Cancer Letters 277 (2009) 91–100

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Recombinant human arginase inhibits proliferation of human hepatocellular carcinoma by inducing cell cycle arrest

T.L. Lam [a,b], G.K.Y. Wong [a,b], H.C. Chong [a,b], P.N.M. Cheng [d], S.C. Choi [a,b], T.L. Chow [d], S.Y. Kwok [a,b], R.T.P. Poon [c], D.N. Wheatley [d], W.H. Lo [a,b,*], Y.C. Leung [a,b,*]

[a] Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China
[b] Lo Ka Chung Centre for Natural Anti-Cancer Drug Development, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China
[c] Centre for Cancer Research, Department of Surgery, Queen Mary Hospital, The University of Hong Kong, Hong Kong, China
[d] Bio-Cancer Treatments International Ltd., Hong Kong Science Park, Bio-informatics Building, 3 Park Avenue West, Shatin, NT, Hong Kong, China

ARTICLE INFO

Article history:
Received 23 July 2008
Received in revised form 31 October 2008
Accepted 22 November 2008

Keywords:
Hepatocellular carcinoma (HCC)
Recombinant human arginase
Ornithine transcarbamylase
G_2/M phase arrest
Combination therapy

ABSTRACT

Human hepatocellular carcinoma (HCC) has an elevated requirement for arginine *in vitro*, and pegylated recombinant human arginase I (rhArg-PEG), an arginine-depleting enzyme, can inhibit the growth of arginine-dependent tumors. While supplementation of the culture medium with ornithine failed to rescue Hep3B cells from growth inhibition induced by rhArg-PEG, citrulline successfully restored cell growth. The data support the roles previously proposed for ornithine transcarbamylase (OTC) in the arginine auxotrophy and rhArg-PEG sensitivity of HCC cells. Expression profiling of argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and OTC in 40 HCC tumor biopsy specimens predicted that 16 of the patients would be rhArg-sensitive, compared with 5 who would be sensitive to arginine deiminase (ADI), another arginine-depleting enzyme with anti-tumor activity. Furthermore, rhArg-PEG-mediated deprivation of arginine from the culture medium of different HCC cell lines produced cell cycle arrests at the G_2/M or S phase, possibly mediated by transcriptional modulation of cyclins and/or cyclin dependent kinases (CDKs). Based on these results, together with further validation of the *in vivo* efficacy of rhArg-PEG against HCC, we propose that the application of rhArg-PEG alone or in combination with existing chemotherapeutic drugs may represent a specific and effective therapeutic strategy against HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a leading cause of death in many parts of the world. Treatment has largely been unsuccessful, mean survival after diagnosis being limited from a few months to several years [1–4]. There is clearly an urgent need to identify new and better therapeutic agents and regimes for HCC. Many lines of evidence have shown that *in vitro* arginine depletion, either with an arginine-degrading enzyme or using arginine-deficient medium, leads to rapid destruction of a wide range of cancer cells. For instance, HCC and malignant melanoma are generally claimed to be auxotrophic for arginine, depletion of which leads to cancer cell death [5–13].

We previously reported that the systemic release of endogenous arginase, induced by transhepatic arterial embolisation, led to remarkable HCC remission in patients [14]. This convinced us that human hepatic arginase (also known as arginase I), after suitable formulation to lengthen its half-life, was a potential drug candidate for the treatment of HCC. Therefore, we developed a recombinant form

* Corresponding authors. Address: Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China. Tel.: +852 34008661; fax: +852 23649932 (Y.C. Leung), Tel.: +852 34008724; fax: +852 23649932 (W.H. Lo).
E-mail addresses: bctlo@polyu.edu.hk (W.H. Lo), bctleung@polyu.edu.hk (Y.C. Leung).

0304-3835/$ - see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2008.11.031

of human arginase I, covalently modified with polyethylene glycol (PEG) of molecular weight 5000 via a succinimidyl propionate (SPA) linker. Pegylation greatly increased the enzyme's half-life without affecting its enzymatic activity [6]. We also reported that pegylated recombinant human arginase (rhArg-PEG) inhibited the proliferation of HCC cell lines (including HepG2, Hep3B, PLC/PRF/5, Huh7 and SK-HEP-1 [6]). In addition, rhArg-PEG inhibited the growth of Hep3B xenografts implanted into nude mice [6]. This strongly suggests that rhArg-PEG has clinical potential as an inhibitor of HCC proliferation, similar to pegylated arginine deiminase (ADI-PEG), another arginine-depleting enzyme undergoing clinical trials for HCC and melanoma [5,9,15].

A major difference between rhArg-PEG and ADI-PEG is the ineffectiveness of the latter against tumors that express ASS, the urea cycle enzyme that converts citrulline to argininosuccinate [16]. We previously showed that a number of ASS-expressing cancer cell lines remained sensitive to rhArg-PEG; on the other hand, the expression of another urea cycle enzyme – ornithine transcarbamylase (OTC) – conferred resistance towards rhArg-PEG [6]. One likely explanation is that OTC converts ornithine, the product of arginase activity, to citrulline, thus allowing the replenishment of arginine through the urea cycle. We wish to elucidate the relationship between rhArg resistance and OTC expression, and to compare the prevalence of ASS and OTC expression in HCC patients sampled from Queen Mary Hospital, Hong Kong. This work should provide an estimation of the proportion of HCC patients for whom rhArg-PEG would be an effective treatment.

ADI arrests the cell cycle and induces apoptosis in cancer cells [17–19], and rhArg works in a similar manner, although it has never been stated that cell death is specifically or entirely related to apoptosis [20]. Cell cycle progression is regulated by a genetically conserved family of protein kinases, the cyclin-dependent protein kinases (CDKs). Vertebrates have four CDKs, which are only active when they form a complex with cyclins. The activity of cyclin/CDK complexes is regulated by varying expression levels of the cyclins: D-type cyclins and Cyclin E are required for the progression through G_1 and S phases, Cyclin A is produced in late G_1 phase and accumulates during S and G_2, whereas the expression of B-type cyclins typically peaks during the G_2/M transition. Cyclin A and B-type cyclins are the major activators of CDK1 (also known as cdc2), and the activated Cyclin B1/cdc2 complex mediates multiple mitotic events [21–25]. Using flow cytometry, we wish to address the question of whether rhArg-PEG inhibits the growth of HCC cell lines by disrupting the cell cycle, and, if it does, what the possible effectors are.

Ultimately, our aim was to verify the efficacy of rhArg-PEG against different types of HCC *in vivo*. We have previously shown that Hep3B solid tumors in nude mice were susceptible to rhArg-PEG treatment [6], and now present further evidence of anti-HCC activity against two other types of tumor xenografts. Through studying its therapeutic mechanism as well as its resistance, we should establish the potential of rhArg-PEG as a treatment for HCC, with a wider therapeutic coverage than ADI-PEG.

2. Materials and methods

2.1. Materials

Procedures for the expression, purification and pegylation of recombinant human arginase have been detailed previously [6]. The specific activity of the purified enzyme was ~400 U/mg, one unit of arginase being defined as the amount of enzyme that produces 1 μmol urea per min at 30 °C, pH 8.5. 5-Fluorouracil (5-FU) was purchased in solution form (250 mg/5 ml; Ebewe Pharma, Austria) and diluted in Dulbecco's modified Eagle's medium (DMEM) to 10 μg/ml before application. The human hepatocellular carcinoma cell lines Hep3B, HepG2 and PLC/PRF/5 (ATCC numbers: HB-8064, HB-8065 and CRL-8024, respectively) were obtained from the American Type Culture Collection. Cells were maintained in DMEM with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin (Gibco).

