의학석사 학위논문

Gemcitabine에 의해 유도된 췌장암 악액질에 미치는 GV1001의 예방 효과

Preventive effect of GV1001 on gemcitabine-induced pancreatic cancer cachexia

2016년 8월

서울대학교 대학원

의학과 해부학 전공

전 재 은

↑
2㎝
↓

Gemcitabine에 의해 유도된
췌장암 환자에게 주는 GV1001의 예방 효과

↑
2.5㎝
↓

2016년

↑
4㎝
↓

전 재 은

↑
3㎝
↓

↑
2㎝
↓

Gemcitabine에 의해 유도된 췌장암 악액질에 미치는 GV1001의 예방 효과

지도 교수 이 왕 재

이 논문을 의학석사 학위논문으로 제출함.

2016년 4월

서울대학교 대학원
의학과 해부학 전공
전 재 은

전재은의 의학석사 학위논문을 인준함.

2016년 7월

위 원 장 _____ **(인)**
부위원장 _____ **(인)**
위 원 _____ **(인)**

Preventive effect of GV1001 on gemcitabine-induced pancreatic cancer cachexia

by

Jane Jeon

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Master of Science in Medicine (Anatomy) at Seoul National University College of Medicine

July 2016

Approved by Thesis Committee:

Professor _____Chairman

Professor _____Vice chairman

Professor _____

ABSTRACT

Preventive effect of GV1001 on gemcitabine-induced pancreatic cancer cachexia

Jane Jeon
Department of Anatomy
Seoul National University College of Medicine

GV1001 derived from the human telomerase reverse transcriptase (hTERT) sequence is a peptide vaccine for the treatment of pancreatic cancer. The preclinical data clearly showed immunogenicity of GV1001 in patients with pancreatic cancer supported by the synergy of gemcitabine with cancer vaccines and the other positive immunomodulatory effects of gemcitabine. Even though it is reported that GV1001 may block weight loss of cancer patients and improve general condition after treatment of gemcitabine, but there are insufficient evidences so far. For this reason, we evaluate the preventive effect of GV1001 on gemcitabine-induced weigh loss in

xenograft animal model. There was definite body weight loss and muscle weight loss of tumor-bearing mice by the treatment of gemcitabine. However, it was recovered by the treatment of gemcitabine with GV1001. In addition, we compared skeletal muscle proliferation and differentiation upon the treatment of gemcitabine with or without GV1001. When skeletal muscle cells were treated with gemcitabine only, the decrease of myogenesis was observed. The decrease is ameliorated by the co-treatment of GV1001. Upon investigation, the results demonstrate that GV1001 effectively prevents the loss of weight and the decrease in skeletal muscle proliferation and regeneration by gemcitabine.

Keywords: cachexia, gemcitabine, GV1001, muscle wasting

Student Number: 2014-22994

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab: antibody

BCA assay: bicinchoninic acid assay

ELISA: enzyme-linked immunosorbent assay

HRP: horse radish peroxidase

hTERT: human telomerase reverse transcriptase

IFN-γ : interferon-gamma

TNF-α : tumor necrosis factor-alpha

PBS: phosphate buffered saline

PBST: PBS containing 0.05% Tween 20

qPCR: real time quantitative polymerase chain reaction

INTRODUCTION

Pancreatic cancer is among the most aggressive and lethal human cancers of which overall survival rate is only 4% regardless of race and stage despite the use of chemotherapy and radiotherapy (1-4). The poor survival rate may be attributable to the high incidence of metastatic disease at the time of diagnosis and resistance to existing chemotherapeutic agents (5). Gemcitabine (2', 2'-difluoro 2'-deoxycytidine), a nucleoside pyrimidine analog, is the most commonly given first-line therapy for pancreatic cancer. It competes with deoxycytidine triphosphate as an inhibitor of DNA polymerase (6-8). Cancer cells proliferate at a higher speed than do normal cells, thus targets of gemcitabine. Gemcitabine demonstrates broad anti-cancer activity in various tumors such as non-small-cell lung cancer (NSCLC), bladder cancer, gallbladder cancer, ovarian cancer, and breast cancer (6, 9). Randomized clinical studies have shown that gemcitabine is well tolerated and clinically effective as an anti-cancer agent that improves survival (10-12). Notwithstanding many benefits, gemcitabine is also well recognized for its serious side effects. Vomiting,

nausea, weight loss, dyspepsia, and cachexia are some of the unwanted secondary effects that unfavorably affect the quality of life (13, 14).

Cachexia is a syndrome characterized by weight loss, inflammation, anorexia and ongoing loss of skeletal muscle mass with or without loss of fat mass (15-17). Since the patients are burdened with incessant asthenia, increased resting energy expenditure, physical weakness, and mental fatigue, the quality of life and tolerance to therapy drastically decline and eventually mortality rises (18, 19). Cachexia is a common complication of cancer and chemotherapy and up to 80% patients develop cachexia before death and up to one third die from complications associated with cachexia (15, 20, 21). Cachexia is not a single-factored disease. Instead, it is a complex multifactorial disorder that is caused by pro-inflammatory response from the host immune system (TNF- α, IL-1, IL-6, and IFN- γ), production of catabolic factors and cytokines by the tumor (lipid mobilizing factor, LMF and proteolysis-inducing factor, PIF), and activation of the neuroendocrine stress response that lead to alterations in lipid, glucose, protein, and energy metabolism (22, 23). Downregulation of the positive regulator of muscle mass (myoD)

or overexpression of negative regulator of muscle mass (myostatin) are also some features observed in patients with cachexia (17). Both impede muscle regeneration further exacerbating the reduction in skeletal muscle mass.

GV1001 is a 16 amino acid sequence (611-626) from the human telomerase reverse transcriptase (hTERT) peptide vaccine designed for various cancers including pancreatic adenocarcinoma, melanoma, non-small cell lung cancer, and hepatocellular carcinoma (24-27). It targets the active site of telomerase and elicits helper T-cell and cytotoxic T-cell responses (28). Based on preclinical data, GV1001 was well tolerated in patients and when 560 μg of GV1001 was administered up to 75% of patients showed detectable immune responses, defined as a positive delayed-type hypersensitivity test or the presence of GV1001 specific T cells. Since telomerase activity appears in approximately 85-90% of human primary cancers and is repressed in the majority of normal human tissues, telomerase is an attractive target for chemotherapy (29). According to a preclinical data from Middleton et al., the overall survival of patients with locally advanced or metastatic pancreatic ductal adenocarcinoma who

received GV1001, gemcitabine, and capecitabine was 8.4 months, while that of group who received gemcitabine and capecitabine was 7.9 months. If GV1001 treatment elicits longer survival, improved general condition, and enhanced quality of life, it may be used as a chemotherapy adjuvant. Therefore, this study was aimed to discover the practicality of GV1001 that acts synergistically with gemcitabine while preventing cachectic symptoms induced by gemcitabine.

MATERIALS AND METHODS

Cell culture

The murine myoblast cell line, C2C12, and human primary skeletal myoblasts, HSkM (Thermo Fisher Scientific, MA, USA), were cultured in DMEM supplemented with 4500 mg/L D-glucose, L-glutamine, 100 mg/L sodium pyruvate (WELGENE, Daegu, Korea), heat-inactivated 10% fetal bovine serum (GE Healthcare HyClone, USA), 100 U/mL of penicillin and 100 μg/mL streptomycin (WELGENE, Daegu, Korea). Human pancreatic cancer cell lines, PANC-1 and AsPC-1 were maintained in RPMI1640 (WELGENE, Daegu, Korea) with heat-inactivated 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL streptomycin. All cells were purchased from Korean Cell Line Bank unless otherwise stated and incubated at 37 ◦C in a humidified 5% CO^2 chamber.

Cell proliferation and cytotoxicity assay

C2C12 cells were seeded in 96-well culture plates ($1x10^3$/well) in quintuplicates and incubated overnight for stabilization. Cells

were treated with the indicated amount of GV1001 (provided by KAEL-Gemvax, Korea) and gemcitabine (Yuhan Corporation, Korea) and were then incubated for another 72 hrs. Cell proliferation was measured with the Cell Viability, Proliferation & Cytotoxicity Assay Kit (EZ-CYTOX) (DoGen, Korea). After 1/10 volume of EZ-CYTOX solution was added to each well of the plate, absorbance values were measured at 450 nm using the microplate reader and SoftmaxPro software (Molecular Devices, CA, USA).

