 and Hep3B cells with BCT-100 (15.6 to 1000 mU/ml) for 3 days. Results are presented as means ± SD of three separate experiments.

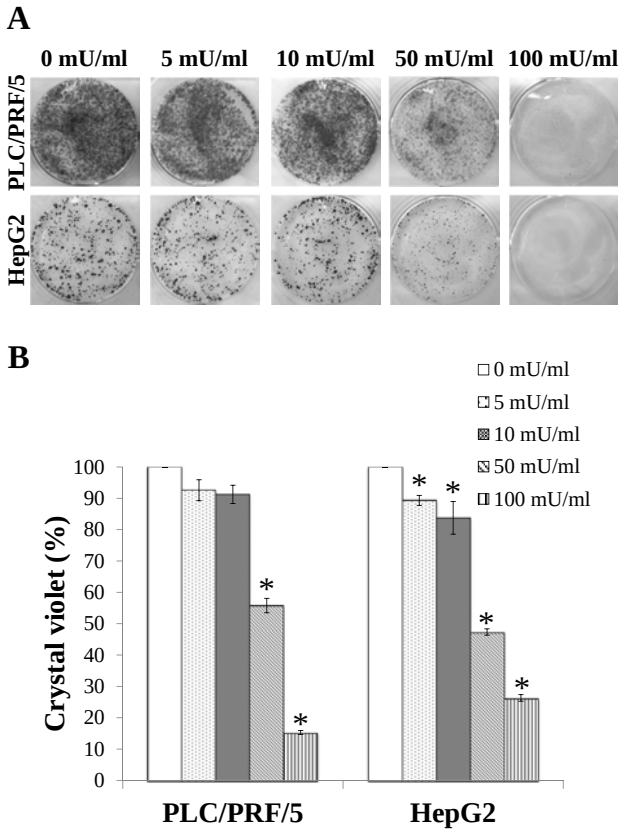

Fig. (3). BCT-100 treatment reduced colony formation of PLC/PRF/5 and HepG2 cells. 5 x 10^3 cells were plated in 6-well plates and treated with BCT-100 at the indicated concentration for 2 weeks. **A.** Colonies were visualized by staining with crystal violet and one representative experiment out of three is shown. **B.** Cells stained with crystal violet were completely dissolved in 0.1% SDS solution and the percentage of crystal violet was determined by dividing the absorbance of BCT-100-treated cells by the average absorbance of control cells. Results are presented as means ± SD of three separate experiments. * indicates statistical significant at $p<0.01$ when compared with control.

expression was undetectable in HepG2 cells, whereas ASL expression was undetectable in PLC/PRF/5 cells. OTC expression was undetectable in these three HCC cell lines. This suggests that all three cell lines can be predicted to be sensitive towards BCT-100 treatment. BCT-100 inhibited cell proliferation in a dose dependant manner with an IC_{50} of 183 mU/ml in PLC/PRF/5 cells, 106 mU/ml in HepG2 cells and 160 mU/ml in Hep3B cells (Fig. **2B**). Dose-dependent reduction in the number of colony formed was also observed (Fig. **3A**), with a reduction to 7.85% ± 0.47% and 11.9% ± 0.27% for PLC/PRF/5 and HepG2 cells when treated with 100 mU/ml BCT100, respectively (Fig. **3B**).

The percentage of live, apoptotic and necrotic cells were determined by the annexin V/7AAD assay. After exposing the cells to the indicated concentrations for 72 h, apoptosis was predominant with a dose-dependent increase in apoptotic cells from 5.00% ± 0.86% to 30.0% ± 5.60% in PLC/PRF/5 cells (Fig. **4A**) and from 5.06% ± 1.06% to 28.7% ± 5.10% in HepG2 cells (Fig. **4B**), when treated with 400 mU/ml BCT-100.

Cell cycle distribution of PLC/PRF/5 (Fig. **5A**) and HepG2 (Fig. **5B**) was measured by staining the DNA content with PI. Decreases in the percentage of cells in the G_0/G_1 phase (PLC/PRF/5: from 71.5% ± 0.74% to 54.5% ± 2.15%; HepG2: from 61.0% ± 1.90% to 34.4% ± 2.08%) and increases in the percentage of cells in the S phase (PLC/PRF/5: from 10.3% ± 0.86% to 31.9% ± 1.65%; HepG2: from 20.1% ± 1.46% to 44.9% ± 0.87%) were observed in both cell lines when treated with 400 mU/ml BCT-100. Therefore, cell cycle arrest at S phase was obtained in both PLC/PRF/5 and HepG2 cells.

BCT-100 Inhibits Wnt/β-catenin Signaling Pathway

We furthered our studies of the mechanism of BCT-100 on the Wnt/β-catenin signaling pathway using PLC/PRF/5 cells and HepG2 cells (Fig. **6**). For the Wnt/β-catenin signaling pathway, BCT-100 treatment had no effect on the intracellular Wnt3a amd Wnt 5a/b expression. However, lower expression of LRP6, one of the members of the low density lipoprotein receptor related protein family in the Wnt signaling pathway and a Wnt co-receptor, was found in a dose dependent manner. In addition, the expressions of Dvl2 and Dvl3 were reduced. β-catenin expression was also found to be downregulated with BCT-100 treatment, suggesting a

