Combined antitumor effects of P-5m octapeptide and 5-fluorouracil on a murine model of H22 hepatoma ascites

XIAO HAN[1], LIPING AN[1], DONGMEI YAN[2], HIROSHI MATSUURA[3], WEIGUANG DING[3], MENGCHUAN ZHANG[1], GUANGYU XU[1], YING SUN[1], GUANGXIN YUAN[1], MANLI WANG[1], NANXI ZHAO[1], JINGBO SUN[1], XUN ZHU[2] and PEIGE DU[1]

[1]Department of Microbial and Biochemical Pharmacy, College of Pharmaceutical Science, Beihua University, Changchun, Jilin 132013; [2]Department of Immunology, Norman Bethune College of Medicine, Jilin University, Changchun, Jilin 130021, P.R. China; [3]Department of Physiology, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan

Received January 4, 2016; Accepted February 10, 2017

DOI: 10.3892/etm.2018.6422

Abstract. The present study has demonstrated that P-5m octapeptide (P-5m) has therapeutic potential in metastatic human hepatocarcinoma, possibly through the modulation of matrix metalloproteinase-2 expression. The purpose of the present study was to evaluate the antitumor effect of P-5m combined with 5-fluorouracil (5-Fu) on the treatment of hepatoma 22 (H22) hepatocarcinoma malignant ascites in a mouse model. The inhibitory effect on the growth of mouse ascites tumors was monitored by measuring body weight gain, survival time, ascites volume, numbers of tumor cells, DNA synthesis and peritoneal capillary permeability analysis. The present data revealed a significant reduction in ascites volume and cell count in mice that were treated with P-5m plus 5-Fu. Furthermore, the median survival time in mice in the combination group was prolonged compared with the disease control group. Moreover, a significant reduction in the total H22 ascites cell count in mice from the combination group was observed when compared with the disease control group. P-5m plus 5-Fu was able to induce the cell cycle arrest and inhibit the peritoneal capillary permeability of the mice. To conclude, the present study indicated that P-5m may have therapeutic potential in ascites caused by hepatocellular carcinoma.

Introduction

Malignant ascites are a common and distressing condition associated with a variety of advanced stage of neoplasms, particularly breast, ovary, stomach, pancreas, liver, and colon cancer (1,2). The majority of patients with malignant ascites experience progressive abdominal swelling and troublesome symptoms such as pain, nausea, dyspnea, constipation, and edema (3). With the exception of ovarian cancer, malignant ascites typically have a poor prognosis and a median survival time of no more than 4 months (4). In contrast, ovarian cancer patients with malignant ascites have a superior survival rate, with a median of ~2 years (4,5).

Treatment of malignant ascites remains challenging. In the majority of cases, systemic chemotherapy is ineffective. Furthermore, diuretics and paracentesis are the most common procedures used; however, intraperitoneal chemotherapy is potentially one of the promising options for future treatment of malignant ascites (2). Intraperitoneal chemotherapy allows for direct exposure of tumor cells to high peritoneal concentrations of cytotoxic drugs without increasing systemic toxicity. By destroying tumor cells at the peritoneal surface, intraperitoneal chemotherapeutic drugs induce a fibrotic reaction and thus will prevent the formation of peritoneal fluid (6). The majority of pharmacological agents with known activity in peritoneal malignant disease, including 5-fluorouracil (5-Fu), cisplatin, melphalan, carboplatin, mytomicin, topotecan, etoposide, doxorubicin, paclitaxel, cytarabine, and methotrexate, have been examined via the intraperitoneal route (7-9).

5-Fu is a molecule that has a pharmacologic advantage for first-line clinical treatment of peritoneal malignant diseases (10,11). In patients who received early postoperative intraperitoneal treatment, the area under the curve (AUC) for intraperitoneal 5-Fu was 422 times more than intravenous 5-Fu and the AUC ratio of plasma to tumor tissues was 5.2 (11). However, the serious side effects of 5-Fu, including hepatotoxicity and bone marrow suppression, may restrict its extensive clinical application (12). Therefore, it is necessary to explore novel types of pharmacological agents to substitute or combine with 5-Fu.

Synthetic antitumor peptides have received an increasing amount of worldwide attention. Modern chemotherapy based on synthetic oligo peptides provides an effective anti-metastasis medication for patients with tumors, exhibiting high affinity and low toxicity (13-16). P-5m is an octapeptide derived from domain

Correspondence to: Dr Peige Du, Department of Microbial and Biochemical Pharmacy, College of Pharmaceutical Science, Beihua University, 3999 East Binjiang Road, Changchun, Jilin 132013, P.R. China
E-mail: dupeige2001@126.com

Key words: 5-fluorouracil, ascites, hepatoma 22 cells, P-5m octapeptide

5 of high-molecular weight kininogen; the P-5m peptide has been indicated to promote the inhibition of metastatic activity exhibited in human melanoma cells (17). The study indicated that significant anti-invasion and anti-migration effects were exerted by the His-Gly-Lys motif of the P-5m peptide *in vitro* and *in vivo* in human melanoma cells (17). However, the results cannot explain the anti-metastatic molecular mechanisms of P-5m octapeptide. In a previous study (18), we measured the anti-metastasis effect of P-5m with HCCLM3 human hepatocarcinoma cells *in vitro* and *in vivo* in order to attempt to detect the underlying mechanisms of any inhibitory effects. The data indicated that P-5m treatment significantly inhibited lung metastasis in nude mice and the expression of metalloproteinase-2 (MMP-2) in the tumor tissues. These observations suggest that therapy with P-5m inhibits invasion and metastasis of hepatocellular carcinoma, at least partially through modulation of MMP-2 expression. Furthermore, the data indicated that P-5m may have therapeutic potential in metastatic human hepatocarcinoma. The present *in vivo* study aimed to determine if P-5m peptide is able to increase the sensitivity of murine ascitic H22 cells to 5-Fu, chemotherapeutically.

