



Antitumor activity and pharmacokinetic properties of ARS-interacting multi-functional protein 1 (AIMP1/p43)

Jung Min Han^{a,b,1}, Heejuon Myung^{c,1}, Sunghoon Kim^{a,*}

^a Center for Medicinal Protein Network and Systems Biology, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

^b Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

^c Department of Bioscience and Biotechnology, Hankuk University of Foreign Studies, Yong-In, Kyung-Gi 449-791, Republic of Korea

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ABSTRACT

Although AIMP1 was identified as a component of the macromolecular aminoacyl tRNA synthetase complex involved in the cellular translation process, it was also found to be secreted as a cytokine having complex physiological functions. Among these, AIMP1's angiostatic and immune stimulating activities suggest its potential use as a novel antitumor therapeutic protein. Here we evaluated its antitumor efficacy in a mouse xenograft model bearing human stomach cancer cells. Intravenous injection of recombinant AIMP1 for 6 days resulted in significant decreases in both tumor volume and weight. Tumor volume decreased 31.1% and 54.0% when treated with AIMP1 at a concentration of 2 mg/kg and 10 mg/kg, respectively. Tumor weight decreased 29.1% and 52.2% when treated with AIMP1 at a concentration of 2 mg/kg and 10 mg/kg, respectively. Proliferating cell nuclear antigen (PCNA) staining of tumor tissues from AIMP1-treated mice (at both 2 mg/kg and 10 mg/kg) showed a 53% reduction of cells exhibiting an active cell cycle progression. Blood levels of tumor-suppressing cytokines such as TNF- α and IL-1 β increased in AIMP1-treated mice, whereas IL-12p40 and IFN- γ levels remained unaltered. Thus, this work suggests that AIMP1 may exert its antitumor activity by inducing tumor-suppressing cytokines. In a pharmacokinetic study in rats after a single intravenous injection, AIMP1 exhibited a low clearance showing a one-compartmental disposition. However, due to a low volume of distribution, AIMP1 had a short half-life of 0.1 h. In a serum stability test, AIMP1 showed a half life of >60 min in human serum, 52 min in dog serum and 32 min in rat serum.

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1. Introduction

AIMP1 was first identified as a component of the mammalian macromolecular tRNA synthetase complex [1]. Since our discovery of extracellular secretion of AIMP1, we have unveiled various functions related to secreted AIMP1, including pro-inflammatory activity [2], apoptosis of endothelial cells [3], fibroblast proliferation [4], and a hormonal activity for glucose homeostasis [5].

Angiogenesis, the process by which new blood vessels are formed, is considered to be a promising target for cancer treatment [6,7]. Many angiostatic agents including endostatin, turnstatin, angiostatin, thrombospondin-1, platelet factor-4, and the 16 kDa N-terminal fragment of prolactin have been evaluated over the last several years and some of these have been approved for the treatment of cancer [8]. Modulation by cytokines is another way to control tumor cell growth. For example, tumor necrosis factor (TNF)- α was reported to induce necrosis of subcutaneous tumors when administered systemically [9]. Interleukin (IL)-1 β is another cytokine reported to eradicate tumor cells at a low dose via local inflammation [10]. IL-12 was reported to induce an antiangiogenic program

* Corresponding author. Tel.: +82 2 880 8180; fax: +82 2 875 2621.

E-mail address: sungkim@snu.ac.kr (S. Kim).

¹ These authors contributed equally to this work.

mediated by IFN- γ -inducible genes and by lymphocyte-endothelial cell crosstalk [11]. Interferon (IFN)- γ was reported to act as an angiostatic cytokine by reducing the secretion of pro-angiogenic IL-8 [12]. Since AIMP1 induces both endothelial cell death and the production of immunity-promoting cytokines, it is expected to control tumor growth via a dual mechanism.

We previously attempted to evaluate AIMP1's potential as an anti-cancer agent in a xenograft mouse model with stomach cancer cells. In these experiments, purified recombinant AIMP1 was systemically delivered into mice via intraperitoneal injection at a dose of 25–50 mg/kg alone or in combination with paclitaxel (5 mg/kg). We found that AIMP1 treatment alone did not cause a significant tumor suppressive effect although 84% and 94% decreases in cancer growth were observed when combined with a low dose of paclitaxel [13].