Cell cycle analysis

C2C12 cells ($2x10^5$, $1x10^5$) were seeded in 6-well culture plates and incubated overnight. Cells were pretreated with GV1001 (100 μM) for 1 hr then treated with gemcitabine (5 nM). After 24 hrs and 48 hrs, cells were harvested, fixed with ice-cold 70% ethanol overnight, incubated with PI/RNase Staining Buffer (BD Biosciences, NJ, USA) according to the manufacturer's instruction, and were analyzed by a FACSCalibur flow cytometer (BD Biosciences, NJ, USA) and FlowJo software (BD Biosciences, NJ, USA).

Real-time Polymerase Chain Reaction (qPCR)

C2C12 cells ($3x10^5$/well) were seeded in 6-well culture plates and incubated overnight for stabilization. Cells were pretreated with GV1001 (100 μM) for 1 hr then treated with gemcitabine (5 nM). Cells were harvested at 3, 6, 12, and 24 hrs after treatment. Total RNA was extracted using TRIzol regent (Life Technologies, CA, USA) and reverse transcription was performed using AMV reverse transcriptase (Promega, WI, USA). The real-time PCR reaction was carried out in Rotor-Gene Q (QIAGEN, Hilden, Germany) with SYBR Green PCR Kit (QIAGEN, Hilden, Germany). Primers used for real-time PCR reaction are listed in Table 1. The PCR amplification process consisted of an initial activation step of 95° C for 5 min and 40 cycles of 95° C for 15 s; 60° C for 30 s; and 72° C for 5 min.

Gene	Primer sequence (5′-3′)		Size
MyH9	Forward Reverse	AGAGCTCACGTGCCTCAACG TGACCACACAGAACAGGCCTG	101 bp
MyoD	Forward Reverse	ACGACTGCTTTCTTCACCACTCCT TCGTCTTAACTTTCTGCCACTCCG	136 bp
Myogenin	Forward Reverse	ACAGCATCACGGTGGAGGATATGT CCCTGCTACAGAAGTGATGGCTTT	131 bp

Table 1. List of primers

Western blot analysis

C2C12 cells (1×10^5/dish) were seeded in 60ø dish and were incubated overnight for stabilization. Cells were pretreated with GV1001 (100 μM) for 1 hr, and then treated with gemcitabine (5 nM). After 48 hrs, cells were lysed and proteins were extracted using lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktails (Sigma). Protein concentration was measured by BCA Assay. An equal amount of protein (15 μg /sample) was dissolved in a 10% polyacrylamide-SDS gel with 50-80 V for 4 hrs and transferred onto a nitrocellulose membrane with 400 mA for 1 hr. To prevent nonspecific binding, blocking was performed with 5% nonfat milk containing 0.05% Tween 20 (PBST) for 1 hr at room temperature (RT) overnight at 4° C. Membranes were then incubated with primary antibodies diluted with 1% nonfat milk in PBST. Primary antibodies used were anti-MyoD Ab (1:200; Santa Cruz Biotechnology, TX, USA), anti-Myogenin Ab (1:250; Abcam, Cambridge, UK) and anti-β-actin (1:5,000; Sigma, MO, USA). After washing 3 times (5 min/each) with PBST, membranes were incubated with secondary antibodies diluted with 1% nonfat milk in PBST: horse

radish peroxidase (HRP)-conjugated anti-rabbit IgG Ab (1:5,000; Cell signaling Technology, MA, USA) for MyoD and HRP-conjugated anti-mouse IgG Ab (1:5,000; Cell signaling Technology, MA, USA) for Myogenin and β-actin for 1 hr at RT. Membranes were washed again 3 times (5 min/each) with PBST and immunoreactive proteins were visualized with EZ-Western Lumi LA (Dogen, Korea). The bands were analyzed for their densities using the Image J software (NIH, MD, USA). Results were expressed as relative intensity and each band was adjusted to that of β-actin.

Enzyme-Linked Immunosorbent Assay (ELISA)

C2C12 cells ($1x10^5$) and hSkM cells ($2.4x10^5$) were cultured in 6-well culture plates for 72 hrs until they reached 80-95% confluency. The medium was then replaced with DMEM supplemented with 2% horse serum (GIBCO, NY, USA) to induce differentiation. After 72 hr, cells were pretreated with GV1001 (100 μM) for 1 hr, treated with 20 ng/mL recombinant TNF-α (R&D systems, CA, USA) and 100 U/mL recombinant IFN-γ (R&D systems, CA, USA). Cell supernatants were collected after 48 hrs of incubation. The concentration of myostatin in the

culture supernatants was measured using an ELISA kit (R&D systems, CA, USA) according to the manufacturer's instruction and the relative absorbance was measured at 450 nm using the SoftmaxPro software (Molecular Devices, CA, USA).

Animals

Six-week-old male BALB/c nude mice (n = 65) were purchased from Orient Bio (Gyeonggi-do, Korea). Mice were housed under specific-pathogen-free conditions in the animal facility at the Seoul National University College of Medicine with the animal experiment protocols reviewed and approved by the IACUC at Seoul National University Hospital (IACUC No. 13-0717).

Establishment of pancreatic cancer xenograft model and treatment

PANC-1 and AsPC-1 cells ($1x10^6$/50 μL PBS) were subcutaneously (s.c.) inoculated with 50 μL Matrigel (BD Biosciences, NJ, USA) in both flanks. The mice were randomly divided into groups of 5: (i) negative control; (ii) control group with tumor inoculation; (iii) scramble peptide (daily s.c. injection of 50 μg/each), (iv) GV1001 (daily s.c. injection of 50 μg/each), (v) gemcitabine (i.p. injection of 125 mg/kg every 3 days), (vi)

gemcitabine and scramble, and (vii) gemcitabine and GV1001. Treatment started after 2 weeks of tumor inoculation when subcutaneous solid tumors became palpable (approximately 60 mm^3) and were maintained for 3 weeks.

Measurement of tumor size, body weight, muscle weight and food intake

Body weight, food intake, and tumor size were measured every 3 days using an electronic scale and a Vernier caliper. Each cages were given known amount of γ-ray-irradiated laboratory rodent chow and the changes in the weight were measured. Tumor volume (V) was calculated by the following formula: $V=(w^2 \times l)/2$, where w is the width and l is the length of the tumor. Hind-limb muscles were isolated from mice after they were sacrificed and the weights of gastrocnemius, quadriceps, and tibialis anterior were measured with an electronic scale.

Statistical analysis

Data are expressed as mean ± SDs. Unpaired two-tailed t test was used to compare two groups. Differences were considered statistically signigicant for P-value ≤ 0.05. Statistical analyses

were carried out using GraphPad InStat version 5.01 (GraphPad Software, CA, USA).

RESULTS

1. GV1001 treatment slightly alleviates cytotoxic effect and inhibition of cell proliferation caused by gemcitabine

The optimal dose of GV1001 and gemcitabine that does not have cytotoxicity or has minimal cytotoxicity was determined by dose titration. C2C12 cells were treated with GV1001 and gemcitabine ranging from 0 to 100 μM and 0 to 100 nM respectively. After 72 hrs of incubation, cytotoxicity was measured. GV1001 dose up to 100 μM did not affect viability, proliferation, and toxicity of C2C12 cells (Fig. 1A), while gemcitabine showed significantly toxic effects from 10 nM (Fig. 1B). Therefore, 100 μM GV1001 and 5 nM gemcitabine were used in the following experiments unless otherwise specified. To examine whether GV1001 overturns cytotoxicity caused by gemcitabine, C2C12 cells were co-treated with GV1001 and gemcitabine. As in Fig. 1C, the adverse effect by 10 and 25 nM gemcitabine on cells was too severe for GV1001 to reverse the response. When C2C12 cells were treated with 100 μM GV1001 and 5 nM gemcitabine, the outcome was nearly significant (Fig.