In the present study, a significant reduction in total H22 ascites cell count in mice from the combination group was observed. Moreover, P-5m plus 5-Fuwas able to induce cell cycle arrest and inhibit the peritoneal capillary permeability of mice. P-5m in combination with 5-Fu may be potentially useful when researching of anti-metastatic agents and cancer intraperitoneal chemotherapy.

Materials and methods

Peptide synthesis. P-5m peptide (GHGKHKNK) was synthesized in our laboratory via a standard fluorenylmethoxycarbonyl solid-phase strategy as described previously (19). The crude peptide was purified by reverse-phase high performance liquid chromatography (>98% purity). The molecular weights were identified by electrospray ionization mass spectrometry (Agilent Technologies GmbH, Waldbronn, Germany).

Animals and cell lines. A total of 180 Female Kunming mice (weight, 18-22 g; age, 6-8 weeks) were obtained from Animal Center of Jilin University (Jilin, China). Mice were bred in-house in the animal care facility of the University of Beihua under standardized specific pathogen-free conditions. Mice were housed at an ambient temperature of 20-23˚C, a relative humidity of 30-40% and a 12 h dark/light cycle). Free access to standard rodent chow and water was allowed for the duration of the study. All experiments using laboratory animals were performed according to the guidelines of the Animal Management Rules of the Ministry of Health of the People's Republic of China and were approved by the Beihua University Committee on Laboratory Animals (Changchun, China). The Mouse hepatoma H22 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in Dulbecco's modified Eagle's medium (cat. no. SH30249.01; Hyclone; GE Healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum (cat. no. 26400044; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and maintained at 37˚C in humidified 5% CO_2. Cells were passaged when they reached 70-80% confluence. Viable H22 cells in ($1x10^7$ cells/0.2 ml 0.9% sodium chloride) were injected into the peritoneal cavity of mice to trigger ascitic cell growth. Transplantation of H22 cells was performed once weekly, only the fluid transplant generations were passaged over 10 times, provoking the formation of regular exudates, were used in the subsequent experiments. Furthermore, tumor transplantation was performed by intraperitoneal injection of $2.5x10^6$/ml H22 cells suspended in 0.2 ml of 0.9% sodium chloride (20-22).

Treatment protocol. Mice (n=180) were randomly allocated into the following groups (all n=24 except the control): Negative control (NC) group, disease group (DG), low-dose P-5m-treated group (LP; 12.5 μg/kg P-5m), medium-dose P-5m group (MP; 50 μg/kg P-5m), high-dose P-5m group (HP; 200 μg/kg P-5m), low-dose 5-Fu group (LF; 5 mg/kg 5-Fu), high-dose 5-Fu group (HF; 20 mg/kg 5-Fu) and combination of P-5m (12.5 μg/kg) and 5-Fu (5 mg/kg) group (PF). Mice in the NC group (n=12) were neither inoculated with H22 cells nor received any treatment and were only used for body weight evaluation and survival analysis. For the other groups (n=24), 12 mice in each group were used for body weight evaluation and survival analysis, another 6 were used for ascitic fluid evaluation, and the remaining 6 were used for peritoneal capillary permeability analysis. Mice in the LF and HF groups were intraperitoneally injected 3 times a week and mice in other groups were intraperitoneally injected 5 times a week for 30 days. The physical state characteristics were monitored daily, and body weight was recorded every 2 days.

Humane endpoints. The endpoint of each experiment was determined either by spontaneous death or by the elective killing of the animal when signs of pain or suffering were shown, according to established criteria (Replication of Animal Models of Human Diseases) (23). The signs, depending on severity, duration and response to therapy, were as follows: rapid or progressive weight loss; sizable abdominal enlargement or ascites with loss of a righting reflex; possessing a volume of ascites (g)/body weight (g) of >20%; anorexia or failure to drink; debilitating diarrhea; dehydration/reduced skin turgor; edema; progressive dermatitis; rough hair coat/unkempt appearance; hunched posture; lethargy or persistent recumbency; coughing; labored breathing; nasal discharge; jaundice; cyanosis; pallor/anemia; neurological signs (including seizures, paresis/paralysis, circling or head tilt, blindness); bleeding from any orifice; and self-induced trauma.

When animals exhibited signs of the aforementioned humane endpoints, they were immediately sacrificed via compressed CO_2 gas. Animals were placed in a chamber, and the gas flow rate was 20% vol/min prior to the loss of consciousness. The flow velocity of CO_2 gas was accelerated to 25% following loss of consciousness. Sacrifice was confirmed by the absence of a heartbeat.

Survival analysis. Tumor inoculation and treatment were performed and the mice were observed daily until fatality or 60 days following the completion of treatments. Results are expressed as percentages of increased life span (ILS%), calculated according to the formula: ILS%=(T/C-1) x100, where T represents mean survival time of treated mice and C represents mean survival time of the control group.