To see whether treatment with AIMP1 alone can generate sufficient tumor suppressive activity, here we decided to try intravenous injection. While monitoring the antitumor activity of AIMP1, we also examined how it would affect tumor angiogenesis and immune response. In addition, we evaluated various pharmacokinetic parameters of AIMP1 in rats.

2. Materials and methods

2.1. Purification of AIMP1

AIMP1 was purified in its native form. pET23C expressing AIMP1 without a histidine tag was overexpressed in *Escherichia coli* BL21(DE3) by adding IPTG at a concentration of 1 mM. Cells were collected via centrifugation at 8000g for 10 min, resuspended in a suspension buffer (20 mM Tris, pH 7.5, 1.0 M NaCl, 0.1 mM EDTA, 2 mM dithiothreitol) and lysed using a homogenizer. Nucleic acids were removed by addition of 10% polyethyleneimine to a final concentration of 0.5%. The precipitates were collected by centrifugation at 15,000g for 20 min. The supernatant was recovered, diluted by addition of a 4 \times volume of dilution buffer (20 mM Tris, pH 7.5, 0.1 mM EDTA, 2 mM DTT) and loaded onto an SP SepharoseTM column (GE Healthcare). The column was washed with an equal volume of washing buffer (20 mM Tris, pH 7.5, 0.1 mM EDTA, 0.2 M NaCl, 2 mM DTT). The active fraction was eluted with elution buffer (20 mM Tris, pH 7.5, 0.1 mM EDTA, 0.33 M NaCl, 2 mM DTT). The eluted sample was loaded on a Heparin-SepharoseTM column (GE Healthcare) and the column was washed with an equal volume of washing buffer (20 mM Tris, pH 7.5, 0.1 mM EDTA, 0.3 M NaCl, 2 mM DTT). The active fraction was eluted with elution buffer (20 mM Tris, pH 7.5, 0.1 mM EDTA, 0.45 M NaCl, 2 mM DTT) and dialyzed against phosphate-buffered saline (pH 7.0) overnight.

2.2. Mice and cell line for xenograft study

Six-week old Balb/C nude mice were obtained from SLC (Seoul, Korea). The human stomach cancer cell line MKN 45 was obtained from Korea Research Institute of Bioscience and Biotechnology (KRIBB).

2.3. Antitumor experiments in a human xenograft mouse

MKN 45 cells were grown and adjusted to a concentration of 1.0×10^8 cells/ml using phosphate buffered saline, pH 7.0. 0.1 ml of these cells was used for subcutaneous injection into the right scapular region of each mouse and tumors were allowed to grow to an average size of 72.7 mm³. Thirty mice were randomly assigned to one of five groups for treatment with vehicle only, two different doses of AIMP1, one dose of paclitaxel or one dose of 5-fluorouracil (FU). Tumor growth was monitored until treatment. Injection of drugs into a group of mice began when the average tumor size in that group reached 72.7 mm³. AIMP1 was injected (IV bolus) at days 0, 1, 2, 3, 4 and 5 at a concentration of either 2 mg/kg or 10 mg/kg. Paclitaxel and 5-FU were injected intraperitoneally at days 0, 2 and 4 at a concentration of 5 mg/kg and 50 mg/kg, respectively. The vehicle used was phosphate buffered saline, pH 7.0.

2.4. Measurement of tumor size and volume

Tumor size was measured 4 times after the average size reached 72.7 mm³ until the 6th day. Tumor volume was measured 3-dimensionally using a vernier caliper and calculated as follows: tumor volume (mm³) = length \times width \times height \times 1/2. Tumor weight was measured after sacrifice and isolation of the tumor mass on day 6.

2.5. Proliferating cell nuclear antigen (PCNA) staining

Tissue blocks were embedded in paraffin and cut with a Minot microtome at a thickness of 10 mm. Sections were incubated for 48 h with mouse anti-proliferating cell nuclear antigen (PCNA) (1:100; BD Biosciences, CA) mixed with 0.2% Triton X-100 and 5% normal horse serum. Then, sections were incubated in biotinylated mouse secondary antibody for 1 h and 30 min at room temperature. Tissues were rinsed in PBS and incubated in avidin–biotin–peroxidase complex for 2 h at room temperature. Finally, sections were rinsed three times in PBS and twice in Tris–HCl buffer (0.1 M, pH 7.6), then incubated in 0.02% diaminobenzidine (DAB) with 0.003% H₂O₂ for 5 min.