1D).

To investigate closer into the proliferation aspect of C2C12 cells, cell cycle analysis was performed. Addition of 5 nM gemcitabine into the media resulted in the suppression of S-phase of the cell cycle in both 24 hrs (Fig. 2A) and 48 hrs (Fig. 2B) of treatment. Co-treatment with 5 nM gemcitabine and 100 μM GV1001 helped C2C12 cells to recover their S-phase to the control level. The results from Fig. 1 and Fig. 2 indicate that gemcitabine reduces the proliferation of C2C12 cells in a dose-dependent manner and GV1001 effectively reverses the effect.

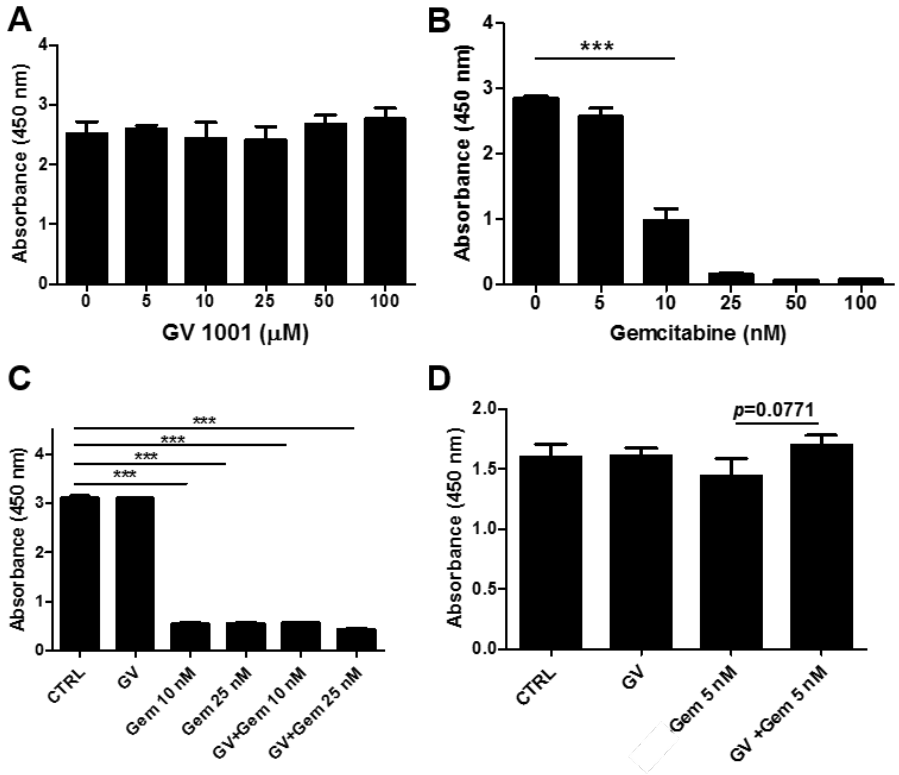


Fig. 1 The effect of GV1001 and gemcitabine on the proliferation and cytotoxicity of C2C12 cells

C2C12 cells ($1x10^3$/well) were seeded in quintuplicates and were treated with indicated amount of GV1001 (A) and gemcitabine (B) for 72 hrs. 100 μM GV1001 was treated in panels (C) and (D). Results are representative of three independent experiments. Each sample is in triplicates and data are presented as the means ± SD. *** $p \leq 0.0001$

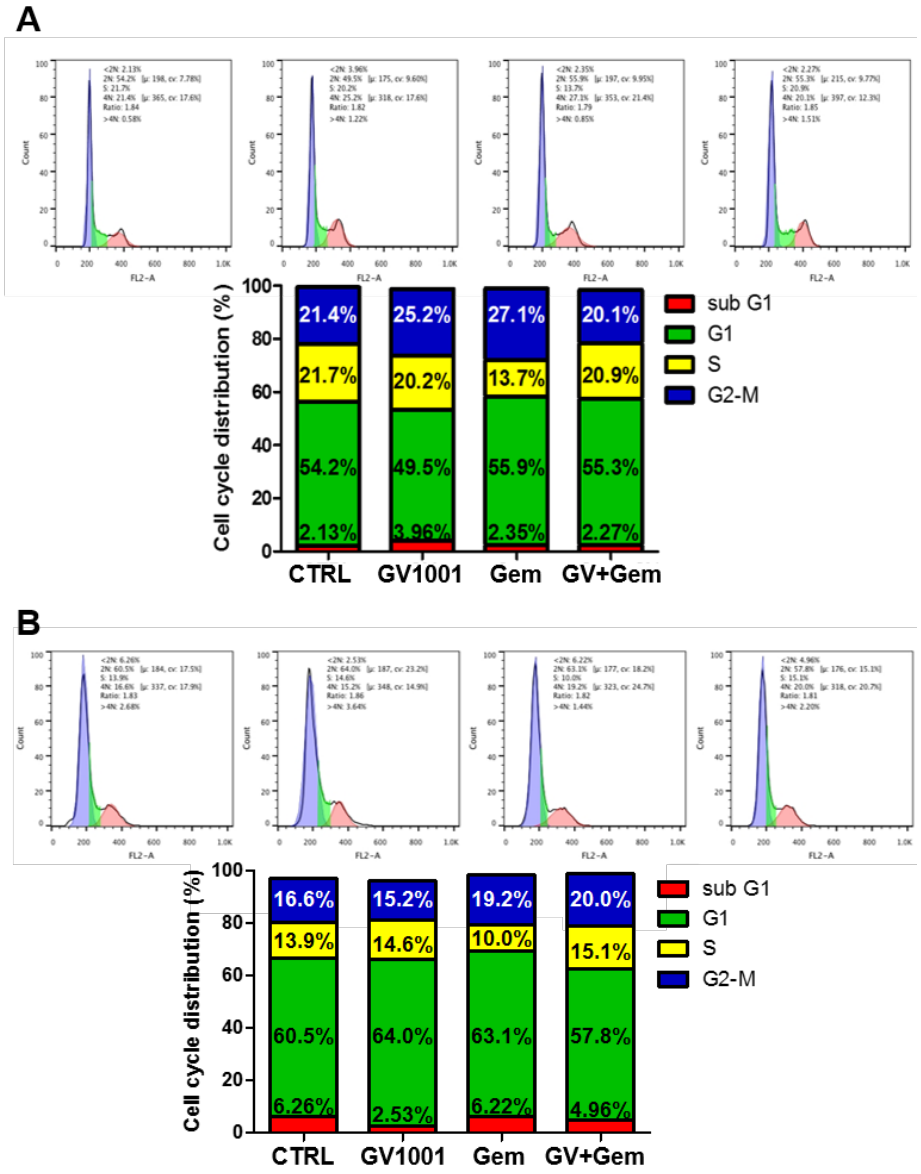


Fig. 2 Cell cycle analysis of C2C12 with GV1001 and gemcitabine treatment

(A) C2C12 cells ($2x10^5$) were pretreated with GV1001 (100 μM) for 1 hr then treated with gemcitabine (5 nM). After 24 hr cells were examined for cell cycle as described in *Materials and*

Methods. (A) C2C12 cells ($1x10^5$) were prepared in the same manner and harvested 48 hrs after for analysis. Results are representative of three independent experiments.

2. The mRNA and protein levels of myogenesis markers were elevated by GV1001

Myogenin, MyoD, and MyH9 are positive regulators of myogenesis, the formation of skeletal muscle. The expression of myogenin turns on the transition from proliferating myoblasts to differentiating myotubes (30). MyoD deficient mice exhibits increased myopathy and displays reduced capacity to repair damage, which suggests MyoD is required for efficient regeneration of skeletal muscle (31). Reports have elucidated that cachectic factors display a remarkably high degree of selectivity for the downregulation of myosin heavy chain (32). Therefore, whether treatment with gemcitabine and GV1001 alters the expressions of the regulators of myogenesis was assessed at time intervals of 3, 6, 12, and 24 hrs. Although, the time of activation of the myogenic factor genes were different, all three genes were upregulated with GV1001 treatment. Interestingly, GV1001 treatment alone upregulated the expression of MyH9, which implies that GV1001 imposes positive impact on the differentiation of C2C12 cells.