2.2. Cell viability

Approximately 2500 Hep3B cells/well were transferred to 96-well plates and incubated with different concentrations of rhArg-PEG for three days at 37 °C, 95% air/5% CO_2, and the metabolically viable cell fraction was determined by the CellTiter 96® AQueous non-radioactive cell proliferation assay (Promega), a colorimetric method for determining the number of viable cells in proliferation assays. Cultures were incubated with [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfoph-enyl)-2H-tetrazolium)] (MTS) reagent for 4 h at 37 °C. MTS is chemically reduced by dehydrogenases in viable cells into formazan, which is soluble in tissue culture medium. Formazan concentration was assayed by measuring the absorbance on a spectrophotometer at 490 nm. For amino acid rescue experiments, after incubation with rhArg-PEG for three days, the medium was removed and the cells washed with PBS. The cultures were given arginine-free DMEM (AFM) (USBiological) supplemented with 0.4 mM arginine, citrulline or ornithine, and incubated for an additional three days. The restoration of Hep3B proliferation was measured using the CellTiter 96® AQueous non-radioactive cell proliferation assay. (Note: DMEM normally contains 1 mM arginine. However, in order to compare the data with most of the previous studies [26,27], arginine was standardized here to 0.4 mM.)

2.3. Gene expression analysis

For tumor profiling, 40 HCC patients who underwent surgery at Queen Mary Hospital, Hong Kong, were studied. mRNA samples were prepared from resected tumor tissues by the Divisions of Hepatobiliary and Pancreatic Surgery and Liver Transplantation in the Department of Surgery, University of Hong Kong. Written informed consent was obtained and patient information was kept strictly confidential. mRNA samples were subjected to RT-PCR analysis: the RNA was first reverse-transcribed into cDNA with iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Briefly, 5 μg of total RNA

was subjected to reverse transcription at 42 °C for 30 min. A 2 μl portion of cDNA was then amplified using 50 μl of reaction mixture containing 0.5 units of iTaq DNA polymerase (Bio-Rad). PCR was performed in a DNA thermal MyCycler (Bio-Rad). The reaction products were electrophoresed in 1% agarose gels, and the product band intensities were analyzed with Lumi-Imager (Boehringer Mannheim). Flanking primers are listed in Table 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal control.

For cell cycle factor expression studies, cells were seeded, grown overnight, and treated with different concentrations of rhArg-PEG for three days. Total cellular RNA was extracted using the Qiagen RNeasy Kit according to the manufacturer's instructions. Approximately 1 μg of total RNA was subjected to reverse transcription with iScript cDNA Synthesis Kit (Bio-Rad) at 42 °C for 30 min. The resulting cDNA was subjected to quantitative real time PCR (Bio-Rad; IQ5 real time PCR) using the QuantiTect SYBR Green PCR Kit (Qiagen). The experimental and GAPDH reactions were done in separate wells in triplicates, and the average threshold cycle (C_T) for the triplicate was used in subsequent calculations. The mRNA level of each sample for each gene was normalized to that of the GAPDH mRNA. Gene expression was calculated by $\Delta\Delta C_T$ analysis, and one-way ANOVA was used to determine statistical significance. Primers are listed in Table 1.

2.4. Flow cytometry

3×10^5 cells/well were seeded in 6-well plates, and were given the appropriate treatment one day later. After three days of incubation, the cells were collected, washed twice with PBS, and fixed with 70% ethanol for >30 min. After a further PBS wash, the cells were stained with propidium iodide (PI) staining solution (PI, 2 mg/ml; RNase A, 10 mg/ml in PBS) for 30 min at 37 °C in darkness. Stained cells were analyzed using fluorescence-activated cell sorting (FACS; Beckton Dickinson Co). The percentages of cells in sub-G_1, G_0/G_1, S and G_2/M phases were analyzed with ModFit LT™ software (Verity Software House). One-way ANOVA was applied to determine statistical significance of changes in cell cycle distribution in response to increasing doses of rhArg-PEG.

Table 1
Primers for gene expression analyses.

Gene	Primers (S: sense; AS: anti-sense)
ASS	S: 5′-GGGGTCCCTGTGAAGGTGACC-3′ AS: 5′-CGTTCATGCTCACCAGCTC-3′
ASL	S: 5′-TGATGCCCCAGAAGAAAAACC-3′ AS: 5-CATCCCTTTGCGGACCAGGTA-3′
OTC	S: 5′-TTTTCAAGGGCATAGAATCGTC-3′ AS: 5′-CTTTTCCCCATAAACCAACTCA-3′
CDK1 (cdc2)	S: 5′-TGGATCTGAAGAAATACTTGGATTCTA-3′ AS: 5′-CAATCCCCTGTAGGATTTGG-3′
CDK2	S: 5′-CCTCCTGGGCTGCAAATA-3′ AS: 5′-CAGAATCTCCAGGGAATAGGG-3′
CDK6	S: 5′- TGATCAACTAGGAAAAATCTTGGAC-3′ AS: 5′- GGCAACATCTCTAGGCCAGT-3′
Cyclin A1	S: 5′-AATGGGCAGTACAGGAGGAC-3′ AS: 5′-CCACAGTCAGGGAGTGCTTT-3′
Cyclin B1	S: 5′-CATGGTGCACTTTCCTCCTT-3′ AS: AGGTAATGTTGTAGAGTTGGTGTCC-3′
Cyclin D1	S: 5′-GAAGATCGTCGCCACCTG-3′ AS: 5′-GACCTCCTCCTCGCACTTCT-3′
GAPDH	S: 5′-AGCCACATCGCTCAGACA-3′ AS: 5′-GCCCAATACGACCAAATCC-3′

2.5. Drug efficacy testing in nude mice

To generate tumor-bearing mice, Balb/c nude mice of the same gender (mean body weight ~20 g) were implanted with 3-mm^3 HepG2 or PLC/PRF/5 solid tumors, which were allowed to grow up to an average diameter of 5 mm. The mice were randomly divided into three groups, which were administered intravenously with (i) saline (negative control) ($n = 8$ for HepG2, 12 for PLC/PRF/5); (ii) 100 U rhArg-PEG ($n = 8$ for HepG2, 6 for PLC/PRF/5) or (iii) 400 U rhArg-PEG ($n = 8$ for HepG2, 6 for PLC/PRF/5) respectively, on a weekly (PLC/PRF/5) or twice weekly (HepG2) basis. The solid tumors were measured *in situ* every 3–4 days with a digital caliper, and tumor volume was estimated using the formula $0.5 \times \text{length} \times (\text{width})^2$.

3. Results

3.1. Citrulline rescue of rhArg-PEG-induced growth inhibition

rhArg-PEG inhibited the *in vitro* proliferation of Hep3B cells in a potent manner consistent with our previous results (Fig. 1A) [6], with half-maximal inhibition at only 0.1 U/ml (~0.35 μg/ml), i.e. much lower than the concentration of ADI that proved effective against Hep3B, since cells were able to grow in the presence of 200 μg/ml of the latter enzyme [6]. Cells treated with rhArg-PEG for three days exhibited a marked reduction in growth (Fig. 1A). Remarkably, removal of the enzyme at the end of the 3-day treatment and subsequent addition of 0.4 mM citrulline in arginine-free culture medium (AFM) rescued the rhArg-treated cells (Fig. 1B). Even after treatment with high doses (up to 12.5 U/ml) of rhArg-PEG, citrulline completely restored cell proliferation in 3 days (data not shown). The same concentration of arginine did restore cell growth when given after treatment with low doses (up to 0.8 U/ml) of rhArg-PEG, but failed to recover 100% of the growth when higher doses of rhArg-PEG were used (Fig. 1B). Ornithine, on the other hand, permitted very little 'rescue' of rhArg-PEG-treated cells (Fig. 1B). As reported earlier, Hep3B cells expressed ASS and ASL but not OTC, whose gene product converts ornithine to citrulline. This provides a satisfactory explanation for the inability of ornithine to rescue the cells effectively, and also supports our hypothesis that normal levels of OTC activity could contribute to resistance towards rhArg [6] (Fig. 2).