2.6. Blood cytokine level measurements

Blood TNF α , IL-1 β , IL-12p40 and IFN- γ were measured with commercial ELISA Kits (R&D Systems, MN, USA; Thermo Scientific Pierce Protein Research Products, IL, USA; PBL InterferonSource, NJ, USA).

2.7. Pharmacokinetic assessments

Male Sprague–Dawley (SD) rats were acclimated to the testing facility in a temperature- and humidity- controlled environment for approximately 1 week prior to the study. The animals (10 weeks old, 285–305 g) were cannulated in the right jugular vein. The test substance dissolved in PBS was given intravenously ($n = 3$) at a dose of 5 mg/kg. About 300 μ l samples of blood were collected in BD Microtainer plasma separator tubes at selected times via the cannula over 24 h post-dosing. Samples were centrifuged at

6000g for 5 min and stored in a deep freezer until analysis. Quantitation of AIMP1 in the plasma samples was carried out using an ELISA Kit (Imagene Co., Ltd., Korea) following the manufacturer's protocol. Pharmacokinetic parameters were obtained by non-compartmental analysis of the plasma concentration-time profiles using KineticTM 4.4.1 (Thermo Fisher Scientific, Inc., Woburn, MA, USA).

2.8. Serum stability

AIMP1 was added to 1 ml of human, dog and rat serum at a concentration of 0.5 µg/ml. The experiment was performed in triplicate. The mixtures were incubated in a water bath at 37 °C and aliquots were taken at 0, 10, 30 and 60 min, then analyzed using an AIMP1 ELISA Kit (Imagene, Korea).

3. Results

3.1. Change in body weight after AIMP1 treatment

No statistically significant body weight change was observed among mice injected with either vehicle, two doses of AIMP1, 5-FU or taxol (Fig. 1A). The general decrease in body weight was thought to be due to the progression of xenografted tumors.

3.2. Change in tumor volume after AIMP1 injection

The change in tumor volume was determined by $V_t - V_0$ where V_t is the tumor volume at days 0, 3, 5 and 6, and V_0 is the tumor volume at day 0 (Fig. 1B). At day 6, the decrease in tumor volume was 31.1% and 54.0% with AIMP1 treatment at a concentration of 2 mg/kg and 10 mg/kg, respectively, whereas a 38.4% and 28.5% decrease in tumor volume was observed when mice were treated with 5-FU (50 mg/kg) and taxol (5 mg/kg), respectively.

3.3. Change in tumor weight after AIMP1 injection

The decrease in tumor weight was 29.1% and 52.2% with AIMP1 treatment at a concentration of 2 mg/kg and 10 mg/kg, respectively, whereas a 36.2% and 24.3% decrease in tumor weight was observed when treated with 5-FU (50 mg/kg) and taxol (5 mg/kg), respectively (Fig. 1C).

3.4. PCNA staining of tumor tissues

A reduction in the number of tumor cells exhibiting an active cell cycle progression were visible following PCNA staining of tumor tissues (Fig. 2A). Tumor tissues from AIMP1-treated mice showed a 53% reduction (Fig. 2B). The reduction was the same for tissues treated with AIMP1 at 2 mg/kg or 10 mg/kg. A similar reduction was observed in tissues from mice treated with 5-FU at 50 mg/kg or taxol at 5 mg/kg.

3.5. Blood cytokine levels

An increase in blood TNF-α levels was readily observed in mice following AIMP1 treatment (Fig. 3A). TNF-α in blood increased 2.5-fold with treatment at 2 mg/kg and 5.5-fold at 10 mg/kg. It also increased 2-fold or 1.5-fold when treated with 5-FU at 50 mg/kg or with taxol at 5 mg/kg, respectively. A 35% or 50% increase in blood IL-1β was seen in tissues from mice treated with AIMP1 at 2 mg/kg or 10 mg/kg, respectively (Fig. 3B). However, only a slight increase in IL-1β level was observed in 5-FU- or taxol-treated tissues. There was no significant increase in IL-12p40 and IFN-γ levels in the blood of mice treated with any of the anti-cancer agents (Fig. 3C and D).

3.6. Pharmacokinetic properties

Results of the pharmacokinetic studies performed in rats are listed in Table 1 and shown in Fig. 4. AIMP1 showed a low clearance (CL) and a low volume of distribution. The half-life ($t_{1/2}$) was 6 min.