The expression of myogenic markers was examined in the protein level as well. Western blot analysis revealed that the

upregulation of myogenin was nearly statistically significant with GV1001 treatment (Fig. 4A). MyoD was downregulated with 5 nM gemcitabine treatment, while its expression was significantly recovered when treated with 100 μM GV1001 (Fig. 4B).

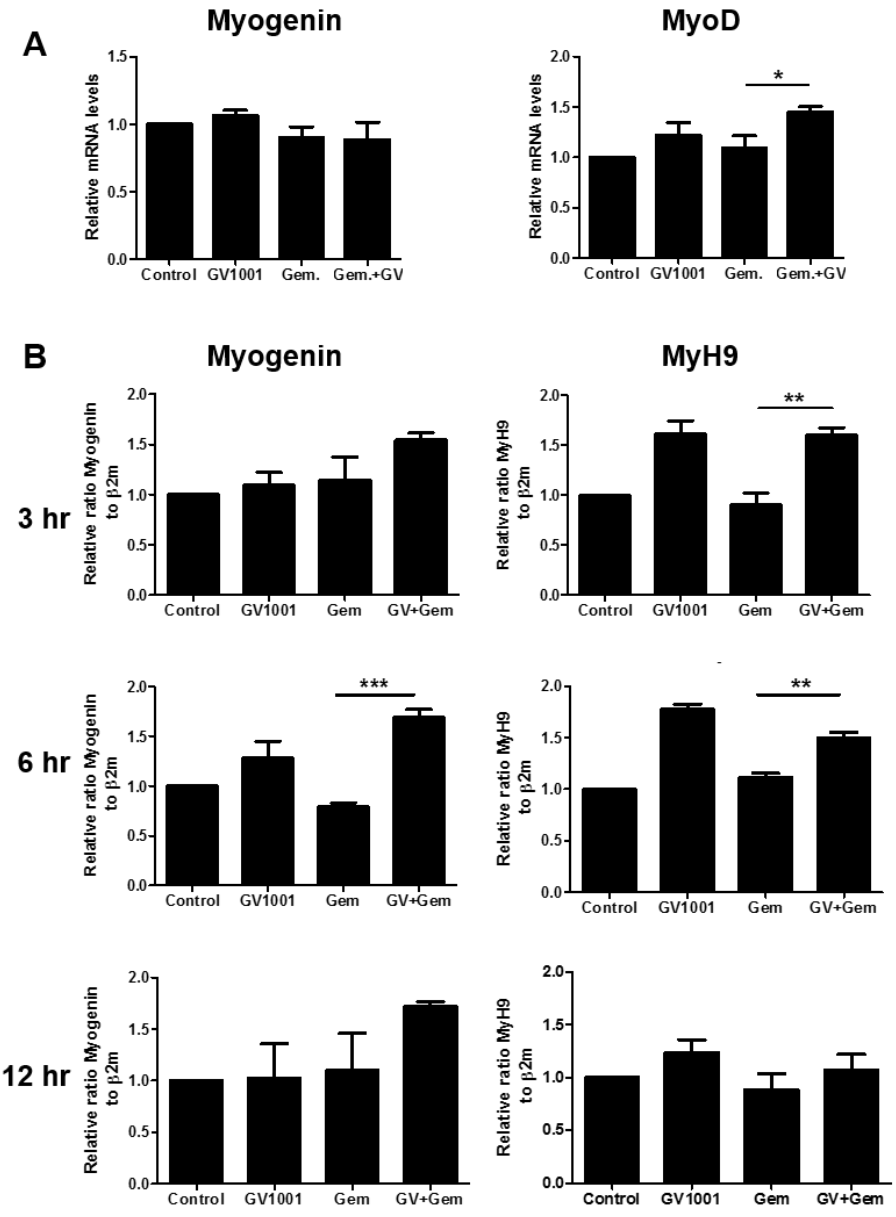


Fig. 3 The mRNA level of myogenesis markers in C2C12 cells treated with GV1001 and gemcitabine

C2C12 cells ($3x10^5$/well) were pretreated with GV1001 (100 μM) for 1 hr then treated with gemcitabine (5 nM). (A) Cells were

harvested at 24 hrs after treatment and real-time PCR was performed using the cDNA obtained from each sample. Real-time PCR was performed using primers for myogenin (left panel) and myoD (right panel). (B) Cells were harvested at 3 (upper panel), 6 (middle panel), and 12 hrs (lower panel) after treatment and real-time PCR was performed using primers for myogenin and MyH9. Results are representative of three independent experiments. Each sample is in triplicates and data are presented as the means ± SD. (* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$)

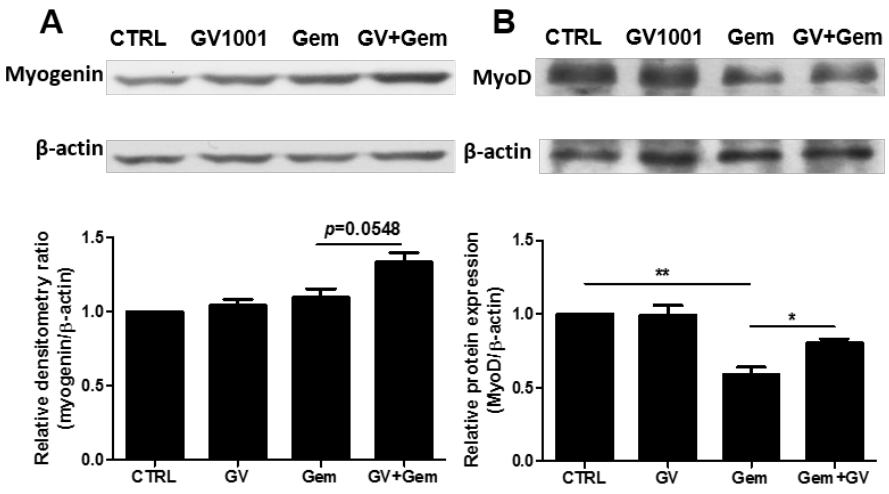


Fig. 4 The protein level of myogenesis markers in C2C12 cells treated with GV1001 and gemcitabine

C2C12 cells (1×10^5/dish) were pretreated with GV1001 (100 μM) for 1 hr, and then treated with gemcitabine (5 nM) for 48 hrs. The protein expression of myogenin (A) and myoD (B) was examined by immunoblotting. The results were expressed as relative fold change against control and were adjusted to corresponding β−actin bands. Results are representative of three independent experiments and data are presented as the means ± SD. (* $p \leq 0.05$, ** $p \leq 0.001$)

3. GV1001 relieves the increase in protein level of myostatin caused by TNF-α and IFN-γ

Myostatin, a member of the transforming growth factor-β (TGF-β) superfamily, acts as a negative regulator of muscle mass by promoting cell cycle arrest. Previously published results show that systemic overexpression of myostatin in mice induces muscle and fat loss analogous to that seen in human cachexia syndromes (33). Lack of myostatin, not surprisingly, induces excessive skeletal muscle growth (33-36). It has been reported that both myostatin mRNA and protein significantly increase in C2C12 cells incubated with TNF-α (37).

To address the question whether GV1001 has an effect on the level of myostatin of C2C12 cells, C2C12 cells were incubated with TNF-α, IFN-γ, and GV1001. After incubation, the cell culture supernatants were collected and analyzed for myostatin concentration by ELISA. The results confirm that when cells were incubated with TNF-α and/or IFN-γ, there was an increase in the level of myostatin as shown in Fig. 6A. Furthermore, the level of myostatin significantly subsided with GV1001 treatment in both cases where C2C12 cells were treated with TNF-α and/or IFN-γ. Interestingly, co-incubation with

TNF−α and IFN−γ did not act synergistically to enhance the production of myostatin. The cell culture supernatant harvested from human skeletal muscle cell, hSkM, in the same scheme demonstrated a similar pattern although not statistically significant (Fig. 5B).