3.2. Incidence of ASS and OTC deficiency in HCC patients

To predict rhArg-PEG's effectiveness against HCC in humans, we analyzed the mRNA samples extracted from the resected tumor tissues of 40 HCC patients, to give a measure of ASS, ASL and OTC expression by RT-PCR (Table 2). Our model, as shown in Fig. 2, predicts rhArg resistance for tumors expressing all of the three enzymes, and ADI resistance for tumors expressing both ASS and ASL. We found that 35 of the 40 tumor biopsies (87.5%) expressed both ASS and ASL, thus predicting ADI resistance in these patients (Table 2). In contrast, only 24 out of 40 patients (60%) expressed all three enzymes in their tumors and are therefore expected to be rhArg-resistant (Table 2). These results suggest that rhArg-PEG might be more effective than ADI in treating (Hong Kong) HCC patients.

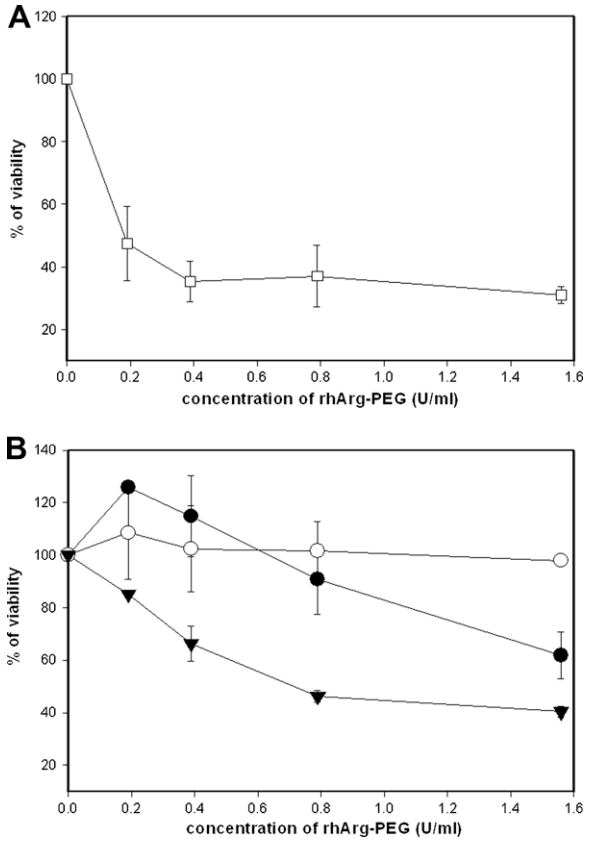

Fig. 1. (A) Hep3B cells showed marked retardation of growth when treated with rhArg-PEG (□) for 3 days, with an IC_{50} of ~0.1 U/ml. (B) Subsequently, the rhArg-PEG was removed from the cultures, and replaced with culture medium containing 0.4 mM citrulline (○), arginine (●) or ornithine (▼). Cell proliferation was assayed three days later. Citrulline rescued cell growth to a level comparable to untreated controls, whereas arginine only partially rescued the cells. In contrast, ornithine had no significant effect on the rhArg-treated cultures. Data shown are means ± SD ($n = 3$).

3.3. Impact on cell cycle distribution upon rhArg-PEG treatment

The effect of rhArg-PEG on cell cycle progression was investigated by exposing Hep3B cells to different concentrations of rhArg-PEG for 3 days, after which their cell cycle profiles were analyzed by flow cytometry (Table 3). In the absence of rhArg-PEG, the cell populations at G_0/G_1, S and G_2/M phases were found to be 59.7%, 32.3%, and 7.99% respectively (Table 3). Following an exposure to 0.5 U/ml rhArg-PEG, the S phase population was reduced to 25.9%, accompanied by a concomitant increase in G_2/M phase cells (22.9%) (Table 3). At 5 U/ml rhArg-PEG, the proportion of cells at G_2/M phase further rose to 25.0% (Table 3). These observations suggest that rhArg-PEG induces G_2/M phase arrest or deceleration in Hep3B cells. A similar G_2/M phase arrest was observed when Hep3B cells were subjected to 72 h of exposure to arginine-free culture medium (AFM) (31.1% at G_2/M phases in AFM-treated group vs 7.99% in control group) (Table 3), implying that arginine depletion was responsible for the effects of rhArg-PEG on the cell cycle. Incidentally, very few late apoptotic cells (<6%), for which the sub-G_1 peak provides an estimate, were observed after rhArg-PEG treatment (Table 3). This is in stark contrast to the ADI-induced G_0/G_1 arrest and substantial apoptosis observed in cultured human umbilical vein endothelial cells and in neuroblastoma and lymphatic leukemia cell lines [17–19]. To explore whether the cell cycle arrest observed was specific to the Hep3B line, we treated HepG2 and PLC/PRF/5 cells, both of HCC origin, with rhArg-PEG and studied their cell cycle distributions. Surprisingly, no significant G_2/M phase arrest was observed in either cell line (Tables 4 and 5). The three cell lines showed distinct profiles even without rhArg-PEG treatment (Tables 3–5). Unlike Hep3B, both HepG2 and PLC/PRF/5 exhibited an S phase arrest in response to rhArg-PEG treatment (Tables 4 and 5). Yet between these two cell lines differences exist: the S phase proportion steadily rose with increasing rhArg-PEG dosage in HepG2, but peaked at 0.5 U/ml rhArg-

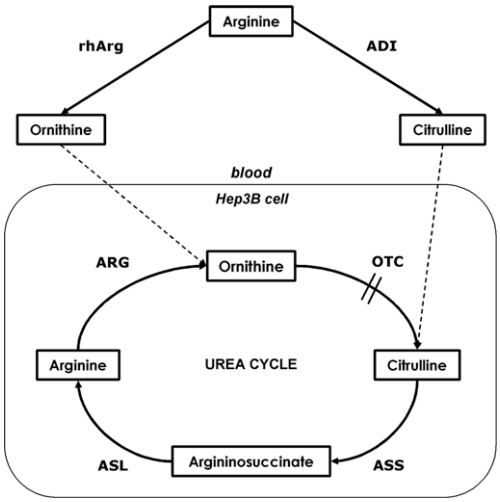

Fig. 2. Model relating urea cycle expression and resistance towards arginine deiminase (ADI) and rhArg. Hep3B cells express the urea cycle enzymes argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG), but lack ornithine transcarbamylase (OTC). rhArg-PEG in the bloodstream depletes arginine and produces ornithine, which enters the cell (indicated by dotted arrow) but fails to be recycled via the urea cycle owing to the absence of OTC. ADI converts arginine to citrulline, which can be readily converted back to arginine by ASS and ASL after uptake into Hep3B cells.

Table 2
Incidence of ASS, ASL and OTC expression in 40 human HCC patients in Hong Kong, with predicted sensitivity to rhArg and ADI. "+": expression detected; "–": expression undetectable; "R": resistant, "S": sensitive.

Patient tumor gene expression			Frequency	Predicted response	
ASS	ASL	OTC		rhArg	ADI
+	+	+	24	R	R
+	+	–	11	S	R
+	–	–	2	S	S
+	–	+	1	S	S
–	+	–	1	S	S
–	–	+	1	S	S

Table 3
Hep3B cell cycle distribution upon rhArg-PEG treatment. Results represent the means ± SD of five independent experiments. One-way ANOVA revealed a significant arrest in G_2/M phase in response to increasing rhArg-PEG dosage ($p < 0.01$). *Post hoc* Dunnett's test: $^{*}p < 0.05$, $^{**}p < 0.01$. Control was treated with arginine-free medium (AFM) only.

rhArg-PEG (U/ml)	Sub-G_1 (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
0	0.39 ± 0.49	59.7 ± 3.45	32.3 ± 1.65	7.99 ± 2.53
0.05	0.33 ± 0.12	57.4 ± 3.59	31.5 ± 4.47	11.1 ± 4.20
0.1	0.57 ± 0.39	56.2 ± 3.65	30.0 ± 6.68	13.9 ± 6.90
0.5	3.62 ± 4.09	51.2 ± 4.35	25.9 ± 7.47	22.9 ± 8.99*
1.0	3.63 ± 1.28	52.3 ± 5.52	31.3 ± 9.53	16.33 ± 7.86
5.0	5.45 ± 2.98	47.1 ± 3.30	27.8 ± 10.1	25.0 ± 9.65**
AFM control	11.6	56.8	12.1	31.1