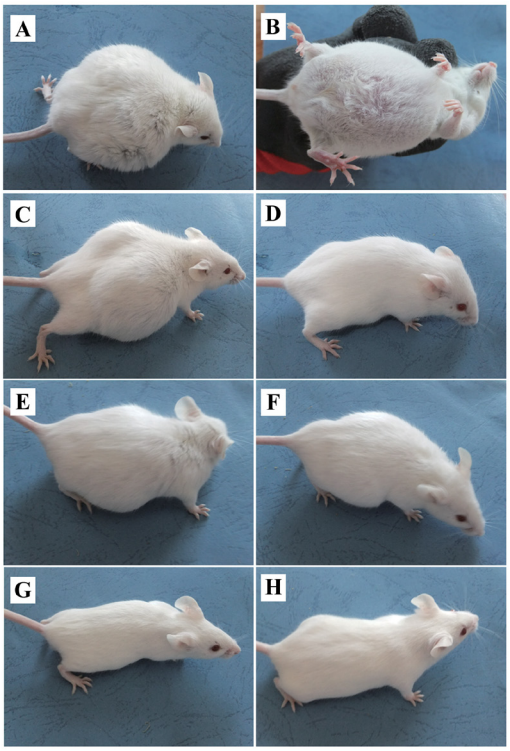


Figure 1. Effect of P-5m and 5-Fu on the production of malignant ascites in a mouse model of hepatoma 22 ascites. Images were captured on day 13, following tumor implantation. (A and B) DG disease group (positive control group without any drug treatment). (C) HP/high-dose P-5m (200 μg/kg) group. (D) MP/medium-dose P-5m (50 μg/kg) group. (E) LP/low-dose P-5m (12.5 μg/kg) group. (F) LF/low-dose 5-Fu (5 mg/kg) group. (G) HF/high-dose 5-Fu (20 mg/kg) group. (H) PF/combination of P-5m (12.5 μg/kg) and 5-Fu (5 mg/kg) group. Mice in these groups were inoculated with H22 cells for 2 days prior to the start of the various therapies. Mice in the LF and HF groups were intraperitoneally injected three times a week, other groups were intraperitoneally injected daily for 30 days. The signs of general physical state were monitored daily. P-5m, P-5m octapeptide; 5-Fu, 5-fluorouracil; DG, positive control group in which mice with ascites did not receive therapeutic treatment; HP, high-dose P-5m (200 μg/kg) group; MP, medium-dose P-5m (50 μg/kg) group; LP, low-dose P-5m (12.5 μg/kg) group; LF, low-dose 5-Fu (5 mg/kg) group; HF, high-dose 5-Fu (20 mg/kg) group; PF, combination of P-5m (12.5 μg/kg) and 5-Fu (5 mg/kg) group.

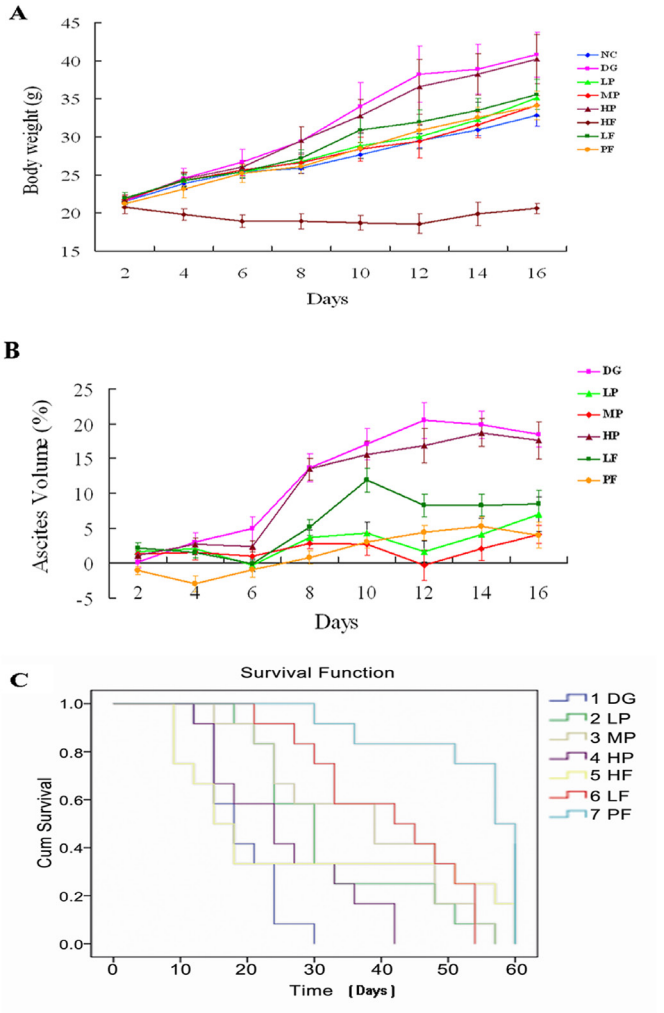


Figure 2. Effects of P-5m and/or 5-Fu on (A) body weight gain, (B) ascites volume (%) and (C) survival time of hepatoma 22-bearing mice. P-5m, P-5m octapeptide; 5-Fu, 5-fluorouracil; NC, negative control group in which mice were not treated; DG, positive control group in which mice with ascites did not receive therapeutic treatment; HP, high-dose P-5m (200 μg/kg) group; MP, medium-dose P-5m (50 μg/kg) group; LP, low-dose P-5m (12.5 μg/kg) group; LF, low-dose 5-Fu (5 mg/kg) group; HF, high-dose 5-Fu (20 mg/kg) group; PF, combination of P-5m (12.5 μg/kg) and 5-Fu (5 mg/kg) group; Cum, cumulative. Ascitic volume are estimate according to the formula: Ascitic volume (%)=(X-N)/X x100, where X represents the body weight of an individual mouse and N represents mean body weight of the Negative control (NC) group. When the animal observed sizable abdominal enlargement or ascites with loss of a righting reflex, or [Volume of ascites (g)/body weight (g)] was >20%, euthanasia would be prompted.