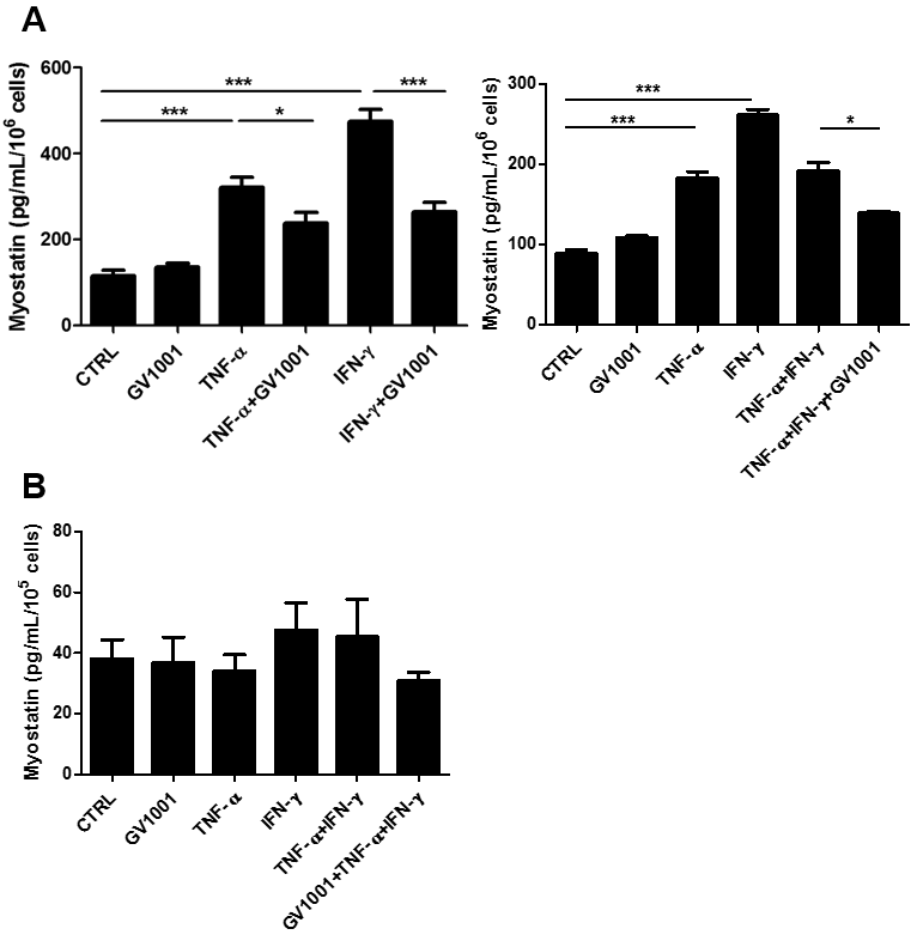


Fig. 5 The protein level of myostatin, the negative regulator of myogenesis, in TNF-α, IFN-γ, and GV1001 treated muscle cells

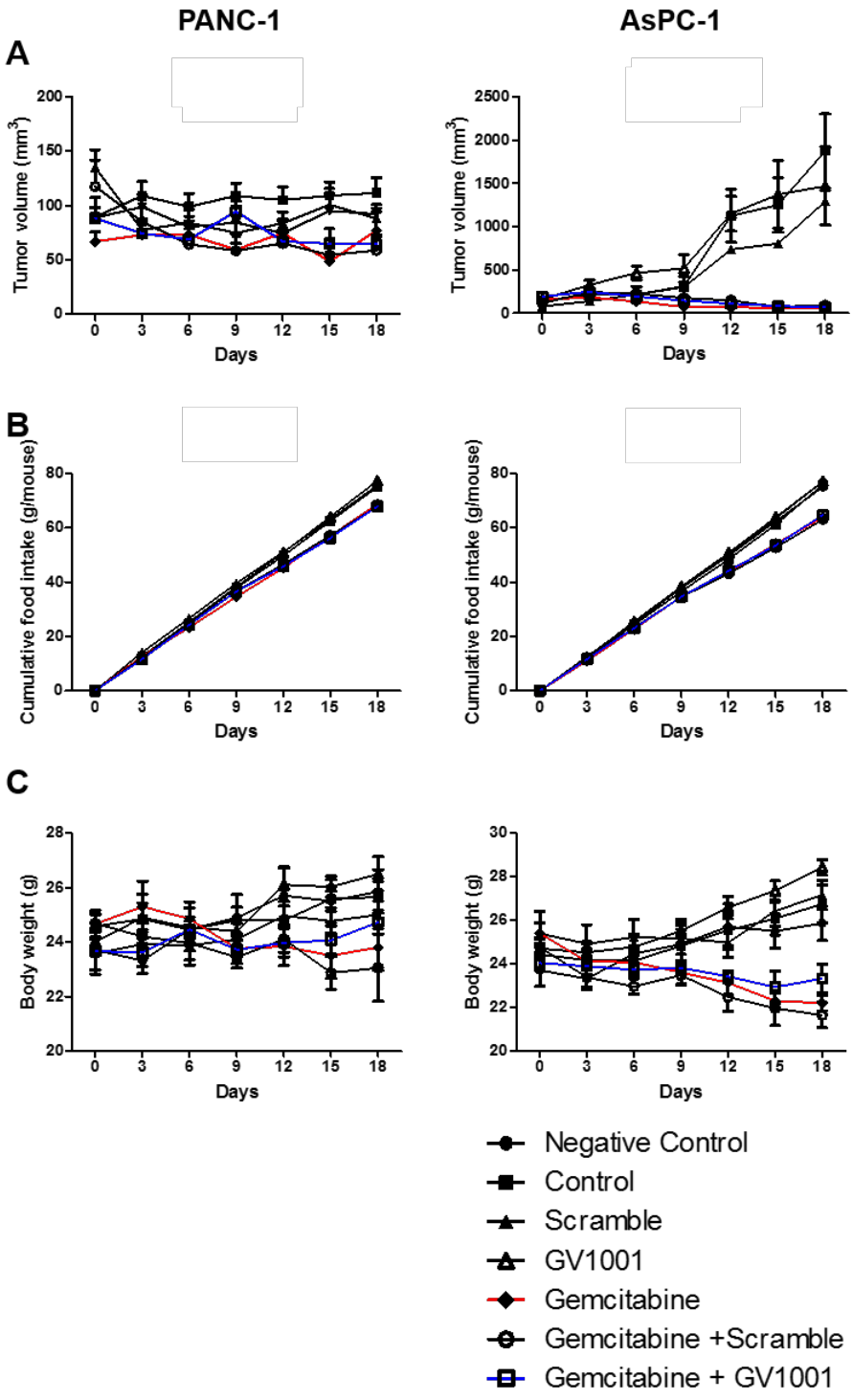
C2C12 cells ($1x10^5$) (A) and hSkM cells ($2.4x10^5$) (B) were cultured until they reached 80-95% confluency. The medium was then replaced with 2% horse serum-supplemented DMEM to induce differentiation. Cells were pretreated with GV1001 (100 μM) for 1 h, treated with recombinant TNF-α (20 ng/mL)

and recombinant IFN-γ (100 U/mL). Cell supernatants were collected after 48 hrs and the level of myostatin was measured by ELISA as specified in *Materials and Methods*. Each sample is in triplicates and results are representative of three independent experiments. Data are presented as the means ± SD. (* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$)

4. Gemcitabine-induced weight and muscle loss was prevented by combined treatment with GV1001 in BALB/c nude mice pancreatic cancer xenograft model