3.7. Serum stability

Since serum is one of the major sites where protein drugs can be degraded before reaching their target, we evaluated the serum stability of AIMP1. In human serum, AIMP1 exhibited a relatively long half-life of >60 min, whereas it exhibited half-lives of 52 min and 32 min in dog and rat serum, respectively (Fig. 5).

4. Discussion

In our previous studies, AIMP1 showed significant anti-tumor activity against stomach cancer cells only in combination with taxol [13]. We suspected that tumor tissues were not exposed to an effective dose of AIMP1 via intraperitoneal injection. In this study, we delivered AIMP1 via intravenous injection instead and found that AIMP1 itself can reduce tumor growth at a much lower dose, suggesting that the method of administration is a critical factor for the antitumor efficacy of this protein. In addition, we examined the primary cause of the AIMP1 antitumor activity shown in this work. AIMP1 was reported to induce apoptosis of endothelial cells *in vitro* and to cause the reduction of blood vessels on the chorioallantoic membrane of fertilized eggs [3]. However, no significant change in vasculature development was observed in this xenograft model (data not shown), suggesting possibilities other than anti-angiogenesis as the primary cause for tumor reduction. Since AIMP1 also induces proinflammatory responses [2], and inflammatory cytokines are known to modulate tumor physiology, we investigated whether blood levels of cytokines were increased by intravenous introduction of AIMP1. In our previous report, AIMP1 upregulated proinflammatory cytokine genes such as TNF-α, MCP-1, IL-1β, IL-8, RANTES, MIP-1α, MIP-1β, and MIP-2α in THP-1 human acute monocytic leukemia cell line [2]. Among these AIMP1-induced cytokines, TNF-α's antitumor activity is well documented [14]. We found that TNF-α was significantly higher in AIMP1-treated xenograft mice than in the control. TNF-α exerts its antitumor activity both directly through killing of tumor cells and destruction of the tumor vasculature, and indirectly at the same time through manipulation of the complex tumor physiology [15]. Since the reduction of blood vessels in the tumor region was not prominent in our observations, we think that the increased innate immunity maybe the main cause of *in vivo* antitumor effect of AIMP1. Intravenous route is generally considered a more efficient way for drug delivery although there may be some exceptional cases [16]. A direct and rapid systemic distribution of AIMP1 could result in more efficient contact with immune cells which produce cytokines including TNF-α. Thus intravenous injection of AIMP1 might have contributed to the improved efficacy compared with the previous one in which AIMP1 was delivered via intraperitoneal injection. This notion is also supported by previous reports describing improvement of TNF-α anticancer activity by endothelial monocyte activating polypeptide (EMAP) II [17–19], which is the 166-amino acid- C-terminal domain of AIMP1. EMAP II induced relocalization of TNF-R1 from the Golgi apparatus to the cell surface, rendering endothelial cells sensitive to TNF-α and inducing membrane expression of the TNF-R1-associated death domain (TRADD) protein [20]. A moderate in-