Table 4
HepG2 cell cycle distribution upon rhArg-PEG treatment. Results represent the means ± SD of three independent experiments. No significant sub-G_1 population was detected in any sample. One-way ANOVA revealed a significant arrest in S phase in response to increasing rhArg-PEG dosage ($p < 0.001$). *Post hoc* Dunnett's test: $^{**}p < 0.01$.

rhArg-PEG (U/ml)	G_0/G_1 (%)	S (%)	G_2/M (%)
0	57.7 ± 3.86	26.4 ± 2.40	15.9 ± 2.30
0.05	52.5 ± 3.60	29.4 ± 6.73	18.0 ± 3.23
0.1	48.4 ± 7.30	34.2 ± 7.03	17.4 ± 1.61
0.5	39.8 ± 11.7	43.8 ± 5.22**	16.5 ± 6.44
1.0	34.8 ± 12.8	49.0 ± 10.4**	16.2 ± 2.94
5.0	37.6 ± 10.7	49.3 ± 6.40**	13.1 ± 4.63

Table 5
PLC/PRF/5 cell cycle distribution upon rhArg-PEG treatment. Results represent the means ± SD of three independent experiments. One-way ANOVA revealed a significant arrest in S phase in response to increasing rhArg-PEG dosage ($p < 0.05$). *Post hoc* Dunnett's test: $^{*}p < 0.05$.

rhArg-PEG (U/ml)	Sub-G_1 (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
0	5.32 ± 3.72	76.5 ± 1.31	3.26 ± 3.42	17.7 ± 4.04
0.05	16.6 ± 16.6	65.2 ± 5.56	6.48 ± 5.80	20.3 ± 15.5
0.1	12.1 ± 7.32	59.7 ± 2.95	19.1 ± 15.1	17.1 ± 10.2
0.5	8.92 ± 0.97	58.1 ± 1.17	23.7 ± 14.0*	17.7 ± 13.2
1.0	10.0 ± 8.44	61.4 ± 5.53	21.3 ± 10.0	14.7 ± 10.5
5.0	12.8 ± 0	68.5 ± 2.40	20.4 ± 12.8	11.7 ± 12.3

PEG in PLC/PRF/5 (Tables 4 and 5). These results suggest that the same treatment could cause growth inhibition in tumor cells via different mechanisms, depending on the genetic background of the tumor. One common feature between the three cell lines, however, is the lack of an rhArg-induced pro-apoptotic effect, as estimated by the sub-G_1 fraction of cells (Tables 3–5).

Given that rhArg-PEG treatment induces cell cycle arrest in Hep3B cells, it was of interest to study the effect of combining rhArg-PEG treatment with cell cycle-disrupting chemotherapeutics, of which 5-fluorouracil (5-FU) was chosen. When rhArg-PEG and 5-FU were applied in combination, the proportion of late apoptotic (sub-G_1) cells reached 15.5%, compared with 6.8% in cells treated with 5-FU only (Fig. 3), or 3.6% in cells treated with 0.5 U/ml rhArg-PEG alone (Table 3). Their synergistic tumoricidal action *in vitro* has also been shown in our *in vivo* experiments [6], and demonstrates the potential of combination therapy for HCC patients.

3.4. Effect of rhArg-PEG on cell cycle-related genes in HCC cell lines

To elucidate the mechanisms involved in the observed cell cycle arrest, we measured the levels of several cell cycle-related transcripts in three HCC cell lines incubated with various concentrations of rhArg-PEG. Hep3B cells treated with ⩾0.5 U/ml rhArg-PEG showed a drastic reduction (>80%) in the mRNA levels of Cyclin B1 as well as cdc2 (Fig. 4A and B). Since the Cyclin B1/cdc2 complex is critical for the G_2/M transition, the decline of expression is consistent with G_2/M arrest, both effects becoming more apparent when cells were given ⩾0.5 U/ml rhArg-PEG (Table 3 and Fig. 4A and B). The results suggest that suppression of Cyclin B1 and cdc2 synthesis by rhArg-PEG is responsible for the arrest of Hep3B cells at G_2/M. On the other hand, expression of Cyclin A1 and CDK2, involved in the entry into S phase, was unaffected by rhArg-PEG (Supplementary Fig. 2). In HepG2 cells, rhArg-PEG elicited a transcriptional upregulation of Cyclin A1, a factor responsible for progression from G_1 to S phase, peaking with a ~7-fold increase when treated with 0.5 U/ml rhArg-PEG (Fig. 4C). Cyclin D1, whose levels are low during G_0 and S phases of the cell cycle, showed ~35% reduction in its expression at the mRNA level (Fig. 4D). High levels of Cyclin A1 and low levels of Cyclin D1 are therefore consistent with the rhArg-PEG-induced S phase arrest observed in HepG2 (Table 4). Respective protein partners of these two cyclins, CDK2 and CDK6, however, did not show altered gene transcription in response to rhArg-PEG (Supplementary Fig. 2). PLC/PRF/5 cells displayed an increase in Cyclin A1 (~5-fold) and CDK6 expression (up to 2.5-fold) after treatment (Fig. 4D and E), although rhArg-PEG had no effect on Cyclin D1 and CDK2 expression in this cell line (Supplementary Fig. 2). High Cyclin A1 in this case also agrees with the observed S phase arrest in this cell line (Table 5). Together with the flow cytometry results, these observations highlight the heterogeneity of HCC cell types, as reflected by their diverse transcriptional and biological responses to the same drug.

3.5. rhArg-PEG attenuates growth of HepG2 and PLC/PRF/5 xenografts in nude mice

Having previously demonstrated that rhArg-PEG is efficacious against Hep3B xenografts in nude mice, we tested the efficacy of the drug against HCC xenografts with different genetic backgrounds. HepG2 or PLC/PRF/5 cells were implanted into nude mice, and when resulting tumors reached the size of 5 mm, the mice were treated with 100 or 400 U rhArg-PEG. At both dosage levels, rhArg-PEG significantly attenuated the growth of HepG2 and PLC/PRF/5 xenografts, compared to control mice treated with saline (Fig. 5). With HepG2 xenografts, the anti-tumor activity of rhArg-PEG was not augmented by the increased dose (Fig. 5A), whereas in PLC/PRF/5 tumor-carrying mice, the higher dose elicited a greater level of tumor growth attenuation, keeping the mean tumor volume at 22% of the saline-treated control group by day 43 (Fig. 5B). We have thus shown that rhArg-PEG is an effective anti-tumor agent against several types of HCC in mice.

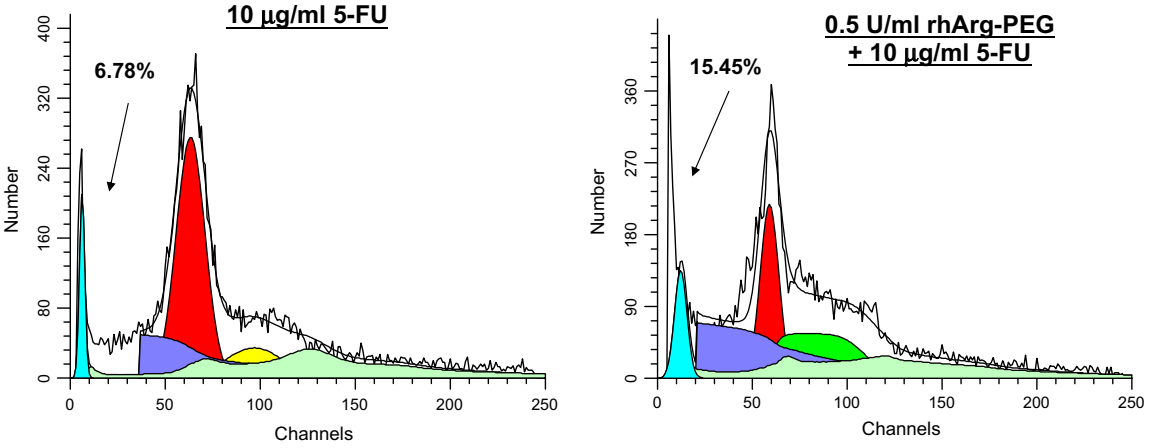

Fig. 3. Flow cytometric analysis of Hep3B treated with 5-FU (10 µg/ml) in the absence (left) or presence (right) of 0.5 U/ml rhArg-PEG. The percentage represented by the sub-G_1 peak (hypodiploidy) provides an estimate of late apoptotic cells, and is shown by an arrow in each panel.