A gemcitabine-induced cachectic pancreatic cancer model was established by intraperitoneal injection of 125 mg/kg gemcitabine on 3-day intervals. Following the administration of gemcitabine, tumor sizes dramatically decreased in xenograft model established by AsPC-1 cells (Fig. 6A). On the other hand, tumor sizes of PANC-1 group were comparably smaller than those of AsPC-1 group and were less effected by gemcitabine treatment. It may be caused by different tumorigenicity between cancer cell lines. The differences in tumor sizes of gemcitabine and GV1001+gemcitabine group were minimal, signifying that GV1001 treatment did not have direct inhibitory role in tumor growth. Regardless of the pancreatic cancer cell inoculated, all six groups injected with gemcitabine showed declined food intake (Fig. 6B). Subcutaneous injection of GV1001 did not inhibit gemcitabine-induced anorexia, meaning that body weight changes shown in Fig. 6C is not due to food intake differences. Body weights measured every third day exhibited differences between the gemcitabine group and GV1001+gemcitabine group. Body weight loss was prominent among mice that were administered gemcitabine. Compared to the lesser body weights than those of gemcitabine group on Day 0, GV1001+gemcitabine group gained more weight as the experiment progressed. The experiment had to be terminated at Day 21 since the sizes of AsPC-1 solid tumors have overgrown to a point where the ethics of the experiment may be questioned. If the body weight

measurement kept going, the gaps between the two groups may have been greater. Muscle weights from gastrocnemius, quadriceps, and tibialis anterior were found to be heavier in the negative control group (Fig. 6D). Tumor development alone was sufficient to reduce muscle mass, a hallmark of cachexia. Mice given gemcitabine and GV1001 together had greater muscle weight than those given gemcitabine only. Taken together, GV1001 effectively inhibited body weight and muscle weight losses from gemcitabine treatment.


PANC-1
AsPC-1
A
Tumor volume (mm3)
Days
B
Cumulative food intake (g/mouse)
Days
C
Body weight (g)
Days
Negative Control
Control
Scramble
GV1001
Gemcitabine
Gemcitabine +Scramble
Gemcitabine + GV1001

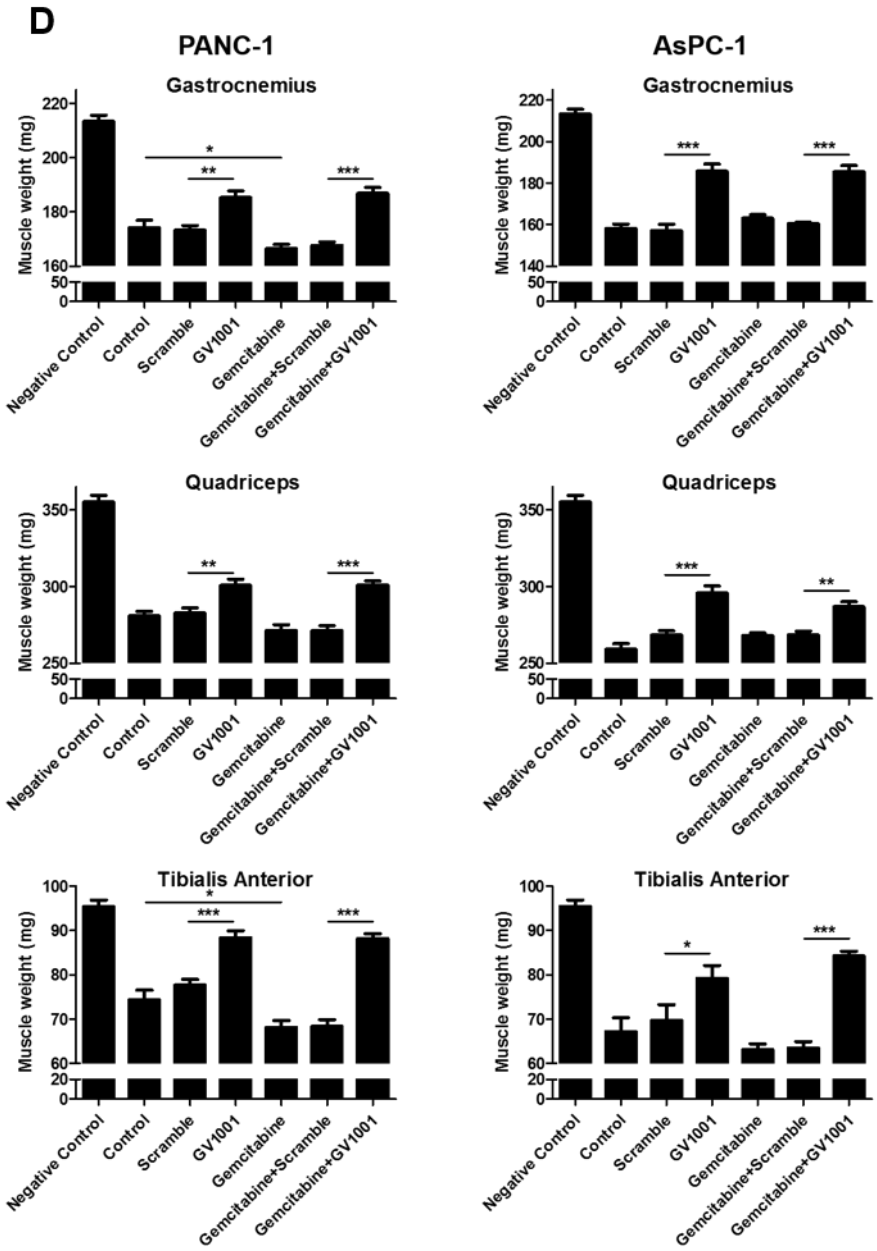

D
PANC-1
AsPC-1
Gastrocnemius
Quadriceps
Tibialis Anterior
Muscle weight (mg)
Negative Control
Control
Scramble
GV1001
Gemcitabine
Gemcitabine+Scramble
Gemcitabine+GV1001

Fig.6 The effect of GV1001 on gemcitabine-induced cachexia in pancreatic cancer xenograft model in BALB/c nude mice

Six-week-old male BALB/c nude mice were subcutaneously inoculated with PANC-1 cells ($1x10^6$) (left panel) and AsPC-1 cells ($1x10^6$) (right panel) in both flanks. Mice were randomly assigned one of the following groups: (i) negative control (no tumor inoculation, closed circle), (ii) control (tumor inoculated, closed square), (iii) scramble (closed triangle), (iv) GV1001 (open triangle), (v) gemcitabine (closed diamond, highlighted in red), (vi) gemcitabine and scramble (open circle), and (vii) gemcitabine and GV1001 (open square, highlighted in blue). Scramble peptide (daily s.c. injection of 50 μg/each), GV1001 (daily s.c. injection of 50 μg/each), and gemcitabine (every 3 days i.p. injection of 125 mg/kg) treatment started 2 weeks after tumor inoculation when tumor sizes were approximately 60 mm^3. Treatment was maintained for 3 weeks. *In vivo* experiments were ceased when xenografts were considered to have approached their ethical endpoints. Food intake (A), tumor size (B), and body weight (C) were measured every 3 days. The weight of hind leg muscles [D, gastrocnemius (upper panel), quadriceps (middle panel), and tibialis anterior (lower panel)]

was obtained after mice were sacrificed. Data are presented as the means ± SD. (* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$)

DISCUSSION

There has been attempts in search of potential targets and effective treatment for cachexia. Different studies have emphasized that a single therapy may not be completely successful in the treatment of cachexia. Since nutritional supplementation alone has failed to fully reverse the condition, multimodal strategies such as combination of nutritional intervention and metabolic management should be taken into consideration (38, 39). Drugs that have anti-inflammatory properties (e.g., suramin, cannabinoids) and/or that increase muscle mass and physical performance (e.g., blockade of myostatin), appetite enhancers (thalidomide, megastrol acetate), and hormonal control (e.g., ghrelin, dopamine, melatonin) are being studied (20, 40). Injection of ActRIIB antagonist to tumor-bearing mice reverses muscle loss, cardiac atrophy, and anorexia. It also extends lifespan even without reducing tumor growth, stimulates muscle regeneration, and suppresses ubiquitin-proteasome system activity in skeletal muscle (41). Potential

target that alter metabolic abnormalities associated with cachexia will improve clinical conditions.