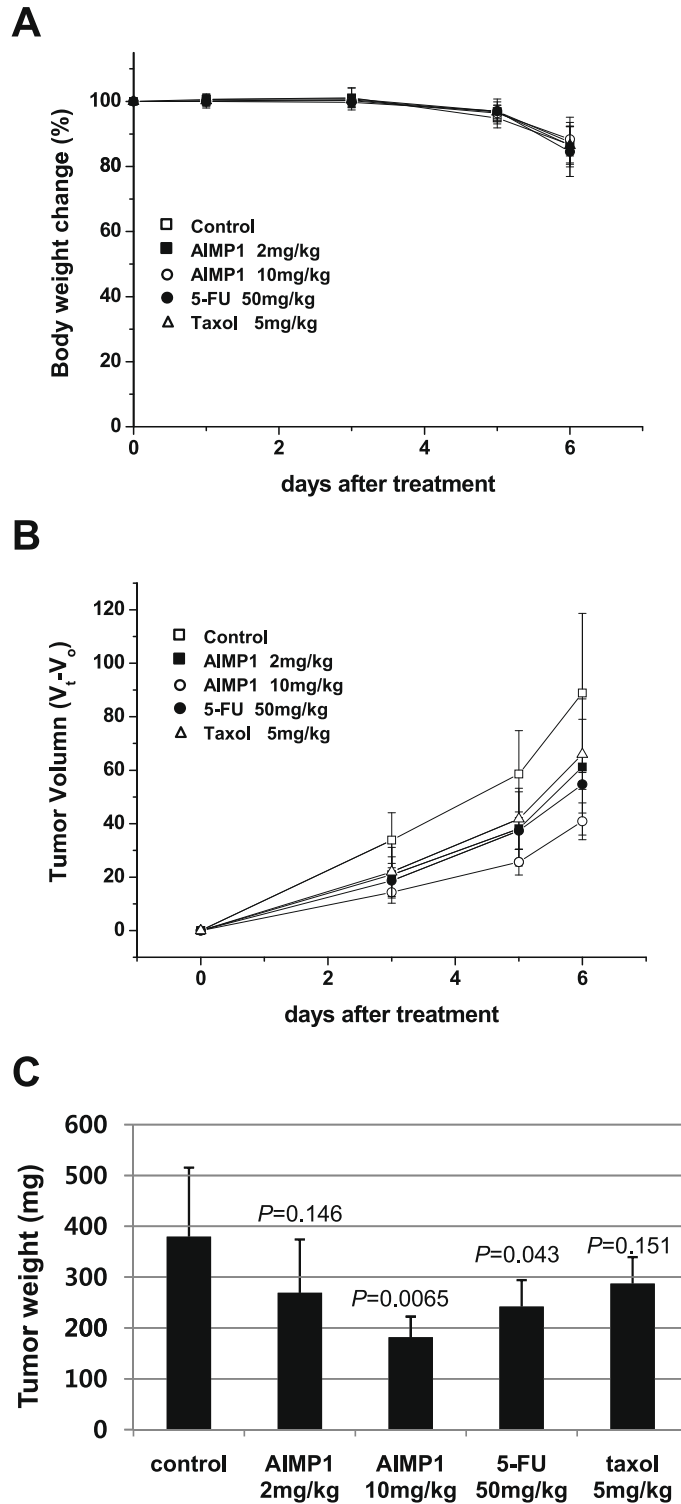


Fig. 1. Effect of AIMP1 on human xenograft mice. MKN45 cells were grown and adjusted to a concentration of 1.0×10^8 cells/ml. 0.1 ml of these cells was used for subcutaneous injection into the right scapular region of each mouse and tumors were allowed to grow to an average size of 72.7 mm^3 . Thirty mice were randomly assigned to one of five groups for treatment with vehicle only, two different doses of AIMP1, one dose of paclitaxel or one dose of 5-FU. Tumor growth was monitored until treatment. AIMP1 was injected (IV bolus) at days 0, 1, 2, 3, 4, and 5 at a concentration of either 2 mg/kg or 10 mg/kg. Paclitaxel and 5-FU were injected intraperitoneally at days 0, 2, and 4 at a concentration of 5 mg/kg and 50 mg/kg, respectively. The vehicle used was phosphate buffered saline, pH 7.0. Mice were treated with vehicle (\square), 2 mg/kg AIMP1 (\blacksquare), 10 mg/kg AIMP1 (\circ), 50 mg/kg 5-FU (\bullet), or 5 mg/kg taxol (\triangle). Values are means. Bars are standard deviations: (A) body weight changes, (B) tumor volume and (C) tumor weight.