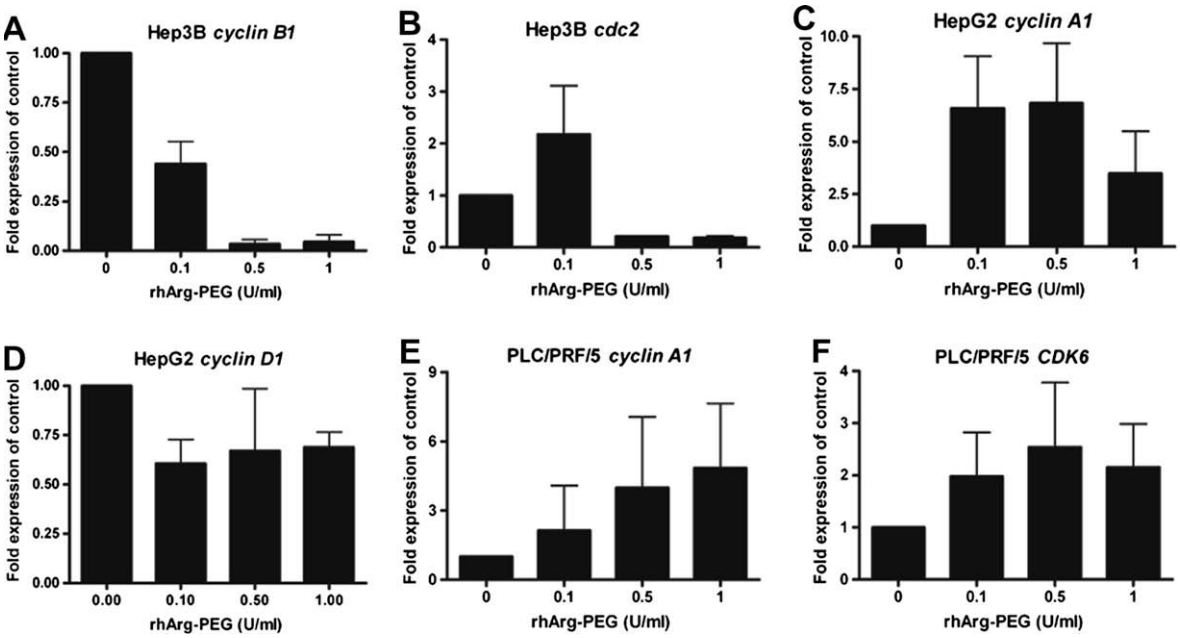

Fig. 4. Effect of rhArg-PEG on expression of cell cycle regulators in HCC cells. Quantitative real time PCR was used to measure amount of transcripts in the cells, expressed as fold increase of (untreated) control. Expression of genes in all panels shown as means ± SD, $n \geqslant 2$. One-way ANOVA: $p < 0.10$ in all cases.

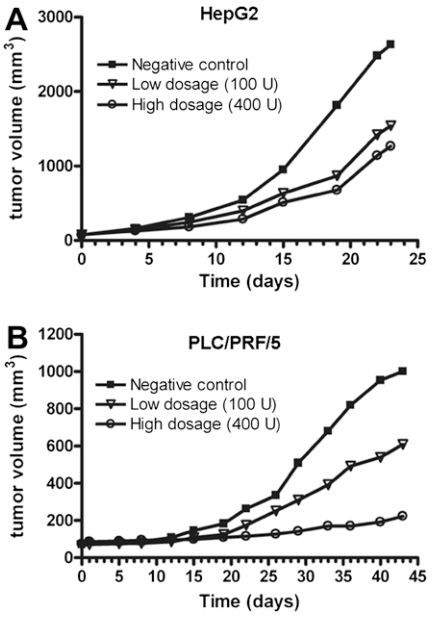

Fig. 5. rhArg-PEG exhibits strong anti-tumor activity against HCC xenografts in nude mice. (A) HepG2 tumors showed significant growth inhibition when treated with either low (100 U) or high (400 U) doses of rhArg-PEG, relative to saline-treated controls ($p < 0.05$) and (B) PLC/PRF/5 tumors showed attenuated growth under both low and high doses of rhArg-PEG, with the latter effect being statistically significant ($p < 0.05$).

4. Discussion

4.1. Therapeutic value of rhArg-PEG

Hepatocellular carcinoma is the third leading cause of cancer-related deaths globally, especially in East/South East Asia and sub-Saharan Africa, with an increasing incidence in the western world. It remains notoriously difficult to treat. Non-surgical therapies, such as transarterial chemoembolization and percutaneous ethanol injection, have limited efficacy or are associated with severe side effects. For patients suffering from advanced HCC, the options are restricted essentially to chemotherapy; however, HCC is generally considered quite resistant to most conventional agents. Even though doxorubicin is commonly prescribed alone or in combination with other agents, its effectiveness is in doubt and the associated toxicity can be severe [1–4]. The demand for an agent that is non-toxic and efficacious against HCC is therefore urgent. rhArg-PEG has been shown to have no apparent side effects in animal studies (data not shown), thus meriting more thorough clinical evaluation. The lack of side effects is of particular relevance since patients with terminal cancer attach a lot of significance to quality of life as well as the outcome of the treatment. Additionally, patients recovering from HCC resection or liver transplantation could be given rhArg-PEG as adjuvant therapy to prevent tumor recurrence. In view of these potential therapeutic applications, a Phase I/II study of rhArg-PEG in the treatment of HCC is now being conducted in Hong Kong, with the preliminary results expected in the near future.

4.2. Gene expression and drug resistance

Even though HCC has proven resilient against conventional chemotherapy, its arginine auxotrophy could be its Achilles' heel: deprivation of arginine by the arginine-depleting enzyme rhArg-PEG has a devastating effect on malignant Hep3B cells and on Hep3B tumor-bearing mice, as previously described [6]. We also proposed that OTC expression could render cancer cells resistant to

rhArg-PEG, by allowing ornithine conversion to citrulline, eventually resulting in the recycling of arginine. Since, according to our previous results [6], Hep3B cells express the urea cycle enzymes argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) but not OTC, citrulline should rescue their growth after rhArg-PEG-induced inhibition, as it would be converted to arginine by ASS and ASL. Ornithine, however, would fail to elicit such a rescue owing to the lack of OTC (Fig. 2). Indeed, citrulline rescued rhArg-PEG-treated Hep3B cultures, whereas ornithine was much less effective in restoring cell growth (Fig. 1B). As expected, arginine was also able to elicit some recovery in the rhArg-PEG-treated cultures (Fig. 1B). However, we were surprised to find that citrulline was significantly better at rescuing the treated cultures (Fig. 1B). If citrulline only serves as a precursor to arginine, upon which the cells depend, we would expect the two amino acids to have similar rescue effects. Citrulline has been shown to rescue arginine-deprived HeLa (human cervical carcinoma) and L1210 (murine lymphoblastic leukemia) cells, in experiments where it was applied in the continued presence of arginase [26]. In the same experiment, arginine, as expected, did not show any rescue effect, since the arginase in the culture medium would degrade it. Indeed, our results could be explained by the persistence of a lower concentration of rhArg-PEG in the culture medium, even though the cultures were washed with PBS before the arginine/citrulline/ornithine was added. This suggests that the enzyme might have adsorbed onto cell surfaces, and if this is also the case *in vivo*, its therapeutic effect could be prolonged as a result of its persistence in the system. Together, these results support the hypothesis that OTC deficiency is probably responsible for the sensitivity of Hep3B towards rhArg-PEG [6]. rhArg-PEG produces ornithine, which, in the absence of active OTC, cannot be recycled via the urea cycle to form arginine, leading to eventual arginine depletion (Fig. 2). On the other hand, ADI converts arginine to citrulline, which is efficiently recycled to form arginine by ASS and ASL, thus explaining why Hep3B cells are ADI-resistant (Fig. 2). In addition, since citrulline and arginine seem likely to promote tumor resurgence after rhArg-PEG treatment, dietary restrictions are called for in conjunction with therapy.