Ascitic fluid evaluation. On day 13 of the study, mice in the control and experimental groups used for ascitic fluid evaluation were sacrificed, ascitic fluid was collected and the volumes were measured. The total collected H22 cells were washed twice with PBS, and viable cells were stained with trypan blue and counted using a hemocytometer (24).

Cell cycle analysis. Flow cytometry (Beckman Coulter, Inc., Brea, CA, USA) was used to analyze the different stages of cell cycle using a cell cycle staining kit (cat. no. 04511-1KT-F; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the manufacturers' protocol. H22 cells ($2x10^6$) collected from each ascites-bearing mouse were fixed using 70% ethanol for 24 h at 4˚C. Cells were washed twice with cold PBS, resuspended in 500 μl PBS and treated with 10 μl RNase A (10 mg/ml; Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. For staining, cells were incubated with 10 μl of propidium iodide/Triton-X 100 (500 μg/ml; Sigma-Aldrich; Merck KGaA) in the dark for 5 min at 37˚C using a previously described method (24). The fraction of cells in G_0/G_1, S, and G_2/M phase were analyzed using a fluorescence activated cell sorting flow cytometer (Beckman Coulter Epis XL; Beckman Coulter, Inc.) and analyzed using Expo32 software (Expo32-ADC; Beckman Coulter, Inc.).

Analyzing peritoneal capillary permeability. Peritoneal capillary permeability was measured as described by Ujioka *et al* (25). On day 13, mice in the control and experimental groups for peritoneal capillary permeability analysis were injected intravenously with 0.2 ml of 0.8% Evans blue solution (EB; cat. no. 18909-100ML-F; Sigma-Aldrich; Merck KGaA), and sacrificed 120 min after the intraperitoneal injection. Concentrations of EB in the peritoneal fluid

Table I. Effects of various treatments on the survival time of hepatoma 22 ascites mice.

Group	N	Survival time (days)	Median survival time ± standard deviation (days)	Increased life span (%)[a]	Long-term survivors[b]	Cure rate (%)
DG	12	13-30	17.33±5.65	-	0	0
LP	12	18-57	30.50±13.21[c]	76.0	0	0
MP	12	15-57	35.00±14.06[d]	102.0	0	0
HP	12	13-42	23.50±10.56	35.6	0	0
LF	12	21-54	39.00±11.932[d]	125.0	0	0
HF	12	10-60	26.00±22.39	50.0	2	16.67
PF	12	30-60	52.83±10.667[d]	204.8	6	50.00

[a]Increased life span% = (T/C-1) x100, where T, survival time of treated mice and C, survival time of the control mice. [b]Long-term survivors, mice survived >60 days after treatment. [c]P<0.05 and [d]P<0.01 vs. DG. P-5m, P-5m octapeptide; 5-Fu, 5-fluorouracil; DG, positive control group in which mice with ascites did not receive therapeutic treatment; HP, high-dose P-5m (200 μg/kg) group; MP, medium-dose P-5m (50 μg/kg) group; LP, low-dose P-5m (12.5 μg/kg) group; LF, low-dose 5-Fu (5 mg/kg) group; HF, high-dose 5-Fu (20 mg/kg) group; PF, combination of P-5m (12.5 μg/kg) and 5-Fu (5 mg/kg) group.

were measured via spectrophotometry at 580 nm wavelength, and expressed as light absorption x ascitic fluid volume.

Statistical analysis. All data were analyzed with SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) and expressed as mean ± standard deviation. Survival of mice was calculated using the Kaplan-Meier method. For multiple comparisons, one-way analysis of variance was applied. Least-significant difference (LSD) or Games-Howell was used according to the homogeneity of variances. P<0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Effects of P-5m and/or 5-Fu on body weight gain and survival time of mice bearing H22 hepatocellular carcinoma. One of our previous studies indicated that P-5m has therapeutic potential in metastatic human hepatocarcinoma, in which the efficacy may function, at least partially, through modulation of MMP-2 expression (18). Therefore, it is meaningful to investigate the combined effects of P-5m and 5-Fu in murine tumor models with hepatocarcinoma H22 malignant ascites. In the present study, a murine H22 hepatoma ascites model was established using Kunming mice (Fig. 1). In the early few days, there were no observable differences in the behavior of the mice in every groups. Up until the sixth day, there was an extensive increase in the abdominal girth and body-weight of the DG group, and revealed malaise of spirit, little movement, poor response (Fig. 1A) and abdominal ectasia (Fig. 1B). LP (Fig. 1C) and LF (Fig. 1F) groups exhibited a reduction in body weight when compared with the DG group. A significant reduction in body weight was also observed in mice that were treated with P-5m plus 5-Fu (LP; Fig. 1H), whereas medium-dose of P-5m (MP; Fig. 1D) had a similar effect. There were no significant differences between the high-dose P-5m (HP) group and the DG group (Fig. 1E). Although a high dose of 5-Fu (HF, 20 mg/kg; Fig. 1G) fully suppressed the development of ascites, this treatment exhibited 5-Fu-associated host toxicities, including a reduction of body weight, reduced food and water uptake, piloerection, hunched posture, lethargy and hypoactivity, which resulted in the mortality of 83.3% of mice.