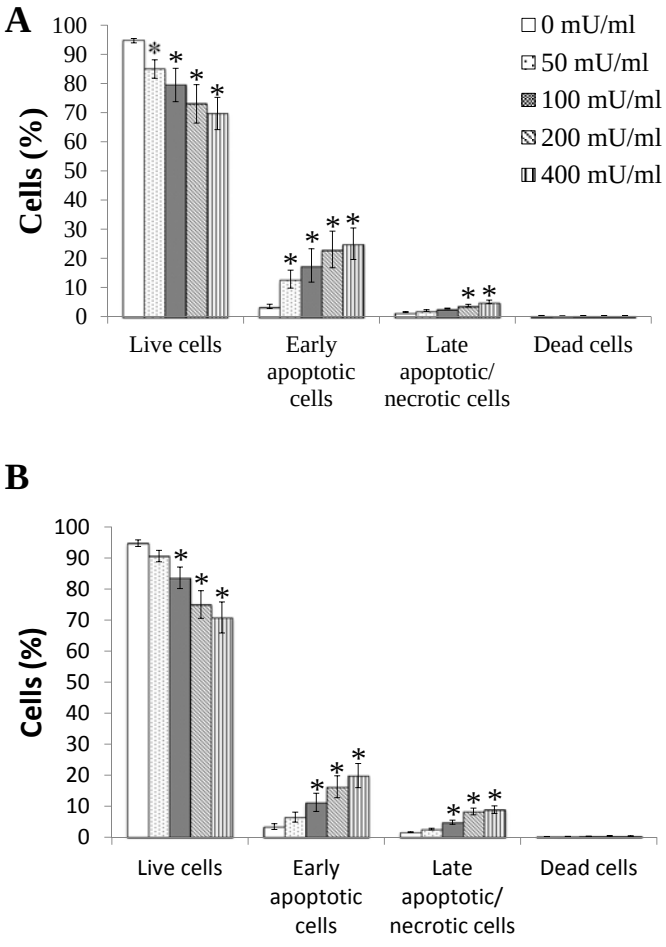

Fig. (4). Increases in apoptosis were observed with the escalating dosage of BCT-100 in **A.** PLC/PRF/5 and **B.** HepG2 cells. Floating and adherent cells were harvested 72 hours after treatment. The percentage of cells stained with annexin and/or 7-AAD was analyzed by flow cytometry. Results are presented as means ± SD of three separate experiments. * indicates statistical significant at $p<0.01$ when compared with control.

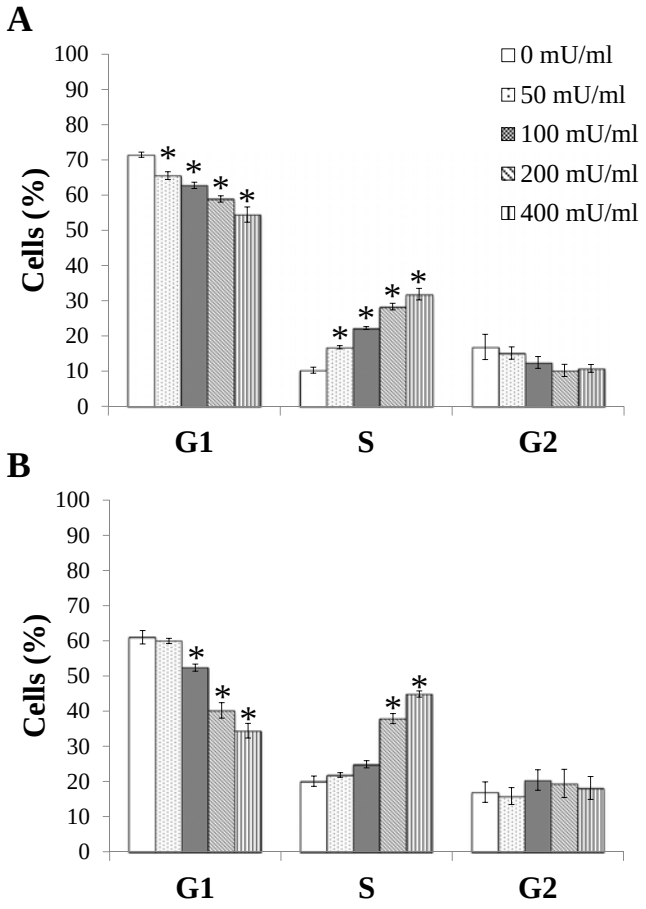

Fig. (5). Dose-dependent increase in S phase population (cell cycle arrest at S phase) was observed with the escalating dosage of BCT-100 in **A.** PLC/PRF/5 and **B.** HepG2 cells. Floating and adherent cells were harvested 72 hours after treatment and stained with PI. The percentage of cells in each phase of the cycle was analyzed by flow cytometry. Results are presented as means ± SD of three separate experiments. * indicates statistical significant at $p<0.01$ when compared with control.