ASS, ASL and OTC expression plays a key role in determining patients' sensitivity to arginine depletion therapy. Assessing ASS expression in tumor biopsies from patients has helped predict the therapeutic potential of ADI in treating renal and pancreatic cancer [28,29]. Our study with HCC tumor biopsies revealed that, unexpectedly, most of the HCC patients sampled (38/40) expressed ASS at a consistently high level (Table 2). According to our model, the 35 out of 40 (87.5%) tumors expressing both ASS and ASL would be insensitive to arginine deiminase (ADI), another arginine-depleting enzyme treatment in development [5,9,15,16,30–32] (Fig. 2 and Table 2). On the other hand, 16 out of 40 (40%) tumors were deficient in at least one of the three transcripts studied, which would render them sensitive to rhArg according to our model (Fig. 2 and Table 2). These findings have two significant implications. First, cases of HCC can be appropriately and quickly prescribed arginine depletion treatment after tumor biopsy and gene expression profiling. Moreover, at least as far as this study shows, rhArg-PEG is more likely to be efficacious against HCC when compared to ADI. Although racial disparity has been known to exist in tumor gene expression [29], and treatments undergone by the subjects prior to the biopsy might have had an impact on the results, the study nonetheless provides a preliminary estimation of the efficacy of rhArg and ADI in Hong Kong HCC patients.

4.3. Mechanism of drug action

Insight into the mechanisms involved in the growth inhibition of rhArg-PEG-treated HCC cells was gained by studying the effect of the enzyme on cell cycle progression and on factors that regulate this process. Flow cytometric analysis of cells labeled with propidium iodide suggests that rhArg-PEG blocks the cell cycle when increasing rhArg-PEG concentrations were applied (Table 3). More importantly, we have demonstrated that rhArg-PEG, or arginine depletion in general, could exert anti-tumor effects via different mechanisms in different cancer cell types. Even though all three cell lines used in this study were of HCC origin, rhArg-PEG induced a G_2/M phase arrest in Hep3B, and an S phase arrest in HepG2 and PLC/PRF/5 (Tables 3–5). The observed arrests may solely be due to the depletion of arginine: we showed that arginine-free medium had a very similar effect on the Hep3B cell cycle to rhArg-PEG (Table 3), and other studies have also shown that arginine deprivation of tumor cells inevitably led to imbalanced growth in a continued unsuccessful cycle attempt, causing cells to die [17,18,33–35]. ADI, on the other hand, consistently elicits a G_0/G_1 arrest in human umbilical vein endothelial cells, as well as in neuroblastoma and lymphatic leukemia cell lines [17–19]. It will therefore be interesting to see if arginine depletion with rhArg-PEG would cause the same type of cell cycle arrest as ADI in these cell lines, or whether distinctions exist between the mechanisms of the two arginine-depleting enzymes.

Cell cycle progression is controlled by several Cyclin/CDK complexes, such as Cyclin D1/CDK4, Cyclin D1/CDK6 and Cyclin E1/CDK2 in the G_1/S transition, and Cyclin B1/cdc2 in the G_2/M transition [21–25,36–39]. To examine whether rhArg-PEG-triggered inhibition of cell proliferation is caused by a modulation in Cyclin or CDK expression, we analyzed the expression of these cell cycle regulators in HCC cells treated with rhArg-PEG. Our studies demonstrate that the transcription of cell cycle-related factors was differentially affected by rhArg-PEG, depending on the cell line. Hep3B showed a significantly decreased level of Cyclin B1 expression when $\geqslant 0.5$ U/ml rhArg-PEG was applied (Fig. 4A). The expression of cdc2 mRNA was also reduced (Fig. 4B), consistent with the Cyclin B1/cdc2 complex being the primary regulator of the transition from G_2 to M phase [22,36]. In response to rhArg-PEG treatment, HepG2 and PLC/PRF/5 cells both showed a transcriptional upregulation of Cyclin A1 (Fig. 4C and E), whose gene product associates with CDK2 to mediate G_1/S phase progression [21]. These effects are therefore consistent with the increase in S phase sub-population we observed in the two cell lines (Tables

4 and 5). Cyclin D1 downregulation was observed in HepG2 but not in PLC/PRF/5 (Fig. 4D and Supplementary Fig. 2): although Cyclin D1 is responsible for G_1 phase progression, its levels decrease during S phase [39]. Moreover, S phase arrest has been reported in cells with downregulated Cyclin D1 and upregulated Cyclin A [40]. Increased CDK6 expression in PLC/PRF/5 cells could be favorable for an S phase arrest by forming a complex with Cyclin D and driving G_1 phase cells into S phase [21].

Our cell cycle distribution analysis and gene expression studies revealed considerable heterogeneity in HCC cell lines, which is unsurprising considering their dissimilar genetic backgrounds. For example, HepG2 expresses wild-type p53, PLC/PRF/5 expresses a point mutant form, whilst Hep3B is p53-null [41–43]. Moreover, Hep3B and PLC/PRF/5 carry integrated hepatitis B virus in their genomes, whereas HepG2 does not [44,45]. Nevertheless, rhArg-PEG has proven to be an effective anti-tumor agent against all three types of HCC, both *in vitro* [6] and *in vivo* (Fig. 5), albeit involving different mechanisms. Understanding these differences will help inform us in customizing treatments for individual cancer patients.

What also remains to be determined is the molecular pathway leading from rhArg-PEG application to transcriptional modulation of these cell cycle factors. One possible candidate is the mammalian target of rapamycin (mTOR) pathway, known to respond to amino acid (particularly leucine and arginine) starvation [46]. Notably, leucine starvation has been shown to inhibit cdc2 activity via the mTOR pathway, thus inducing a G_2/M phase arrest in HeLa cells [47]. It would therefore be of great interest to see whether arginine depletion also exerts its effects via the same pathway.

4.4. Potential for combination therapy

The inhibitory action of rhArg-PEG on cell cycle progression in Hep3B could shed light on our previous results showing synergistic effects of the enzyme with 5-fluorouracil (5-FU) on tumor growth *in vivo* [6]. In the present study, we also observed a synergy in the pro-apoptotic effects of 5-FU and rhArg-PEG (Fig. 3). 5-FU is an antimetabolite that exerts its anti-cancer effects through inhibition of thymidylate synthase and the incorporation of its metabolites into RNA and DNA, thus preventing DNA synthesis and inhibiting cell cycle progression [48,49]. However, it has been shown to be ineffective against cancer cells which do not express wild type p53 [50]. Hep3B cells are p53-deficient [41], but there have been conflicting reports concerning their resistance to 5-FU [51–55].

A recent study suggests that 5-FU could cause cell death by primary necrosis as opposed to apoptosis, but only at high doses [56]. We found that Hep3B cells showed very little late apoptosis (~6.8%) after treatment with 10 μg/ml 5-FU (Fig. 3), and when injected into nude mice, the tumors formed did not respond to 5-FU [6]. Since both 5-FU and rhArg-PEG have cell cycle-related mechanisms of action, their synergy could be a result of cross-talking signaling pathways, details of which remain to be investigated. Nevertheless, it does suggest that 5-FU and rhArg-PEG might be suitable as a combination therapy for HCC. Moreover, given that the latter induces cell cycle arrest in all three HCC cell lines tested, it could potentiate the cytotoxicity of cell cycle-disrupting chemotherapeutics in general. There is, therefore, potential for the use of rhArg-PEG in conjunction with cycle-dependent cancer drugs, similar to the way acute lymphoblastic leukemia is treated with L-asparaginase together with other cytotoxic agents [57] or methioninase and 5-FU, which can selectively eliminate tumor cells in mouse models [58]. There are now ongoing pre-clinical studies on nude mice in our laboratory to test different chemotherapy combinations together with rhArg-PEG in order to establish the most effective treatment combination. The results can inform the design of future phase III clinical studies.