LP, MP and LF groups significantly exhibited reduced body weight and ascites volume (%) in mice compared with the DG group. However, the combination of P-5m and 5-Fu (PF) further reduced the tumor load (Fig. 2A and B). There were no significant differences between the HP group and the DG group. Although a high dose of 5-Fu (HF, 20 mg/kg) fully suppressed the development of ascites, this treatment had 5-Fu-associated host toxicities (Fig. 2C). However, LP treatment significantly prolonged survival time of mice. Although mice in MP and PF groups gained weight at every time point from day 2 to 16, these changes may reflect health status rather than the development of ascites, since these mice exhibited flat abdomens. Therefore, the order of body weight/ascites volume (%) reduction of mice compared with DG group was: HF>PF>MP>LP>LF>HP.

Survival analysis indicated that while all mice in the disease control group succumbed to disease within 30 days, a significantly prolonged survival time and delayed development of malignant ascites were demonstrated in mice treated with low- or medium-doses of P-5m, low doses of 5-Fu, and particularly in the groups of mice treated with a combination of P-5m and 5-Fu (Fig. 2C; Table I). However, this was not exhibited in mice treated with high-doses of P-5m or 5-Fu. The median survival time in mice in the group treated with a combination of P-5m and 5-Fu was 52.8 days, which corresponds to an increase of 204.8% when compared with the disease control group (Table I). Taking the results of 5 and 20 mg/kg 5-Fu into account in the ascites model, the cure rate (no ascites symptom 30 days after stopping of intraperitoneal chemotherapy) of 5-Fu alone was 16.67%. Conversely, the combination of 5-Fu with P-5m increased the cure rate up to 50% (Table I). Therefore, the order of life span prolongation of mice with ascites was: PF>LF>MP>LP>HF>HP.

Effects of P-5m and/or 5-Fu on accumulation of the ascites, number of red blood cells and tumor cells, and the cell cycle of H22 hepatocellular carcinoma cells. On day 13 of the present study, the transplanted H22 cells and fluid from ascites were

Table II. Effects of various treatments on the accumulation of ascites, numbers of tumor cells and red blood cells.

Group	N	Volume of ascites (ml)	Tumor cells (10^7/ml)	Red blood cells (10^6/ml)
DG	5	7.9±1.91	8.06±1.48	13.52±7.47
LP	6	4.13±2.80[a]	5.47±2.03	2.57±2.85[a]
MP	6	1.6±1.55[a]	2.43±2.3[a]	1.6±1.74[a]
HP	4	5.4±3.91	7.6±3.58	8.98±5.89
LF	6	4.38±2.65[b]	5.38±2.95	2.77±2.17[a]
HF	4	0.1±0.14[a]	0.00±0.00[a]	0.00±0.00[a]
PF	6	0.38±0.48[a]	0.58±0.94[a]	0.00±0.00[a]

[a]P<0.01 and [b]P<0.05 vs. DG. Data are presented as the mean ± standard deviation. P-5m, P-5m octapeptide; 5-Fu, 5-fluorouracil; DG, positive control group in which mice with ascites did not receive therapeutic treatment; HP, high-dose P-5m (200 μg/kg) group; MP, medium-dose P-5m (50 μg/kg) group; LP, low-dose P-5m (12.5 μg/kg) group; LF, low-dose 5-Fu (5 mg/kg) group; HF, high-dose 5-Fu (20 mg/kg) group; PF, combination of P-5m (12.5 μg/kg) and 5-Fu (5 mg/kg) group.