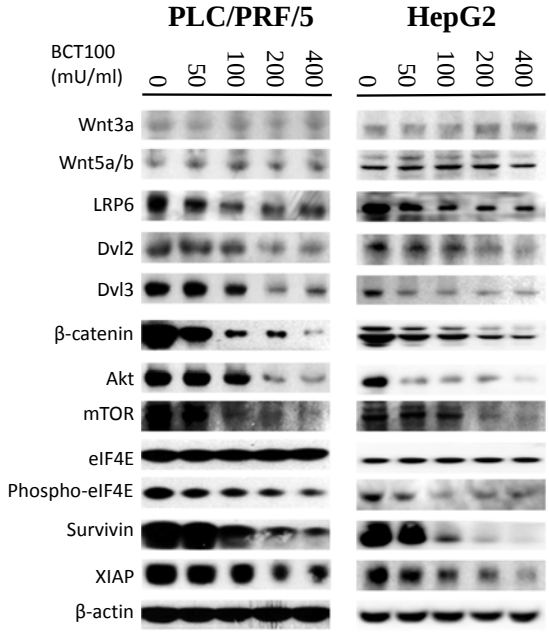

Fig. (6). BCT-100 reduced expression of proteins indicated in PLC/PRF/5 and HepG2 cells. After treatment with BCT-100 with increasing concentration for 72 hours, cells were lysed and protein expression was evaluated by Western Blot analysis. One representative experiment out of three is shown. β-actin was used as a loading control.

reduction in the activity of the canonical Wnt/β-catenin signaling pathway (which enhances ubiquitination of intracellular β-catenin). The reduction in β-catenin expression was also found in HepG2 cells, which expresses a truncated form of β-catenin [22].

As Wnt signaling has been found to regulate Akt activity [23], the expression levels of Akt, mTOR and eIF4E were also investigated (Fig. **6**). BCT-100 treatment of cells led to reduced expressions of both Akt and mTOR in a dose-dependent manner; and had no effect on the expression of the total eIF4E, but led to reduced phosphorylation of eIF4E.

Some of the downstream effectors of these pathways were also studied (Fig. **6**). As survivin is a direct target gene of the Wnt signaling pathway [24] and Akt phosphorylation increases survivin expression [25] and stabilizes XIAP (a member of the inhibitor of apoptosis protein family) from ubiquitination [26], both survivin and XIAP expression were also studied. BCT-100 treatment was found to reduce the protein expression levels of both survivin and XIAP.

Apoptosis Induced by BCT-100 is Caspase-dependent

As survivin and XIAP function through direct interactions to inhibit the activity of several caspases [27], cellular apoptosis caused by BCT-100 treatment may be a caspase-dependent process. Therefore, flow cytometry was used to study the activities of caspase-9, -8 and -3 in PLC/PRF/5 and HepG2 cells (Fig. **7**). After treatment for 2 days, the activities of caspase-9 (PLC/PRF/5: from 3.48% ± 0.75% to 10.7% ± 0.20%; HepG2: 1.48% ± 0.40% to 4.69% ± 0.47%), caspase-8 (PLC/PRF/5: from 21.1% ± 1.98% to 47.9% ± 1.3%; HepG2: 11.6% ± 0.18% to 55.2% ± 3.87%) and caspase-3 (PLC/PRF/5: from 4.38% ± 0.44% to 11.5% ± 1.22%; HepG2: 1.90% ± 0.11% to 10.4% ± 0.46%) increased when treated with 400 mU/ml BCT-100.

BCT-100 Inhibits Xenograft Tumor Growth in Combination with Oxaliplatin

PLC/PRF/5 cells were injected s.c. in the flank of nude mice. BCT-100 at 400 U/kg body weight and/or oxaliplatin