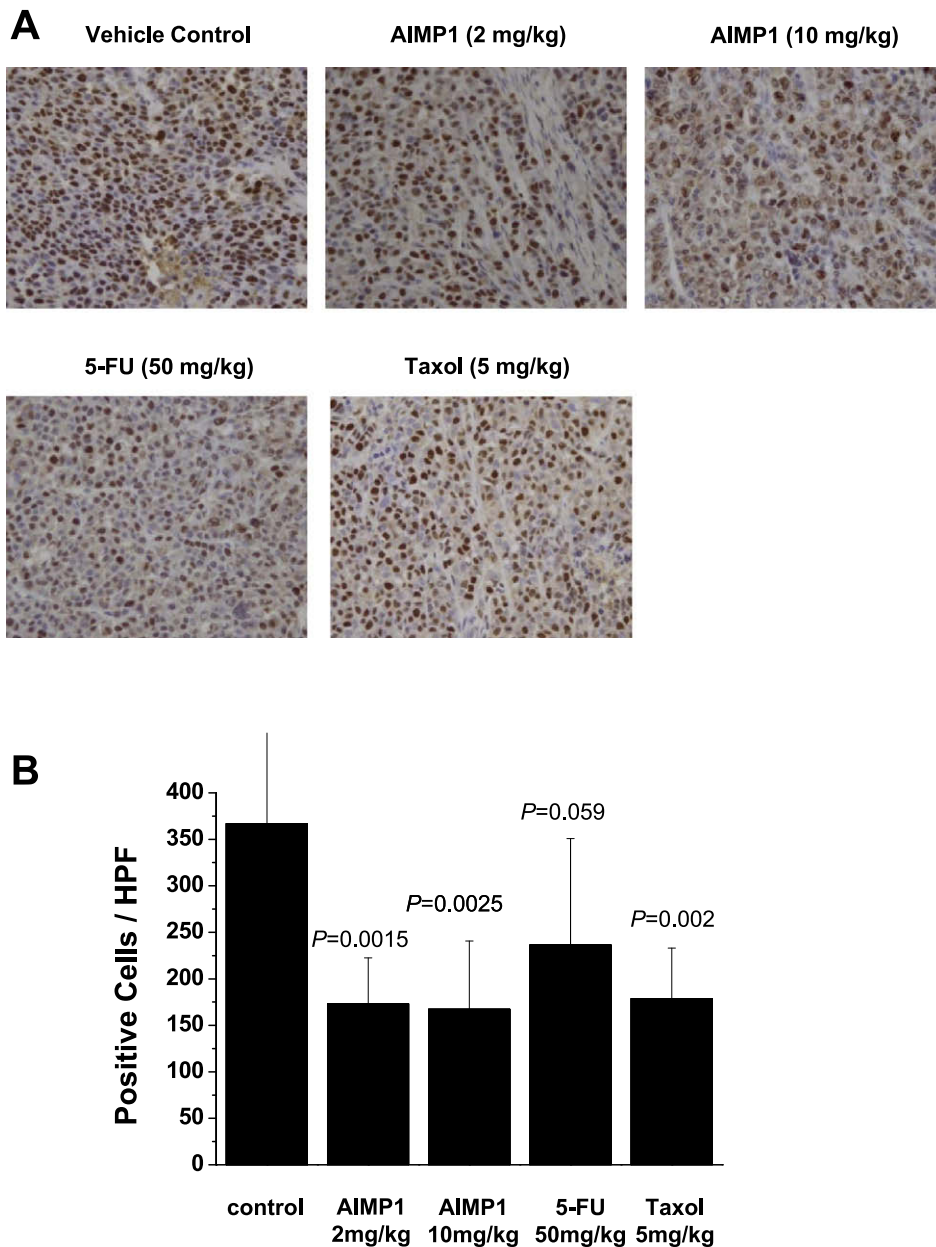


Fig. 2. Proliferating cell nuclear antigen (PCNA) staining of tumor tissues. Tumor tissue blocks were embedded in paraffin and cut with a Minot microtome at a thickness of 10 μ m. Sections were incubated for 48 h with mouse anti-PCNA mixed with 0.2% Triton X-100 and 5% normal horse serum. Then, sections were incubated in biotinylated mouse secondary antiserum for 1 h and 30 min at room temperature. Tissues were rinsed in PBS and incubated in avidin-biotin-peroxidase complex for 2 h at room temperature. Finally, sections were rinsed three times in PBS, twice in Tris-HCl buffer (0.1 M, pH 7.6) and incubated in 0.02% diaminobenzidine (DAB) with 0.003% H_2O_2 for 5 min. (A) Photographs showing proliferating cell nuclear antigens in each tumor tissue and (B) number of PCNA-positive cells counted in each tumor tissue. HPF: high power field.

crease in blood IL-1 β levels by treatment with AIMP1 also suggests induction of antitumor activity. Although no increase in IL-12 or IFN- γ was observed in the blood of xenograft mice treated with AIMP1 in this study, these cytokines may play a role in other cancer models.