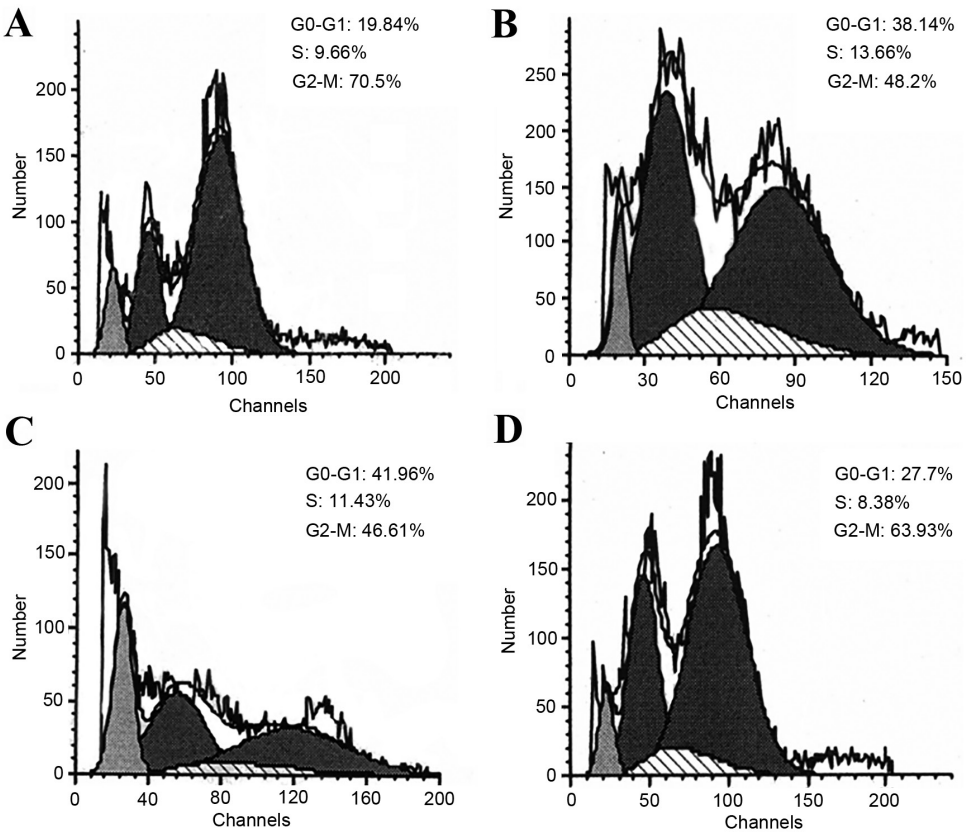


Figure 3. Effects of various treatments on the ascites cell cycle analyzed by flow cytometry in the (A) DG, (B) LP, (C) MP and (D) HP groups. P-5m, P-5m octapeptide; 5-Fu, fluorouracil; DG, positive control group in which mice with ascites did not receive therapeutic treatment; HP, high-dose P-5m (200 μg/kg) group; MP, medium-dose P-5m (50 μg/kg) group; LP, low-dose P-5m (12.5 μg/kg) group.

harvested from the peritoneal cavity of the mice in all experimental groups. Results indicated that low- and medium-doses of P-5m alone (12.5 and 50 μg/kg, respectively) significantly decreased tumor growth, whereas the combination of 12.5 μg/kg P-5m and 5 mg/kg 5-Fu further reduced the tumor load (Table II). Furthermore, 5-Fu-associated host toxicities were observed, although 20 mg/kg of 5-Fu produced a significant inhibitory effect on the growth of ascites. Additionally, H22 cell viability was decreased when compared with untreated controls. Among the groups, the lowest viability of malignant cells was observed in mice in the combination treatment group (Table II).

These results suggested that P-5m increased the antitumor effect of 5-Fu *in vivo* without triggering mice to succumb to disease early. Furthermore, P-5m acted as a biochemical modulator of 5-Fu, as P-5m may neutralize the toxicity caused by 5-Fu. However, high-dose P-5m administration did not exert any antitumor effects in the present study. One potential explanation is that the high doses of P-5m may induce internalization of its

Table III. Cell cycles of hepatoma 22 ascites mice in different groups.

Group	N	Cell cycle stage		
		G_0-G_1 (%)	S (%)	G_2-M (%)
DG	5	20.04±3.74	11.90±3.60	68.06±3.06
LP	5	32.46±8.75[a]	15.03±3.76	52.73±10.47[a]
MP	5	42.44±6.74[a]	11.94±3.87	45.63±2.97[a]
HP	4	27.3±3.35	10.27±4.31	62.43±7.52
LF	5	23.00±3.69	28.34±5.27[a]	48.66±4.16[a]

The high-dose 5-Fu (20 mg/kg) group and the combination of P-5m (12.5 μg/kg) and 5-Fu (5 mg/kg) group were not able to be statistically analyzed for cycle distributions due to insufficient amounts of cell sample numbers. Data are presented as ± standard deviation. [a]P<0.01 vs. DG. P-5m, P-5m octapeptide; 5-Fu, 5-fluorouracil; DG, positive control group in which mice with ascites did not receive therapeutic treatment; HP, high-dose P-5m (200 μg/kg) group; MP, medium-dose P-5m (50 μg/kg) group; LP, low-dose P-5m (12.5 μg/kg) group; LF, low-dose 5-Fu (5 mg/kg) group.

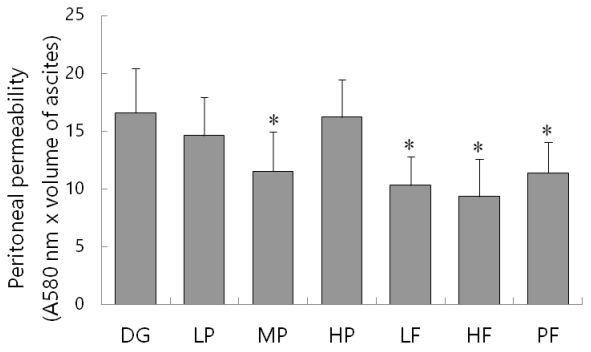


Figure 4. Effect of different treatments on the peritoneal permeability of mice (n=4-6). Evans blue dye was used to evaluate the peritoneal capillary permeability of mice in DG, LP, MP, HP, LF, HF and PF groups. Mice were injected via the caudal vein with 8% Evans blue, ascites were collected and the light absorption at the wavelength of 580 nm was analyzed using a spectrophotometer. Data are presented as the mean + standard deviation. *P<0.05 vs. DG. P-5m, P-5m octapeptide; 5-Fu, 5-fluorouracil; DG, positive control group in which mice with ascites did not receive therapeutic treatment; HP, high-dose P-5m (200 μg/kg) group; MP, medium-dose P-5m (50 μg/kg) group; LP, low-dose P-5m (12.5 μg/kg) group; LF, low-dose 5-Fu (5 mg/kg) group; HF, high-dose 5-Fu (20 mg/kg) group; PF, combination of P-5m (12.5 ug/kg) and 5-Fu (5 mg/kg) group.

receptor. It has been shown that most biologically active peptides have receptors on the plasma membrane of cells and binding to ligands will trigger receptor internalization and downregulation, although the fate of these receptors within cells differ (26,27). Optimal doses of P-5m used in the present study were chosen according to our preliminary experiments in which a medium dose of P-5m (50 μg/kg) had the most effective antitumor activities (data not shown).