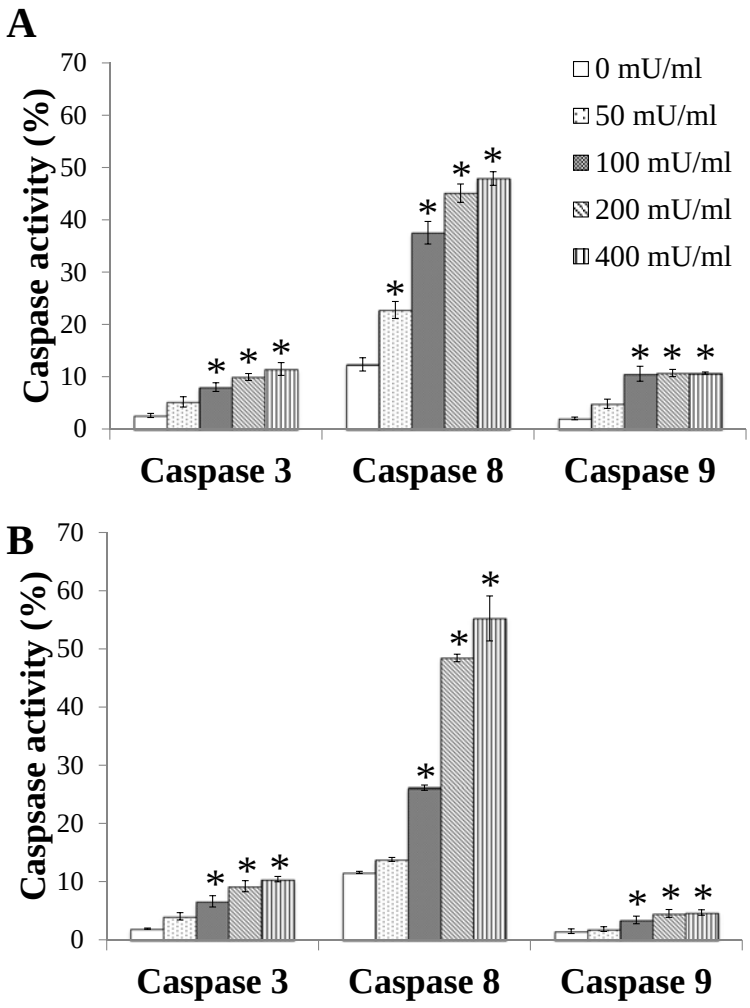

Fig. (7). Dose-dependent increase in caspase-3, -8, and -9 activities was observed with the escalating dosage of BCT-100 in **A.** PLC/PRF/5 and **B.** HepG2 cells. Floating and adherent cells were harvested 48 hours after treatment. Cells were then incubated with specific caspase inhibitor conjugated to sulfo-rhodamine (Red-DEVD-FMK, Red-IETD-FMK, and Red-LEHD-FMK for detection of caspase-3, caspase-8, and caspase-9, respectively). The percentage of cells stained was analyzed by flow cytometry. Results are presented as means ± SD of three separate experiments. * $P<0.01$ when compared with control.

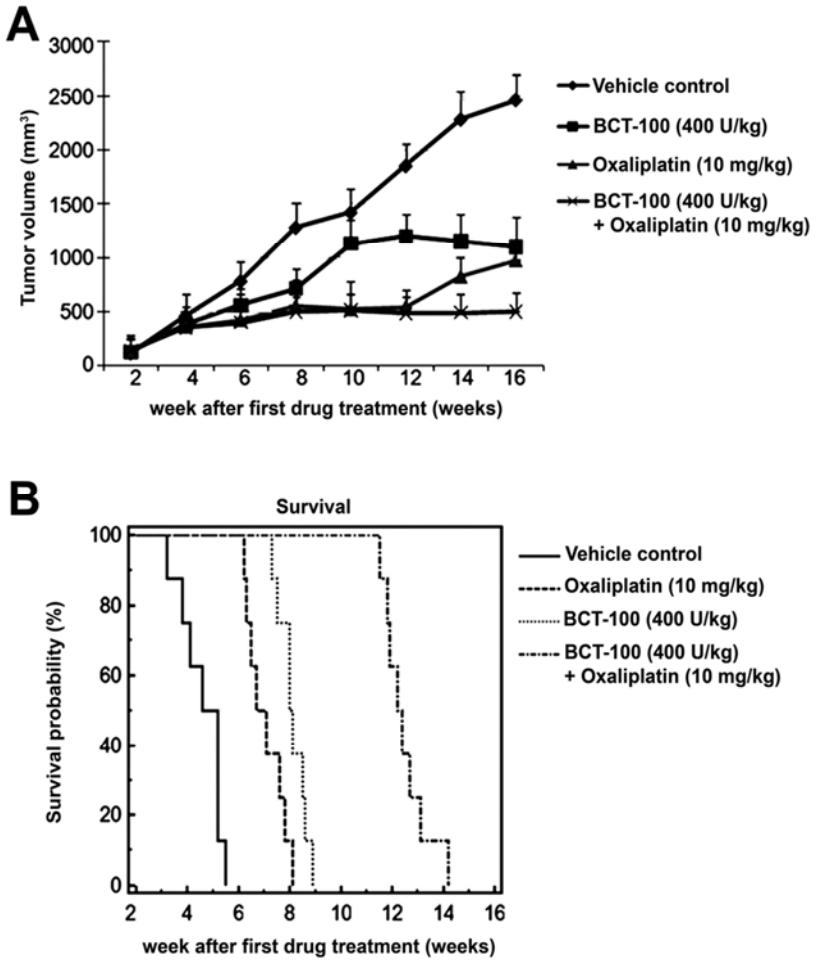

Fig. (8). Combination treatment of BCT-100 and Oxaliplatin demonstrates significant anti-tumor efficacy and improved survival when compared to vehicle control group in xenograft tumors of PLC/PRF/5 cells. **A.** Tumor growth between different treatment groups was statistically analyzed with one-way ANOVA. Animals in all three treatment groups showed significant suppression of tumor growth when compared to the vehicle control group, and combination treatment with BCT-100 and Oxaliplatin resulted in the most significant tumor growth retardation (*P < 0.05 starting from week 12; ** P < 0.01 starting from week 10). **B.** Animal survival between different groups in B was compared by log-rank test. Animals in all three treatment groups showed significantly improved survival as compared with the vehicle control group ($P < 0.01$); and animals of the combination treatment group showed significantly improved survival when compared to either BCT-100 or oxaliplatin single treatment group ($P < 0.05$).

at 10 mg/kg body weight were administered i.p., twice weekly for 16 weeks, starting when all animals in the study had established tumors averaging 200 mm^3 with 10 mice per group. Tumor dimensions and body weights were recorded twice a week, starting from the first day of treatment. Mice were sacrificed on week 16 or when tumor sizes exceeded 30% of their body weight. Tumor volumes were calculated using the equation L x W^2 x 3.14 / 6, where L and W refer to the larger and smaller dimensions collected at each measurement. BCT-100 inhibited the subcutaneous tumor growth in Nude mice (Fig. **8A**). Significant reductions in tumor volumes were recorded starting from week 12 after injection with 400 U/kg body weight BCT-100 alone. However, oxaliplatin treatment alone demonstrated an increase in tumor volume starting from week 12 after injection; whereas combined treatment of 400 U/kg body weight BCT-100 and 10 mg/kg Oxaliplatin significantly abrogated this effect (P=0.041). The group with combined treatment demonstrated a significant increase in survival rate as compared to single treatment with BCT-100 or oxaliplatin alone (Fig. **8B**).