Previously, it was reported that the anti-angiogenic agent TNP-470 reduced peritoneal dissemination of human MKN45 stomach cancer cells in a mouse xenograft

experiment [21]. However, MKN45 cells with the vascular endothelial growth factor (VEGF) (-/-) genotype showed the same microvessel perfusion as wild type cells in another mouse xenograft experiment [22]. Thus, anti-angiogenic treatment of MKN45 cells showed different results depending on the particular experimental conditions. In the current study, AIMP1 exerted its antitumor activity through immune activation rather than an anti-angio-

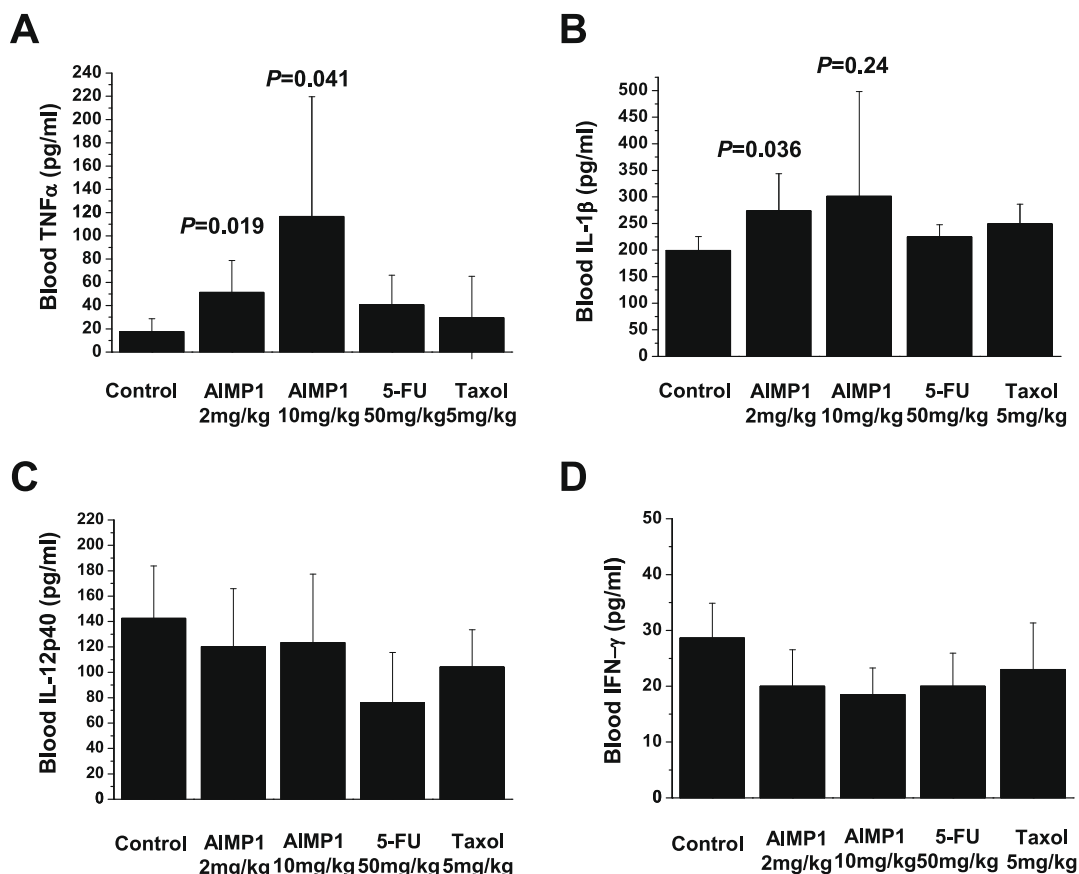


Fig. 3. Blood cytokine levels. Cytokine levels were measured from blood collected after sacrifice of xenograft mice treated with vehicle, 2 mg/kg AIMP1, 10 mg/kg AIMP1, 50 mg/kg 5-FU or 5 mg/kg taxol. Each value is the mean of three independent experiments. (A) Blood TNF- α level was measured using TNF- α ELISA, (B) blood IL-1 β level was measured using IL-1 β ELISA, (C) blood IL-12p40 level was measured using IL-12p40 ELISA and (D) blood IFN- γ level was measured using IFN- γ ELISA.

genic effect. It is conceivable that AIMP1 may exert its antitumor activity through the control of angiogenesis

Table 1

Pharmacokinetic parameters of AIMP1.

Parameter	IV			N
	Mean	SD		
Dose (mg/kg)	5			3
AUC _{last} (h ng/ml)	10970.3	3396.6		3
AUC _{inf} (h ng/ml)	11080.4	3395.9		3
CL (l/h/kg)	0.5	0.1		3
V _{ss} (l/kg)	0.1	0.04		3
V _z (l/kg)	0.1	0.03		3
t _{1/2} (h)	0.1	0.01		3
MRT _{inf} (h)	0.2	0.1		3

AUC_{last}: area under the plasma concentration-time curve, last.