Loss of muscle mass is critical in cancer patients since it limits the ability to perform daily activities and consequently compromises the patient's quality of life. Physical exercise may seem a plausible treatment that increases both muscle strength and endurance (42, 43). Unfortunately, however, chronic asthenia that follows cachexia, is a confounding factor that limits exercise practice. Therefore, we should change our view to the molecular scale. Due to changes in protein metabolism, physical exercise alone does not reverse cachectic symptoms. Increased muscle protein breakdown and downregulation of muscle differentiation are features of muscular abnormalities (44). The main pathway contributing to skeletal muscle loss during cachexia is degradation of intracellular skeletal muscle proteins by ubiquitin-proteasome pathway (45).

There are reports concerning the pro-inflammatory environment created by gemcitabine treatment (9, 46, 47). C57BL/6J mice that received gemcitabine (120 mg/kg) and thoracic irradiation had clearly higher expression of TNF-α, IL-1α, and IL-6 than did control mice. Increased expression of

pro-inflammatory cytokines then upregulate myostatin, which then inhibits myogenesis (37). Inflammation cytokines provide protection to the host by directly attacking proliferating tumor cells but they also evoke adverse effects on normal host tissues and cells. If the pro-inflammatory environment created by gemcitabine treatment can be modulated without affecting the anti-tumor effect, the use of gemcitabine can be broadened.

GV1001 is a telomerase peptide vaccine that has been studied in various preclinical trials. Lee et al. verified its ability to cross the cellular membrane. (48) Unlike gemcitabine, GV1001 did not show toxicity to skeletal muscle cells *in vitro* or *in vivo* and direct anti-tumor effect *in vivo*. Xenograft mice model showed that gemcitabine, gemcitabine+GV1001, and gemcitabine+scramble peptide had significant tumor reduction compared to other groups. Seen from the fact that GV1001 or scramble peptide treatment group did not have reduction in tumor sizes, the anti-tumor effect came from gemcitabine. Phase I/II clinical studies with GV1001 showed the prolonged survival and tolerability (49). Enhancing the quality of life of patients who would be suffering from chemotherapy-induced cachexia is essential.

This study showed novel data that correlates GV1001 with muscle generation. GV1001 does not disturb the anti-cancer effect of gemcitabine and effectively boosts the regeneration of skeletal muscle.

As a whole, GV1001 may be a useful chemotherapy adjuvant that blocks gemcitabine-induced muscle toxicity and gemcitabine-induced pro-inflammatory environment.

REFERENCES

1. Collins, A., and M. Bloomston. 2009. Diagnosis and management of pancreatic cancer. *Minerva gastroenterologica e dietologica* 55: 445.
2. Li, J., and M. W. Saif. 2009. Any progress in the management of advanced pancreatic cancer. *Jop* 10: 361-365.
3. Li, D., K. Xie, R. Wolff, and J. L. Abbruzzese. 2004. Pancreatic cancer. *The Lancet* 363: 1049-1057.
4. Fazal, S., and M. W. Saif. 2007. Supportive and palliative care of pancreatic cancer. *Jop* 8: 240-253.
5. Sakamoto, H., M. Kitano, Y. Suetomi, Y. Takeyama, H. Ohyanagi, T. Nakai, C. Yasuda, and M. Kudo. 2006. Comparison of standard-dose and low-dose gemcitabine regimens in pancreatic adenocarcinoma patients: a prospective randomized trial. *Journal of gastroenterology* 41: 70-76.
6. Ueno, H., K. Kiyosawa, and N. Kaniwa. 2007. Pharmacogenomics of gemcitabine: can genetic studies

lead to tailor-made therapy? *British journal of cancer* 97: 145-151.

7. Plunkett, W., P. Huang, Y.-Z. Xu, V. Heinemann, R. Grunewald, and V. Gandhi. 1995. Gemcitabine: metabolism, mechanisms of action, and self-potentiation. In *Seminars in oncology*. 3-10.

8. Plunkett, W., P. Huang, C. E. Searcy, and V. Gandhi. 1996. Gemcitabine: preclinical pharmacology and mechanisms of action. In *Seminars in oncology*. 3-15.

9. Friedlander, P. A., R. Bansal, L. Schwartz, R. Wagman, J. Posner, and N. Kemeny. 2004. Gemcitabine-related radiation recall preferentially involves internal tissue and organs. *Cancer* 100: 1793-1799.

10. Von Hoff, D. D., T. Ervin, F. P. Arena, E. G. Chiorean, J. Infante, M. Moore, T. Seay, S. A. Tjulandin, W. W. Ma, and M. N. Saleh. 2013. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *New England Journal of Medicine* 369: 1691-1703.

11. Neri, B., G. Cipriani, R. Grifoni, E. Molinara, P. Pantaleo, S. Rangan, A. Vannini, P. Tonelli, A. Valeri, and D. Pantalone. 2009. Gemcitabine plus irinotecan as first-line weekly

therapy in locally advanced and/or metastatic pancreatic cancer. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics* 17: 559-564.

12. Burris, H. r., M. J. Moore, J. Andersen, M. R. Green, M. L. Rothenberg, M. R. Modiano, M. C. Cripps, R. K. Portenoy, A. M. Storniolo, and P. Tarassoff. 1997. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *Journal of clinical oncology* 15: 2403-2413.

13. Mini, E., S. Nobili, B. Caciagli, I. Landini, and T. Mazzei. 2006. Cellular pharmacology of gemcitabine. *Annals of Oncology* 17: v7-v12.

14. Jiang, S.-M., J.-H. Wu, and L. Jia. 2012. Intervention of Mirtazapine on gemcitabine-induced mild cachexia in nude mice with pancreatic carcinoma xenografts. *World journal of gastroenterology: WJG* 18: 2867.

15. Fearon, K. C., A. C. Voss, D. S. Hustead, and C. C. S. Group. 2006. Definition of cancer cachexia: effect of weight loss, reduced food intake, and systemic inflammation on functional status and prognosis. *The American journal of*

clinical nutrition 83: 1345-1350.

16. Dodson, S., V. E. Baracos, A. Jatoi, W. J. Evans, D. Cella, J. T. Dalton, and M. S. Steiner. 2011. Muscle wasting in cancer cachexia: clinical implications, diagnosis, and emerging treatment strategies. *Annual review of medicine* 62: 265-279.

17. Penet, M.-F., and Z. M. Bhujwalla. 2015. Cancer cachexia, recent advances, and future directions. *The Cancer Journal* 21: 117-122.

18. Argilés, J. M., S. Busquets, R. Moore-Carrasco, and F. J. López-Soriano. 2006. The role of cytokines in cancer cachexia. In *Cachexia and Wasting: A Modern Approach*. Springer. 467-475.

19. Takahashi, Y., K. Yasumoto, and M. Mai. 2005. Chemotherapy under cachectic conditions and the possibility of cachexia-controlled chemotherapy. *Oncology reports* 14: 135-140.

20. Stewart, G. D., R. J. Skipworth, and K. C. Fearon. 2006. Cancer cachexia and fatigue. *Clinical medicine* 6: 140-143.

21. Bachmann, J., K. Ketterer, C. Marsch, K. Fechtner, H. Krakowski-Roosen, M. W. Büchler, H. Friess, and M. E.

Martignoni. 2009. Pancreatic cancerrelated cachexia: influence on metabolism and correlation to weight loss and pulmonary function. *BMC cancer* 9: 1.

22. Fearon, K. C., D. J. Glass, and D. C. Guttridge. 2012. Cancer cachexia: mediators, signaling, and metabolic pathways. *Cell metabolism* 16: 153-166.

23. Tan, C. R., P. M. Yaffee, L. H. Jamil, S. K. Lo, N. Nissen, S. J. Pandol, R. Tuli, and A. E. Hendifar. 2015. Pancreatic cancer cachexia: a review of mechanisms and therapeutics. *Risk Factors for Pancreatic Cancer: Underlying Mechanisms and Potential Targets*: 90.

24. Brunsvig, P. F., J. A. Kyte, C. Kersten, S. Sundstrøm, M. Møller, M. Nyakas, G. L. Hansen, G. Gaudernack, and S. Aamdal. 2011. Telomerase peptide vaccination in NSCLC: a phase II trial in stage III patients vaccinated after chemoradiotherapy and an 8-year update on a phase I/II trial. *Clinical Cancer Research* 17: 6847-6857.