DISCUSSION

HCC is one of the major killer cancers in the world [28]. Treatment options of HCC depend on liver function, tumor size and the presence or absence of metastatic lesions or vascular invasion. As the majority of HCC patients are associated with cirrhosis and impaired liver function, treatment of HCC is more difficult than many other cancers. In most cases, liver resection, radiofrequency ablation or liver transplantation is not feasible especially in advanced HCC. Even after successful surgical procedures, poor prognosis with recurrence and metastasis still occur, which greatly reduces survival rate. Traditional chemotherapy is also found to have no or little benefit in prolonging survival of HCC patients [2-4, 29]. Therefore, new effective

treatment is essential for HCC patients. In this study, we have shown that BCT-100, a recombinant human argianse, effectively inhibits cell proliferation through enhanced caspase-dependent apoptosis and cell cycle arrest at S phase *in vitro*. Besides, antitumor efficacy of BCT-100 is demonstrated by the partial tumor regression of the HCC xenografts *in vivo*. Indeed, the anti-proliferative and anti-tumor efficacy of human recombinant arginase has been demonstrated in HCC [8, 19, 20-21, 30], pancreatic cancer [30], leukemia [18] and melanoma [9]. These results suggest the potential use of BCT-100 as a novel agent for treatment of HCC. Combined treatment of BCT-100 and oxaliplatin exerted an additive tumor growth inhibition in the xenograft model and the survival rate was further enhanced. Oxaliplatin is a platinum-based drug, which inhibits DNA synthesis in cancer cells, and is one of the commonly used chemotherapeutic agents for HCC patients. Low response rate has always been demonstrated with monotherapy of molecular targeting agents; the combination of BCT-100 and oxaliplatin provides an alternative solution for HCC treatment. This may also allow lower concentrations of each drug to be used, thereby reducing the risk of toxicity and enhancing the efficacy of conventional chemotherapeutic agents. This may also prevent the development of chemoresistance, which may develop after pro-longed use of a single agent during treatment.

Upon intravenous administration of BCT-100, arginase metabolizes arginine to ornithine and urea, which in turn depletes intracellular arginine level. Arginine is a precursor for initiation of a variety of metabolic pathways including protein synthesis, polyamines, nucleotides, proline and glutamate, which have been shown to promote tumor growth, survival and invasion [13, 31]. As arginine can be biosynthesized from citrulline and ornithine in the urea cycle by ASS-1, ASL and OTC, the expression of these enzymes is able to predict the sensitivity of a tumor towards BCT-100 treatment. Our study demonstrated that 76% of HCC tumors do not express ASS-1, ASL and/or OTC and thus, the urea cycle is blocked and arginine is unable to be biosynthesized. This is comparable with the findings published previously, where 40% of HCC tumors were deficient in the RNA expression of either one of these enzymes [8]. Therefore, by first screening the expression of these enzymes, the efficacy of BCT-100 can be predicted in HCC patients. It is also suggested by a recent study that human recombinant arginase 1 in combined with L-citrulline supplementation may be a good therapeutic agent for tumors with ASS-1 deficient [32].

Our study demonstrated a reduction in protein expression of LRP6, Dvl2, Dvl3 and β-catenin in the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling is frequently deregulated in HCC, causing uncontrolled cell proliferation, malignant transformation and tumor development [33-36]. β-catenin accumulation was also found in HCC [37]. Blockage of the Wnt/β-catenin signaling pathway by antibodies or antagonists, or suppression with microRNA was also found to be effective in inhibiting HCC tumor growth [38-40]. In addition, the expression of Wnt or Dvl regulates Akt activity [23]. We also demonstrated in this study that a reduction in Akt and mTOR expression, which suggests that a reduction in Akt activity might occur, further reduces the β-catenin accumulation in cells [23]. Reduction in Akt/mTOR pathway reduced the phosphorylation of eIF4E, leading to a reduction in protein translation. Akt phosphorylation has been found to increase survivin expression [25] and stabilize XIAP from ubiquitination [26]. Therefore, we found that reduced Akt expression might lead to the reduction of survivin and XIAP expression levels. Survivin and XIAP has been implicated in the suppression of apoptosis [41]. Survivin is over-expressed in most human cancers, especially HCC, breast and gastric carcinomas, but its expression is undetectable in normal differentiated tissues [42, 43]. Both survivin and XIAP inhibit caspase activity by binding caspase-9 and suppressing its activity, leading to the inhibition of apoptosis [27, 41]. Sorafenib, an effective multi-kinase inhibitor in HCC treatment, was found to reduce the expression of survivin which partly explain the apparent superior anti-tumor activity of sorafenib compared to sunitinib [44]. Therefore, with the decrease in survivin expression pursuant to BCT-100 treatment, enhanced caspase activities driven apoptosis may occur, promoting cancer cell death as demonstrated in this study. Thus, the anti-tumor activity of BCT-100 may be due to the inactivation of the Wnt/β-catenin signaling pathway. However, we cannot confirm whether the reduction in expression of proteins is due to the inhibition of the Wnt/β-catenin signaling pathway or deprivation of arginine (which impairs protein synthesis). It is worthwhile to further the study by using arginine-deprived medium to distinguish between these two mechanisms.