AUC_{inf}: area under the plasma concentration-time curve, from time zero extrapolated to infinite time.

CL: clearance.

V_{ss}: volume in the steady state.

V_z: volume in the terminal state.

t_{1/2}: half life.

MRT_{inf}: mean residence time, from time zero extrapolated to infinite time.

as well as immune activation, and these two functions may contribute to cancer suppression to varying degrees depending on conditions such as the concentration of the drug in the vicinity of the target and the type of tumor cell.

One reason why AIMP1 did not show vascular remodeling in this case could be its short half-life when injected into the body system in a naked form. A half-life of 6 min may be too short for AIMP1 to reach the tumor vasculature at an effective dose. It has been shown that the increased circulation time of antitumor drugs encapsulated in liposomes directly correlates with better accumulation in solid tumors [23–26], and this approach may improve the anti-angiogenic effect of AIMP1. In contrast, a small amount of a primary cytokine (e.g. AIMP1) can trigger an amplifying cascade, leading to the induction of secondary cytokines (e.g. TNF- α) up to a level sufficient for tumor suppression. Despite the ability of TNF- α to function as a vascular disrupting agent [27], its cytotoxicity has prevented it from being used as an antitumor drug [28]. In this study, we did not see any significant toxicity due to the systemic administration of AIMP1. Perhaps AIMP1 induces TNF- α and other antitumor cytokines

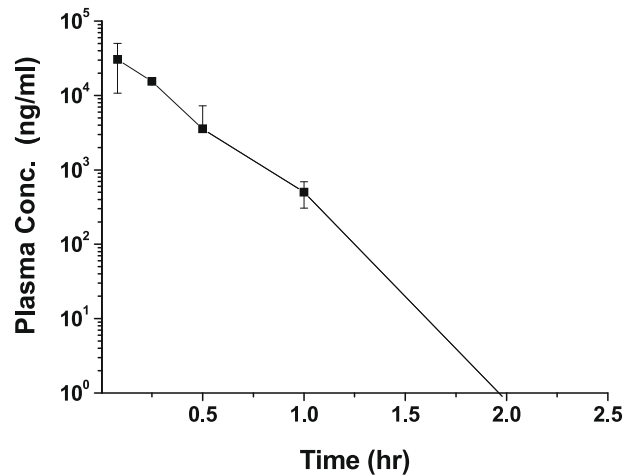


Fig. 4. Plasma concentration–time curve. AIMP1 was administered to SD rats at a concentration of 5 mg/kg via single intravenous injection and its concentration in serum was checked every hour.

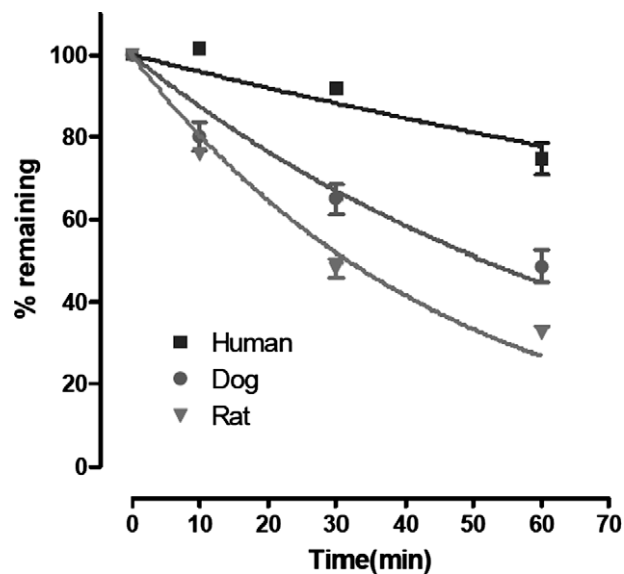


Fig. 5. Serum stability of AIMP1. AIMP1 was added to 1 ml each of human, dog and rat serum at a concentration of 0.5 µg/ml. The experiment was performed in triplicate. The mixtures were incubated in a water bath at 37 °C and aliquots were taken at 0, 10, 30, and 60 min, then analyzed using an AIMP1 ELISA kit.

at a level that is adequate for significant antitumor activity. Indirect modulation of tumor growth by inducing tumor-suppressing cytokines may serve as a novel tumor therapy method.

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