25. Hunger, R. E., K. K. Lang, C. J. Markowski, S. Trachsel, M. Møller, J. A. Eriksen, A.-M. Rasmussen, L. R. Braathen, and G. Gaudernack. 2011. Vaccination of patients with cutaneous melanoma with telomerase-specific peptides.

Cancer Immunology, Immunotherapy 60: 1553-1564.

26. Shay, J., and W. Keith. 2008. Targeting telomerase for cancer therapeutics. *British journal of cancer* 98: 677-683.

27. Staff, C., F. Mozaffari, J.-E. Frödin, H. Mellstedt, and M. Liljefors. 2014. Telomerase (GV1001) vaccination together with gemcitabine in advanced pancreatic cancer patients. *International journal of oncology* 45: 1293-1303.

28. Brower, V. 2010. Telomerase-based therapies emerging slowly. *Journal of the National Cancer Institute* 102: 520-521.

29. Hiyama, E., T. Kodama, K. Shinbara, T. Iwao, M. Itoh, K. Hiyama, J. W. Shay, Y. Matsuura, and T. Yokoyama. 1997. Telomerase activity is detected in pancreatic cancer but not in benign tumors. *Cancer research* 57: 326-331.

30. Faralli, H., and F. J. Dilworth. 2012. Turning on myogenin in muscle: a paradigm for understanding mechanisms of tissue-specific gene expression. *Comparative and functional genomics* 2012.

31. Megeney, L. A., B. Kablar, K. Garrett, J. E. Anderson, and M. A. Rudnicki. 1996. MyoD is required for myogenic stem

cell function in adult skeletal muscle. *Genes & development* 10: 1173-1183.

32. Acharyya, S., K. J. Ladner, L. L. Nelsen, J. Damrauer, P. J. Reiser, S. Swoap, and D. C. Guttridge. 2004. Cancer cachexia is regulated by selective targeting of skeletal muscle gene products. *The Journal of clinical investigation* 114: 370-378.

33. Zimmers, T. A., M. V. Davies, L. G. Koniaris, P. Haynes, A. F. Esquela, K. N. Tomkinson, A. C. McPherron, N. M. Wolfman, and S.-J. Lee. 2002. Induction of cachexia in mice by systemically administered myostatin. *Science* 296: 1486-1488.

34. McPherron, A. C., A. M. Lawler, and S.-J. Lee. 1997. Regulation of skeletal muscle mass in mice by a new TGF-p superfamily member.

35. Lin, J., H. B. Arnold, M. A. Della-Fera, M. J. Azain, D. L. Hartzell, and C. A. Baile. 2002. Myostatin knockout in mice increases myogenesis and decreases adipogenesis. *Biochemical and biophysical research communications* 291: 701-706.

36. Thomas, M., B. Langley, C. Berry, M. Sharma, S. Kirk, J.

Bass, and R. Kambadur. 2000. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *Journal of Biological Chemistry* 275: 40235-40243.

37. Zhang, L., V. Rajan, E. Lin, Z. Hu, H. Han, X. Zhou, Y. Song, H. Min, X. Wang, and J. Du. 2011. Pharmacological inhibition of myostatin suppresses systemic inflammation and muscle atrophy in mice with chronic kidney disease. *The FASEB Journal* 25: 1653-1663.

38. Bauer, J. D., and S. Capra. 2005. Nutrition intervention improves outcomes in patients with cancer cachexia receiving chemotherapy—a pilot study. *Supportive care in cancer* 13: 270-274.

39. Chlebowski, R. T. 1985. Critical evaluation of the role of nutritional support with chemotherapy. *Cancer* 55: 268-272.

40. von Haehling, S., and S. D. Anker. 2015. Treatment of cachexia: an overview of recent developments. *International journal of cardiology* 184: 736-742.

41. Zhou, X., J. L. Wang, J. Lu, Y. Song, K. S. Kwak, Q. Jiao, R. Rosenfeld, Q. Chen, T. Boone, and W. S. Simonet. 2010.

Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. *Cell* 142: 531–543.

42. Aulino, P., E. Berardi, V. M. Cardillo, E. Rizzuto, B. Perniconi, C. Ramina, F. Padula, E. P. Spugnini, A. Baldi, and F. Faiola. 2010. Molecular, cellular and physiological characterization of the cancer cachexia–inducing C26 colon carcinoma in mouse. *BMC cancer* 10: 363.

43. Argilés, J. M., S. Busquets, F. J. López-Soriano, P. Costelli, and F. Penna. 2012. Are there any benefits of exercise training in cancer cachexia? *Journal of cachexia, sarcopenia and muscle* 3: 73–76.

44. Bruera, E., and C. Sweeney. 2000. Cachexia and asthenia in cancer patients. *The lancet oncology* 1: 138–147.

45. Mueller, T. C., J. Bachmann, O. Prokopchuk, H. Friess, and M. E. Martignoni. 2016. Molecular pathways leading to loss of skeletal muscle mass in cancer cachexia–can findings from animal models be translated to humans? *BMC cancer* 16: 1.

46. ARDAVANIS, A. S., G. N. IOANNIDIS, and G. A. RIGATOS. 2005. Acute myopathy in a patient with lung

adenocarcinoma treated with gemcitabine and docetaxel. *Anticancer research* 25: 523-525.

47. Rübe, C. E., F. Wilfert, D. Uthe, J. König, L. Liu, A. Schuck, N. Willich, K. Remberger, and C. Rübe. 2004. Increased expression of pro-inflammatory cytokines as a cause of lung toxicity after combined treatment with gemcitabine and thoracic irradiation. *Radiotherapy and oncology* 72: 231-241.

48. Lee, S.-A., B.-R. Kim, B.-K. Kim, D.-W. Kim, W.-J. Shon, N.-R. Lee, K.-S. Inn, and B.-J. Kim. 2013. Heat shock protein-mediated cell penetration and cytosolic delivery of macromolecules by a telomerase-derived peptide vaccine. *Biomaterials* 34: 7495-7505.

49. Bernhardt, S., M. Gjertsen, S. Trachsel, M. Møller, J. Eriksen, M. Meo, T. Buanes, and G. Gaudernack. 2006. Telomerase peptide vaccination of patients with non-resectable pancreatic cancer: a dose escalating phase I/II study. *British journal of cancer* 95: 1474-1482.

국 문 초 록

Gemcitabine에 의해 유도된 췌장암 악액질에 미치는 GV1001의 예방 효과

서울대학교 의과대학

해부학 전공 전 재 은

사람 telomerase 역전사 효소 (hTERT) 서열에서 유래된 GV1001은 췌장암 및 여러 암에 대한 항암효과를 보이는 단백질 백신이다. 임상 결과에 따르면 췌장암 환자에서 GV1001의 면역원성을 보이며, gemcitabine과의 synergy 효과와 함께 여러 긍정적인 면역 조절 영향을 보인다고 보고된 바 있다. GV1001이 암환자의 체중 감소를 막고 gemcitabine 치료 후 일반적인 상태를 호전시킨다고 보고되었지만 현재까지는 증거가 불충분하다. 따라서, gemcitabine에 의한 체중 감소에 대한 GV1001의 예방 효과를 골격근 세포주와 이종 이식 동물 모델에서 보고자 하였다. Gemcitabine을 투여 받은 쥐들에서는 몸무게와 근육 무게가 현저하게 감소하였고, gemcitabine과 GV1001를 혼합 복용한 경우, 감소되었던 무게가 회복됨이 관찰되었다. 더불어, 골격근 세포의 증식과 분화를 생체 외 실험을 통해 보았을 때, gemcitabine만이

처리된 경우, 세포 분열 주기의 S기와 근육 형성이 mRNA와 단백질 수준에서 감소된 반면 GV1001와 gemcitabine의 혼합 요법으로 의미 있게 회복됨을 확인하였다. 따라서 GV1001이 gemcitabine에 의한 골격근 증식 및 분화 감소, 그리고 몸무게 및 근육 무게 감소를 효과적으로 막는다는 것을 알 수 있다.

주요어: 악액질, gemcitabine, GV1001, 근손실

학 번: 2014-22994