CONCLUSIONS

BCT100 reduced cell proliferation and enhanced the caspase-dependant apoptosis in HCC cells *via* the inhibition of Wnt/β-catenin signalin pathway. Cell cycle arrest in the S phase was observed upon treatment with BCT100. It also reduced tumor growth in the *in-vivo* study. These pre-clinical data demonstrate robust anti-tumour activity of BCT100 in HCC, providing the basis for exploiting its potential use as a therapeutic agent for arginine-driven tumors. In combination with oxaliplatin, reduction in tumor growth was found. This additive effect of BCT100 with oxaliplatin in HCC supports the rationale of combining BCT-100 and oxaliplatin in the clinical treatment of HCC.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Bio-Cancer Treatment International Limited (Hong Kong) for providing us with BCT-100. This study is supported by the Central Allocation Group Research Grant "Molecular Pathology of Liver Cancer – A Multidisciplinary Study" from the Research Grant Council of Hong Kong (HKU-7-CRF09).

ABBREVIATIONS

ASL = argininosuccinate lyase

ASS = argininosuccinate synthetase

Dvl	=	dishevelled (dsh) homolog
eIF4E	=	eukaryotic translation initiation factor 4E
HCC	=	hepatocellular carcinoma
LRP	=	low-density-lipoprotein receptor protein
MAPK	=	mitogen-activated protein kinase
mTOR	=	mammalian target of rapamycin
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
OTC	=	ornithine transcarbamylase
PEG	=	polyethylene glycol
PI	=	propodium iodide
XIAP	=	x-linked inhibitor of apoptosis protein

REFERENCES

[1] Hong Kong Cancer Registry, Hospital Authority. Hong Kong Cancer Stat 2007. 2009. http://www3.ha.org.hk/cancereg/e_canstat2007.pdf. (Accessed Apr 2, **2012**)

[2] Fuchs, C. S.; Clark, J. W.; Ryan, D. P.; Kulke, M. H.; Kim, H.; Earle, C. C.; Vincitore, M.; Mayer, R. J.; Stuart, K. E. A phase II trial of gemcitabine in patients with advanced hepatocellular carcinoma. *Cancer* **2002**, *94* (12), 3186-3191.

[3] Lopez, P. M.; Villanueva, A.; Llovet, J. M. Systematic review: evidence-based management of hepatocellular carcinoma--an updated analysis of randomized controlled trials. *Aliment. Pharmacol. Ther.* **2006**, *23* (11), 1535-1547.

[4] Yeo, W.; Mok, T. S.; Zee, B.; Leung, T. W.; Lai, P. B.; Lau, W. Y.; Koh, J.; Mo, F. K.; Yu, S. C.; Chan, A. T.; Hui, P.; Ma, B.; Lam, K. C.; Ho, W. M.; Wong, H. T.; Tang, A.; Johnson, P. J. A randomized phase III study of doxorubicin versus cisplatin/interferon alpha-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. *J. Natl. Cancer Inst.* **2005**, *97* (20), 1532-1538.

[5] Chan, S. L.; Yeo, W. Targeted therapy of hepatocellular carcinoma: present and future. *J. Gastroenterol. Hepatol.* **2012**, *27* (5), 862-872.

[6] Zhang, Y. W.; Yan, D. L.; Wang, W.; Zhao, H. W.; Lu, X.; Wu, J. Z.; Zhou, J. R. Knockdown of insulin-like growth factor 1 receptor inhibits the growth and enhances chemo-sensitivity of liver cancer cells. *Curr. Cancer Drug Targets* **2012**, *12* (1), 74-84.

[7] Pang, R. W.; Poon, R. T. From molecular biology to targeted therapies for hepatocellular carcinoma: the future is now. *Oncology* **2007**, *72* (Suppl 1), 30-44.

[8] Lam, T. L.; Wong, G. K.; Chong, H. C.; Cheng, P. N.; Choi, S. C.; Chow, T. L.; Kwok, S. Y.; Poon, R. T.; Wheatley, D. N.; Lo, W. H.; Leung, Y. C. Recombinant human arginase inhibits proliferation of human hepatocellular carcinoma by inducing cell cycle arrest. *Cancer Lett.* **2009**, *277* (1), 91-100.

[9] Lam, T. L.; Wong, G. K.; Chow, H. Y.; Chong, H. C.; Chow, T. L.; Kwok, S. Y.; Cheng, P. N.; Wheatley, D. N.; Lo, W. H.; Leung, Y. C. Recombinant human arginase inhibits the *in vitro* and *in vivo* proliferation of human melanoma by inducing cell cycle arrest and apoptosis. *Pigment Cell Melanoma Res.* **2011**, *24* (2), 366-376.

[10] Morris, S.M. Jr. Recent advances in arginine metabolism. *Curr. Opin. Clin. Nutr. Metab. Care* **2004**, *7* (1), 45-51.

[11] Morris, S.M. Jr. Recent advances in arginine metabolism: roles and regulation of the arginases. *Br. J. Pharmacol.* **2009**, *157* (6), 922-930.

[12] Dillon, B. J.; Prieto, V. G.; Curley, S. A.; Ensor, C. M.; Holtsberg, F. W.; Bomalaski, J. S.; Clark, M. A. Incidence and distribution of argininosuccinate synthetase deficiency in human cancers: a method for identifying cancers sensitive to arginine deprivation. *Cancer* **2004**, *100* (4), 826-833.