In summary, our results suggest that rhArg-PEG is a desirable agent for the treatment of HCC, whose anti-cancer activity is mediated by the efficient depletion of arginine in culture medium, resulting in a cell cycle arrest in HCC cells, possibly by effecting up- or down-regulation of Cyclin and CDK expression. This effect reveals a potential for combination therapy, using rhArg-PEG alongside other cell cycle-disrupting chemotherapeutics. It has no apparent side effects in mice, and gene profiling data suggest a relatively low frequency of resistance amongst patients. We therefore believe that rhArg-PEG is a good candidate for treating advanced HCC as well as for adjuvant therapy after surgery.

Conflicts of interest statement

P.N.M. Cheng and D.N. Wheatley have a financial interest in rhArg-PEG.

Acknowledgements

This work was supported by Innovation and Technology Fund project no. UIM/124 and UIM/66. We state that the study sponsors had no involvement in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. We thank F.C. Tsang, C.Y. Fung, L.M. Hui, S.M. Tsui, and K.K. Cheung for assisting in the preparation of rhArg-PEG. We also thank Dr. J.S. Lee for discussion. We thank Beijing Joinn Pharmaceutical Center for their assistance in efficacy testing of rhArg-PEG in nude mice.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2008.11.031.

References

[1] S.A. Curley, J.C. Cusack Jr., K.K. Tanabe, O. Stoelzing, L.M. Ellis, Advances in the treatment of liver tumors, Curr. Prob. Surg. 39 (2002) 449–571.
[2] S.D. Ryder, Guidelines for the diagnosis and treatment of hepatocellular carcinoma (HCC) in adults, Gut 52 (Suppl. 3) (2003) iii1–iii8.

[3] J.L. Raoul, Natural history of hepatocellular carcinoma and current treatment options, Semin. Nucl. Med. 38 (2008) S13–S18.
[4] M. Varela, M. Sala, J.M. Llovet, J. Bruix, Treatment of hepatocellular carcinoma: is there an optimal strategy?, Cancer Treat. Rev. 29 (2003) 99–104.
[5] P.A. Ascierto, S. Scala, G. Castello, A. Daponte, E. Simeone, A. Ottaiano, G. Beneduce, V. De Rosa, F. Izzo, M.T. Melucci, C.M. Ensor, A.W. Prestayko, F.W. Holtsberg, J.S. Bomalaski, M.A. Clark, N. Savaraj, L.G. Feun, T.F. Logan, Pegylated arginine deiminase treatment of patients with metastatic melanoma: results from phase I and II studies, J. Clin. Oncol. 23 (2005) 7660–7668.
[6] P.N. Cheng, T.L. Lam, W.M. Lam, S.M. Tsui, A.W. Cheng, W.H. Lo, Y.C. Leung, Pegylated recombinant human arginase (rhArg-peg5,000 mw) inhibits the in vitro and in vivo proliferation of human hepatocellular carcinoma through arginine depletion, Cancer Res. 67 (2007) 309–317.
[7] G.A. Currie, L. Gyure, L. Cifuentes, Microenvironmental arginine depletion by macrophages in vivo, Brit. J. Cancer 39 (1979) 613–620.
[8] G.G. Gonzalez, C.V. Byus, Effect of dietary arginine restriction upon ornithine and polyamine metabolism during two-stage epidermal carcinogenesis in the mouse, Cancer Res. 51 (1991) 2932–2939.
[9] F. Izzo, P. Marra, G. Beneduce, G. Castello, P. Vallone, V. De Rosa, F. Cremona, C.M. Ensor, F.W. Holtsberg, J.S. Bomalaski, M.A. Clark, C. Ng, S.A. Curley, Pegylated arginine deiminase treatment of patients with unresectable hepatocellular carcinoma: results from phase I/II studies, J. Clin. Oncol. 22 (2004) 1815–1822.
[10] S.M. Morris Jr., Regulation of enzymes of the urea cycle and arginine metabolism, Annu. Rev. Nutr. 22 (2002) 87–105.
[11] J.M. Storr, A.F. Burton, The effects of arginine deficiency on lymphoma cells, Brit. J. Cancer 30 (1974) 50–59.
[12] K. Sugimura, T. Ohno, T. Kusuyama, I. Azuma, High sensitivity of human melanoma cell lines to the growth inhibitory activity of mycoplasmal arginine deiminase in vitro, Melanoma Res. 2 (1992) 191–196.
[13] H. Takaku, M. Matsumoto, S. Misawa, K. Miyazaki, Anti-tumor activity of arginine deiminase from *Mycoplasma argini* and its growth-inhibitory mechanism, Jpn. J. Cancer Res. 86 (1995) 840–846.
[14] P.N. Cheng, Y.C. Leung, W.H. Lo, S.M. Tsui, K.C. Lam, Remission of hepatocellular carcinoma with arginine depletion induced by systemic release of endogenous hepatic arginase due to transhepatic arterial embolisation, augmented by high-dose insulin: arginase as a potential drug candidate for hepatocellular carcinoma, Cancer Lett. 224 (2005) 67–80.
[15] Y. Ni, U. Schwaneberg, Z.H. Sun, Arginine deiminase, a potential anti-tumor drug, Cancer Lett. 261 (2008) 1–11.
[16] L.J. Shen, W.C. Lin, K. Beloussow, W.C. Shen, Resistance to the anti-proliferative activity of recombinant arginine deiminase in cell culture correlates with the endogenous enzyme, argininosuccinate synthetase, Cancer Lett. 191 (2003) 165–170.
[17] H. Gong, F. Zolzer, G. von Recklinghausen, J. Rossler, S. Breit, W. Havers, T. Fotsis, L. Schweigerer, Arginine deiminase inhibits cell proliferation by arresting cell cycle and inducing apoptosis, Biochem. Biophys. Res. Commun. 261 (1999) 10–14.
[18] H. Gong, F. Zolzer, G. von Recklinghausen, W. Havers, L. Schweigerer, Arginine deiminase inhibits proliferation of human leukemia cells more potently than asparaginase by inducing cell cycle arrest and apoptosis, Leukemia 14 (2000) 826–829.
[19] E.J. Noh, S.W. Kang, Y.J. Shin, S.H. Choi, C.G. Kim, I.S. Park, D.N. Wheatley, B.H. Min, Arginine deiminase enhances dexamethasone-induced cytotoxicity in human T-lymphoblastic leukemia CCRF-CEM cells, Int. J. Cancer 112 (2004) 502–508.
[20] D.N. Wheatley, E. Campbell, Arginine catabolism, liver extracts and cancer, Pathol. Oncol. Res. 8 (2002) 18–25.
[21] M. Arellano, S. Moreno, Regulation of CDK/cyclin complexes during the cell cycle, Int. J. Biochem. Cell Biol. 29 (1997) 559–573.
[22] L.H. Hartwell, M.B. Kastan, Cell cycle control and cancer, Science 266 (1994) 1821–1828.
[23] D.O. Morgan, Principles of CDK regulation, Nature 374 (1995) 131–134.
[24] A.B. Pardee, A restriction point for control of normal animal cell proliferation, Proc. Natl. Acad. Sci. USA 71 (1974) 1286–1290.
[25] A.B. Pardee, G1 events and regulation of cell proliferation, Science 246 (1989) 603–608.
[26] R. Philip, E. Campbell, D.N. Wheatley, Arginine deprivation, growth inhibition and tumour cell death: 2. Enzymatic degradation of arginine in normal and malignant cell cultures, Brit. J. Cancer 88 (2003) 613–623.
[27] L. Scott, J. Lamb, S. Smith, D.N. Wheatley, Single amino acid (arginine) deprivation: rapid and selective death of cultured transformed and malignant cells, Br. J. Cancer 83 (2000) 800–810.
[28] T.L. Bowles, R. Kim, J. Galante, C.M. Parsons, S. Virudachalam, H.J. Kung, R.J. Bold, Pancreatic cancer cell lines deficient in argininosuccinate synthetase are sensitive to arginine deprivation by arginine deiminase, Int. J. Cancer 123 (2008) 1950–1955.
[29] C.Y. Yoon, Y.J. Shim, E.H. Kim, J.H. Lee, N.H. Won, J.H. Kim, I.S. Park, D.K. Yoon, B.H. Min, Renal cell carcinoma does not express argininosuccinate synthetase and is highly sensitive to arginine deprivation via arginine deiminase, Int. J. Cancer 120 (2007) 897–905.
[30] K. Sugimura, T. Kimura, H. Arakawa, T. Ohno, Y. Wada, Y. Kimura, T. Saheki, I. Azuma, Elevated argininosuccinate synthetase activity in adult T leukemia cell lines, Leukemia Res. 14 (1990) 931–934.
[31] C.M. Ensor, F.W. Holtsberg, J.S. Bomalaski, M.A. Clark, Pegylated arginine deiminase (ADI-SS PEG20,000 mw) inhibits human melanomas and hepatocellular carcinomas in vitro and in vivo, Cancer Res. 62 (2002) 5443–5550.
[32] L. Feun, N. Savaraj, Pegylated arginine deiminase: a novel anticancer enzyme agent, Expert Opin. Investig Drugs 15 (2006) 815–822.
[33] J. Lamb, D.N. Wheatley, Single amino acid (arginine) deprivation induces G1 arrest associated with inhibition of cdk4 expression in cultured human diploid fibroblasts, Exp. Cell Res. 255 (2000) 238–249.
[34] A.S. Weissfeld, H. Rouse, Arginine deprivation in KB cells. I. Effect on cell cycle progress, J. Cell Biol. 75 (1977) 881–888.
[35] D.N. Wheatley, L. Scott, J. Lamb, S. Smith, Single amino acid (arginine) restriction: growth and death of cultured HeLa and human diploid fibroblasts, Cell Physiol. Biochem. 10 (2000) 37–55.
[36] C.J. Sherr, Cancer cell cycles, Science 274 (1996) 1672–1677.
[37] C.J. Sherr, D-type cyclins, Trends Biochem. Sci. 20 (1995) 187–190.
[38] C.J. Sherr, D1 in G2, Cell Cycle 1 (2002) 36–38.
[39] D.W. Stacey, Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells, Curr. Opin. Cell Biol. 15 (2003) 158–163.
[40] F. Wolter, B. Akoglu, A. Clausnitzer, J. Stein, Downregulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines, J. Nutr. 131 (2001) 2197–2203.
[41] B. Bressac, K.M. Galvin, T.J. Liang, K.J. Isselbacher, J.R. Wands, M. Ozturk, Abnormal structure and expression of p53 gene in human hepatocellular carcinoma, Proc. Natl. Acad. Sci. USA 87 (1990) 1973–1977.
[42] Y. Lin, C.Y. Shi, B. Li, B.H. Soo, S. Mohammed-Ali, A. Wee, C.J. Oon, P.O. Mack, S.H. Chan, Tumour suppressor p53 and Rb genes in human hepatocellular carcinoma, Ann. Acad. Med. Singapore 25 (1996) 22–30.
[43] Y. Murakami, K. Hayashi, T. Sekiya, Detection of aberrations of the p53 alleles and the gene transcript in human tumor cell lines by single-strand conformation polymorphism analysis, Cancer Res. 51 (1991) 3356–3361.
[44] B.B. Knowles, C.C. Howe, D.P. Aden, Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen, Science 209 (1980) 497–499.
[45] J.P. Monjardino, M.J. Fowler, H.C. Thomas, Defective hepatitis B virus DNA molecules detected in a stable integration pattern in a hepatoma cell line, and in induced tumours and derived cell lines, J. Gen. Virol. 64 (10) (1983) 2299–2303.
[46] D.D. Sarbassov, S.M. Ali, D.M. Sabatini, Growing roles for the mTOR pathway, Curr. Opin. Cell Biol. 17 (2005) 596–603.
[47] E.M. Smith, C.G. Proud, cdc2-Cyclin B regulates eEF2 kinase activity in a cell cycle- and amino acid-dependent manner, EMBO J. 27 (2008) 1005–1016.
[48] J.F. Holland, M.I. Frei, G. Pizzorno, Y.C. Cheng, Pyrimidine and Purine Antimetabolites, Williams & Wilkins, London, 1997.
[49] D.B. Longley, D.P. Harkin, P.G. Johnston, 5-Fluorouracil: mechanisms of action and clinical strategies, Nat. Rev. Cancer 3 (2003) 330–338.
[50] F. Bunz, P.M. Hwang, C. Torrance, T. Waldman, Y. Zhang, L. Dillehay, J. Williams, C. Lengauer, K.W. Kinzler, B. Vogelstein, Disruption of p53 in human cancer cells alters the responses to therapeutic agents, J. Clin. Invest. 104 (1999) 263–269.
[51] H.E. Wang, H.C. Wu, S.J. Kao, F.W. Tseng, Y.S. Wang, H.M. Yu, S.L. Chou, S.H. Yen, K.H. Chi, Modulation of 5-fluorouracil cytotoxicity through thymidylate synthase and NF-kappaB down-regulation and its application on the radiolabelled iododeoxyuridine therapy on human hepatoma cell, Biochem. Pharmacol. 69 (2005) 617–626.
[52] S.T. Eichhorst, M. Muller, M. Li-Weber, H. Schulze-Bergkamen, P. Angel, P.H. Krammer, A novel AP-1 element in the CD95 ligand promoter is required for induction of apoptosis in hepatocellular carcinoma cells upon treatment with anticancer drugs, Mol. Cell Biol. 20 (2000) 7826–7837.