Effects of P-5m and/or 5-Fu on the cell cycle of H22 hepatocellular carcinoma cells. Cell cycle analysis revealed that the proportions of H22 cells in G_0-G_1 phase significantly increased from 20.04±3.74% (DG group; Fig. 3A) to 32.46±8.75% (LP group; Fig. 3B) and 42.44±6.74% (MP group; Fig. 3C), respectively, while the proportion of cells in G_2-M phase significantly decreased from 68.06±3.06% to 52.73±10.47 and 45.63±2.97% (Table III), suggesting that P-5m may induce a G_1-cell cycle arrest in the ascites model. However, high doses of P-5m (200 mg/kg; Fig. 3D; Table III) did not exhibit a significant difference when compared with the disease control group, suggesting this dose did not affect the cell cycle distributions. In contrast to P-5m, low-doses of 5-Fu significantly increased the proportion of cells in S-phase (disease group vs. LF group, 11.90±3.60 vs. 28.34±5.27%); however, a significantly decreased proportion of cells was exhibited in G2-M phase (68.06±3.06 vs. 48.66±4.16%) (Table III), suggesting that 5-Fu induced S-cell cycle arrest. The later observations are consistent with previous studies, which demonstrated that 5-Fu exerts antitumor effects by inducing S-phase arrest (28-30). Therefore, it is likely that both P-5m and 5-Fu have important and varying roles in regulating cell cycle distributions in combined treatments. Although in the present study it was not possible to statistically analyze cycle distributions in HF and PF groups due to insufficient amounts of cell sample numbers, the data of one sample in PF group indicated that the proportion of cells in G_2-M phase was merely 14.66%, implying the decline of meiosis competence (Fig. 3; Table III).

Effects of P-5m and/or 5-Fu on the permeability peritoneal capillaries of mice bearing H22 hepatocellular carcinoma. EB dye was used to evaluate the peritoneal capillary permeability of mice. Concentrations of EB in the peritoneal fluid were measured via spectrophotometry at 580 nm wavelength, and expressed as light absorption x ascitic fluid volume. The peritoneal concentrations of EB in fluid from ascites of MP (11.53±3.44), PF (11.37±2.67), LF (10.3±2.49) and HF (9.32±3.29) groups were significantly lower than the disease control group (16.58±3.85; P<0.05; Fig. 4). The data suggested that P-5m enhances the anti-tumor effect of 5-Fu on H22 bearing mice and antagonizes its toxicity, markedly.

Collectively, the findings of the present study suggest that the combination of P-5m octapeptide with 5-Fu may provide an alternative therapeutic strategy in the treatment of tumors, specifically ascites caused by H22 hepatocellular carcinoma. Further studies are required to investigate the intensive mechanisms of this combined therapy.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 31201061); Jilin Province Sci-tech Department, China (grant nos. 20110729 and 20130522051JH); Jilin Province Development and Reform Commission, China (grant no. 2013G019); Jilin Province Health Bureau Development (grant no. 2017J082); and Jilin City Sci-tech Bureau, China (grant no. 2013625030).

Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XH, XZ and PD designed the study. XH, LA, DY, MZ performed the experiments. XH, LA, MW, NZ collected and analyzed the data. GX, GY and JS contributed to sample collection and intellectual input. XH and LA drafted and wrote the manuscript. MH, WD and YS gave advice on the experimental design, interpreted the results and critically revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experiments using laboratory animals were performed according to the guidelines of the Animal Management Rules of the Ministry of Health of the People's Republic of China and were approved by the Beihua University Committee on Laboratory Animals (Changchun, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ayantunde AA and Parsons SL: Pattern and prognostic factors in patients with malignant ascites: A retrospective study. Ann Oncol 18: 945-949, 2007.
2. Barni S, Cabiddu M, Ghilardi M and Petrelli F: A novel perspective for an orphan problem: Old and new drugs for the medical management of malignant ascites. Crit Rev Oncol Hematol 79: 144-153, 2011.
3. Coupe NA, Cox K, Clark K, Boyer M and Stockler M: Outcomes of permanent peritoneal ports for the management of recurrent malignant ascites. J Palliat Med 16: 938-940, 2013.
4. Woopen H and Sehouli J: Current and future options in the treatment of malignant ascites in ovarian cancer. Anticancer Res 29: 3353-3359, 2009.
5. Chung M and Kozuch P: Treatment of malignant ascites. Curr Treat Options Oncol 9: 215-233, 2008.
6. Akgol G, Yildiz C, Karakus S, Koc M, Dogan M, Turan M and Karadayi K: The effects of intraperitoneal chemotherapeutic agents on adhesion formation. European J Gynaecol Oncol 37: 781-785, 2016.
7. Cashin PH, Mahteme H, Graf W, Karlsson H, Larsson R and Nygren P: Activity ex vivo of cytotoxic drugs in patient samples of peritoneal carcinomatosis with special focus on colorectal cancer. BMC Cancer 13: 435, 2013.
8. Parson EN, Lentz S, Russell G, Shen P, Levine EA and Stewart JH IV: Outcomes after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for peritoneal surface dissemination from ovarian neoplasms. Am J Surg 202: 481-486, 2011.
9. Guarneri V, Piacentini F, Barbieri E and Conte PF: Achievements and unmet needs in the management of advanced ovarian cancer. Gynecol Oncol 117: 152-158, 2010.
10. Oh SY, Kwon HC, Lee S, Lee DM, Yoo HS, Kim SH, Jang JS, Kim MC, Jeong JS and Kim HJ: A Phase II study of oxaliplatin with low-dose leucovorin and bolus and continuous infusion 5-fluorouracil (modified FOLFOX-4) for gastric cancer patients with malignant ascites. Jpn J Clin Oncol 37: 930-935, 2007.
11. Van der Speeten K, Stuart OA, Mahteme H and Sugarbaker PH: Pharmacology of perioperative 5-fluorouracil. J Surg Oncol 102: 730-735, 2010.
12. Wyllie E and Wyllie R: Routine laboratory monitoring for serious adverse effects of antiepileptic medications: The controversy. Epilepsia 32 (Suppl 5): S74-S79, 1991.
13. Jang JH, Kim MY, Lee JW, Kim SC and Cho JH: Enhancement of the cancer targeting specificity of buforin IIb by fusion with an anionic peptide via a matrix metalloproteinases-cleavable linker. Peptides 32: 895-899, 2011.
14. Lo A, Lin CT and Wu HC: Hepatocellular carcinoma cell-specific peptide ligand for targeted drug delivery. Mol Cancer Ther 7: 579-589, 2008.
15. Xiao W, Wang Y, Lau EY, Luo J, Yao N, Shi C, Meza L, Tseng H, Maeda Y, Kumaresan P, *et al*: The use of one-bead one-compound combinatorial library technology to discover high-affinity αvβ3 integrin and cancer targeting arginine-glycine-aspartic acid ligands with a built-in handle. Mol Cancer Ther 9: 2714-2723, 2010.
16. Yao Z, Lu R, Jia J, Zhao P, Yang J, Zheng M, Lu J, Jin M, Yang H and Gao W: The effect of tripeptide tyroserleutide (YSL) on animal models of hepatocarcinoma. Peptides 27: 1167-1172, 2006.
17. Kawasaki M, Maeda T, Hanasawa K, Ohkubo I and Tani T: Effect of His-Gly-Lys motif derived from domain 5 of high molecular weight kininogen on suppression of cancer metastasis both in vitro and in vivo. J Biol Chem 278: 49301-49307, 2003.
18. Han X, Yan DM, Zhao XF, Matsuura H, Ding WG, Li P, Jiang S, Du BR, Du PG and Zhu X: GHGKHKNK octapeptide (P-5m) inhibits metastasis of HCCLM3 cell lines via regulation of MMP-2 expression in in vitro and in vivo studies. Molecules 17: 1357-1372, 2012.
19. Yamaguchi N and Kiick KL: Polysaccharide-poly (ethylene glycol) star copolymer as a scaffold for the production of bioactive hydrogels. Biomacromolecules 6: 1921-1930, 2005.
20. Zhang J, Wang X and Lu H: Amifostine increases cure rate of cisplatin on ascites hepatoma 22 via selectively protecting renal thioredoxin reductase. Cancer Lett 260: 127-136, 2008.
21. Santos FM, Latorre AO, Hueza IM, Sanches DS, Lippi LL, Gardner DR and Spinosa HS: Increased antitumor efficacy by the combined administration of swainsonine and cisplatin in vivo. Phytomedicine 18: 1096-1101, 2011.
22. Ghosh P, Sur P and Bag SP: Enhancement of antitumor effects of a new boron compound combined with ultrasound on the mouse ascites tumor. Med Chem 8: 1026-1031, 2012.
23. Li C and Ren LQ: Replication of Animal Models of Human Diseases. People's Medical Publishing House, Beijing, p61, 2008 (In Chinese).
24. Su ZQ, Liu YH, Guo HZ, Sun CY, Xie JH, Li YC, Chen JN, Lai XP, Su ZR and Chen HM: Effect-enhancing and toxicity-reducing activity of usnic acid in ascitic tumor-bearing mice treated with bleomycin. Int Immunopharmacol 46: 146-155, 2017.
25. Ujioka T, Matsuura K, Tanaka N and Okamura H: Involvement of ovarian kinin-kallikrein system in the pathophysiology of ovarian hyperstimulation syndrome: Studies in a rat model. Hum Reprod 13: 3009-3015, 1998.
26. Morel G: Internalization and nuclear localization of peptide hormones. Biochem Pharmacol 47: 63-76, 1994.
27. Finch AR, Caunt CJ, Armstrong SP and McArdle CA: Agonist-induced internalization and downregulation of gonadotropin-releasing hormone receptors. Am J Physiol Cell Physiol 297: C591-C600, 2009.
28. Kim GD, Rhee GS, Chung HM, Chee KM and Kim GJ: Cytotoxicity of 5-fluorouracil: Effect on endothelial differentiation via cell cycle inhibition in mouse embryonic stem cells. Toxicol In Vitro 23: 719-727, 2009.
29. Chen XX, Lai MD, Zhang YL and Huang Q: Less cytotoxicity to combination therapy of 5-fluorouracil and cisplatin than 5-fluorouracil alone in human colon cancer cell lines. World J Gastroenterol 8: 841-846, 2002.
30. Liang SR, Hu GR, Fang LJ, Huang SJ, Li JS, Zhao MY and Meng MJ: CpG oligodeoxynucleotides enhance chemosensitivity of 5-fluorouracil in HepG2 human hepatoma cells via downregulation of the antiapoptotic factors survivin and livin. Cancer Cell Int 13: 106, 2013.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.