[13] Wu, G.; Morris, S.M. Jr. Arginine metabolism: nitric oxide and beyond. *Biochem. J.* **1998**, *336 (Pt 1)*, 1-17.

[14] Bach, S. J.; Lasnitzki, I. Some aspects of the role of arginine and arginase in mouse carcinoma 63. *Enzymologia* **1947**, *12* (3), 198-205.

[15] Bach, S. J.; Lasnitzki, I. Some aspects of the metabolism of arginine in malignant tissue. *Biochem. J.* **1946**, *40* (4), xlviii.

[16] Philip, R.; Campbell, E.; Wheatley, D.N. Arginine deprivation, growth inhibition and tumour cell death: 2. Enzymatic degradation of arginine in normal and malignant cell cultures. *Br. J. Cancer* **2003**, *88* (4), 613-623.

[17] Wheatley, D.N.; Philip, R.; Campbell, E. Arginine deprivation and tumour cell death: arginase and its inhibition. *Mol. Cell Biochem.* **2003**, *244* (1-2), 177-185.

[18] Hernandez, C.P.; Morrow, K.; Lopez-Barcons, L.A.; Zabaleta, J.; Sierra, R.; Velasco, C.; Cole, J.; Rodriguez, P.C. Pegylated arginase I: a potential therapeutic approach in T-ALL. *Blood* **2010**, *115* (25), 5214-5221.

[19] Tsui, S.M.; Lam, W.M.; Lam, T.L.; Chong, H.C.; So, P.K.; Kwok, S.Y.; Arnold, S.; Cheng, P.N.; Wheatley, D.N.; Lo, W.H.; Leung, Y.C. Pegylated derivatives of recombinant human arginase (rhArg1) for sustained *in vivo* activity in cancer therapy: preparation, characterization and analysis of their pharma-codynamics *in vivo* and *in vitro* and action upon hepatocellular carcinoma cell (HCC). *Cancer Cell Int.* **2009**, *9*, 9.

[20] Cheng, P.N.; Lam, T.L.; Lam, W.M.; Tsui, S.M.; Cheng, A.W.; Lo, W.H.; Leung, Y.C. Pegylated recombinant human arginase (rhArg-peg5,000mw) inhibits the *in vitro* and *in vivo* proliferation of human hepatocellular carcinoma through arginine depletion. *Cancer Res.* **2007**, *67* (1), 309-317.

[21] Yau, T.; Cheng, P.N.; Chan, P.; Chan, W.; Chen, L.; Yeun, J.;Pang, R.; Fan, S.T.; Poon, R.T. A phase 1 dose-escalating study of pegylated recombinant human arginasae 1 (Peg-rhArg1) in patients with advanced hepatocellular carcinoma. *Invest New Drugs* **2012**, [Epub ahead of print].

[22] Carruba, G.; Cervello, M.; Miceli, M.D.; Farruggio, R.; Notarbartolo, M.; Virruso, L.; Giannitrapani, L.; Gambino, R.; Montalto, G.; Castagnetta, L. Truncated form of beta-catenin and reduced expression of wild-type catenins feature HepG2 human liver cancer cells. *Ann. N. Y. Acad. Sci.* **1999**, *886*, 212-216.

[23] Fukumoto, S.; Hsieh, C.M.; Maemura, K.; Layne, M.D.; Yet, S.F.; Lee, K.H.; Matsui, T.; Rosenzweig, A.; Taylor, W.G.; Rubin, J.S.; Perrella, M.A.; Lee, M.E. Akt participation in the Wnt signaling pathway through Dishevelled. *J. Biol. Chem.* **2001**, *276* (20), 17479-17483.

[24] You, L.; He, B.; Xu, Z.; Uematsu, K.; Mazieres, J.; Mikami, I.; Reguart, N.; Moody, T.W.; Kitajewski, J.; McCormick, F.; Jablons, D.M. Inhibition of Wnt-2-mediated signaling induces programmed cell death in non-small-cell lung cancer cells. *Oncogene* **2004**, *23* (36), 6170-6174.

[25] Papapetropoulos, A.; Fulton, D.; Mahboubi, K.; Kalb, R.G.; O'Connor, D.S.; Li, F.; Altieri, D.C.; Sessa, W.C. Angiopoietin-1 inhibits endothelial cell apoptosis *via* the Akt/survivin pathway. *J. Biol. Chem.* **2000**, *275* (13), 9102-9105.

[26] Dan, H.C.; Sun, M.; Kaneko, S.; Feldman, R.I.; Nicosia, S.V.; Wang, H.G.; Tsang, B.K.; Cheng, J.Q. Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). *J. Biol. Chem.* **2004**, *279* (7), 5405-5412.

[27] O'Connor, D.S.; Grossman, D.; Plescia, J.; Li, F.; Zhang, H.; Villa, A.; Tognin, S.; Marchisio, P.C.; Altieri, D.C. Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc. Natl. Acad. Sci. USA* **2000**, *97* (24), 13103-13107.

[28] Ferenci, P.; Fried, M.; Labrecque, D.; Bruix, J.; Sherman, M.; Omata, M.; Heathcote, J.; Piratsivuth, T.; Kew, M.; Otegbayo, J.A.; Zheng, S.S.; Sarin, S.; Hamid, S.; Modawi, S.B.; Fleig, W.; Fedail, S.; Thomson, A.; Khan, A.; Malfertheiner, P.; Lau, G.; Carillo, F.J.; Krabshuis, J.; Le Mair, A. World Gastroenterology Organisation Guideline. Hepatocellular carcinoma (HCC): a global perspective. *J. Gastrointestin. Liver Dis.* **2010**, *19* (3), 311-317.