[53] K. Koike, A. Takaki, M. Tatsukawa, M. Suzuki, H. Shiraha, Y. Iwasaki, K. Sakaguchi, Y. Shiratori, Combination of 5-FU and IFNalpha enhances IFN signaling pathway and caspase-8 activity, resulting in marked apoptosis in hepatoma cell lines, Int. J. Oncol. 29 (2006) 1253–1261.
[54] H. Schulze-Bergkamen, B. Fleischer, M. Schuchmann, A. Weber, A. Weinmann, P.H. Krammer, P.R. Galle, Suppression of Mcl-1 via RNA interference sensitizes human hepatocellular carcinoma cells towards apoptosis induction, BMC Cancer 6 (2006) 232.
[55] S. Jiang, M.J. Song, E.C. Shin, M.O. Lee, S.J. Kim, J.H. Park, Apoptosis in human hepatoma cell lines by chemotherapeutic drugs via Fas-dependent and Fas-independent pathways, Hepatology 29 (1999) 101–110.
[56] O. Brenes, F. Arce, O. Gatjens-Boniche, C. Diaz, Characterization of cell death events induced by anti-neoplastic drugs cisplatin, paclitaxel and 5-fluorouracil on human hepatoma cell lines: Possible mechanisms of cell resistance, Biomed. Pharmacother. 61 (2007) 347–355.
[57] O. Rosen, H.J. Muller, N. Gokbuget, W. Langer, N. Peter, S. Schwartz, D. Hahling, F. Hartmann, T.H. Ittel, R. Muck, F. Rothmann, R. Arnold, J. Boos, D. Hoelzer, Pegylated asparaginase in combination with high-dose methotrexate for consolidation in adult acute lymphoblastic leukaemia in first remission: a pilot study, Brit. J. Haematol. 123 (2003) 836–841.
[58] T. Yoshioka, T. Wada, N. Uchida, H. Maki, H. Yoshida, N. Ide, H. Kasai, K. Hojo, K. Shono, R. Maekawa, S. Yagi, R.M. Hoffman, K. Sugita, Anticancer efficacy in vivo and in vitro, synergy with 5-fluorouracil, and safety of recombinant methioninase, Cancer Res. 58 (1998) 2583–2587.