[29] Llovet, J.M.; Di Bisceglie, A.M.; Bruix, J.; Kramer, B.S.; Lencioni, R.; Zhu, A.X.; Sherman, M.; Schwartz, M.; Lotze, M.; Talwalkar, J.; Gores, G.J. Design and endpoints of clinical trials in hepatocellular carcinoma. *J. Natl. Cancer Inst.* **2008**, *100* (10), 698-711.

[30] Glazer, E.S.; Kaluarachchi, W.D.; Massey, K.L.; Zhu, C.; Curley, S.A. Bioengineered arginase I increases caspase-3 expression of hepatocellular and pancreatic carcinoma cells despite

induction of argininosuccinate synthetase-1. *Surgery* **2010**, *148* (2), 310-318.

[31] Delage, B.; Fennell, D.A.; Nicholson, L.; McNeish, I.; Lemoine, N.R.; Crook, T.; Szlosarek, P.W. Arginine deprivation and argininosuccinate synthetase expression in the treatment of cancer. *Int. J. Cancer* **2010**, *126* (12), 2762-2772.

[32] Agrawal, V.; Woo, J.H.; Mauldin, J.P.; Jo, C.; Stone, E.M.; Georgiou, G.; Frankel, A.E. Cytotoxicity of humna recombinant arginase 1 (Co)-PEG5000 in the presence of supplemental L-citrulline is dependent on decreased argininosuccinate synthetase expression in human cells. *Anticancer drugs* **2012**, *23* (1), 51-64.

[33] Nhieu, J.T.; Renard, C.A.; Wei, Y.; Cherqui, D.; Zafrani, E.S.; Buendia, M.A. Nuclear accumulation of mutated beta-catenin in hepatocellular carcinoma is associated with increased cell proliferation. *Am. J. Pathol.* **1999**, *155* (3), 703-710.

[34] He, T.C.; Sparks, A.B.; Rago, C.; Hermeking, H.; Zawel, L.; da Costa, L.T.; Morin, P.J.; Vogelstein, B.; Kinzler, K.W. Identification of c-MYC as a target of the APC pathway. *Science* **1998**, *281* (5382), 1509-1512.

[35] Shtutman, M.; Zhurinsky, J.; Simcha, I.; Albanese, C.; D'Amico, M.; Pestell, R.; Ben-Ze'ev, A. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. USA* **1999**, *96* (10), 5522-5527.

[36] Tetsu, O.; McCormick, F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **1999**, *398* (6726), 422-426.

[37] Pang, R.; Yuen, J.; Yuen, M.F.; Lai, C.L.; Lee, T.K.; Man, K.; Poon, R.T.; Fan, S.T.; Wong, C.M.; Ng, I.O.; Kwong, Y.L.; Tse, E. PIN1 overexpression and beta-catenin gene mutations are distinct oncogenic events in human hepatocellular carcinoma. *Oncogene* **2004**, *23* (23), 4182-4186.

[38] Xu, J.; Zhu, X.; Wu, L.; Yang, R.; Yang, Z.; Wang, Q.; Wu, F. MicroRNA-122 suppresses cell proliferation and induces cell apoptosis in hepatocellular carcinoma by directly targeting Wnt/β-catenin pathway. *Liver Int.* **2012**, 32 (5), 752-760.

[39] Wei, W.; Chua, M.S.; Grepper, S.; So, S.K. Blockade of Wnt-1 signaling leads to anti-tumor effects in hepatocellular carcinoma cells. *Mol. Cancer* **2009**, *8*, 76.

[40] Wei, W.; Chua, M.S.; Grepper, S.; So, S. Small molecule antagonists of Tcf4/beta-catenin complex inhibit the growth of HCC cells *in vitro* and *in vivo. Int. J. Cancer* **2010**, *126* (10), 2426-2436.

[41] Li, F.; Ambrosini, G.; Chu, E.Y.; Plescia, J.; Tognin, S.; Marchisio, P.C.; Altieri, D.C. Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* **1998**, *396* (6711), 580-584.

[42] Sah, N.K.; Khan, Z.; Khan, G.J.; Bisen, P.S. Structural, functional and therapeutic biology of survivin. *Cancer Lett.* **2006**, *244* (2), 164-171.

[43] Ito, T.; Shiraki, K.; Sugimoto, K.; Yamanaka, T.; Fujikawa, K.; Ito, M.; Takase, K.; Moriyama, M.; Kawano, H.; Hayashida, M.; Nakano, T.; Suzuki, A. Survivin promotes cell proliferation in human hepatocellular carcinoma. *Hepatology* **2000**, *31* (5), 1080-1085.

[44] Huynh, H.; Choo, S.P.; Toh, H.C.; Tai, W.M.; Chung, A.Y.; Chow, P.K.; Ong, R.; Soo, K.C. Comparing the efficacy of sunitinib with sorafenib in xenograft models of human hepatocellular carcinoma: mechanistic explanation. *Curr. Cancer Drug Targets* **2011**, *11* (8), 944-953.

Received: March 30, 2011 **Revised: September 24, 2011** **Accepted: March 11, 2012**