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Original Article

Toxicity testing of epidermal growth factor receptor-targeted hybrid peptide for preclinical study

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Abstract: Background: We previously reported that EGFR2R-lytic hybrid peptide was generated from the chemical conjugation of an epidermal growth factor receptor (EGFR)-targeted peptide and a cell-killing lytic peptide. This peptide had effective cytotoxic and anti-tumor activities *in vitro* and *in vivo* against EGFR-expressing cancers, suggesting it may be a novel candidate for a molecular-targeted anti-cancer drug. Methods: In this preclinical study, the toxicity of this hybrid peptide was examined in mice by monitoring body weight, performing clinical and biochemical examinations of blood samples, and observing histological changes. Results: No remarkable toxicity after intravenous injection of the hybrid peptide was observed up to a dose of 15 mg/kg, although a decrease in the alveolar space of the lung was identified from histological observations. Toxicity of the EGFR2R-lytic hybrid peptide in mice was also tested after oral administration. A significant change in body weight was not observed until a single dose of 300 mg/kg, but a decrease in body weight was observed at repeated doses of 75 or 150 mg/kg. However, the tendency for body weight increase was similar between the saline (control) and hybrid peptide groups after the completion of the administration period. In the case of oral administration, the dosage of the hybrid peptide could be increased to a significantly higher range compared with intravenous administration without serious toxicity. Conclusion: The results of this study should facilitate further preclinical studies on the EGFR2R-lytic hybrid peptide and its application in future cancer therapies.

Keywords: Hybrid peptide, molecular-targeted therapy, toxicity, preclinical study, peptide drug

Introduction

Molecular-targeted approaches have recently been a focus of drug discovery for cancer treatment [1]. Molecular targeted anti-cancer drugs currently in clinical use have some serious problems such as the development of drug resistance, although some mechanisms of this drug resistance have been reported [2]. To resolve these problems, it is necessary to identify as many candidates for molecular-targeted anti-cancer drugs as possible, including not only small molecules but also proteins and peptides, including antibodies and immunotoxins. We previously reported that epidermal growth factor receptor (EGFR)-targeted hybrid peptides (EGFR-lytic and EGFR2R-lytic), in which an EGFR-targeting peptide was conjugated with a lytic-type peptide that can rapidly disintegrate cancer cell membranes, are potent and novel anti-cancer drug candidates [3-5]. Furthermore, these hybrid peptides could induce anti-tumor activity against cancer cells with *KRAS* mutations [3, 4], which are resistant to molecular-targeted anti-cancer drugs such as anti-EGFR antibodies and tyrosine kinase inhibitors [6, 7].

The previous studies also revealed the potency of the EGFR2R-lytic hybrid peptide as a drug delivery system through its combination with a biodegradable gelatin hydrogel and carboxymethyl dextran via disulfide linkers *in vitro* and *in vivo* [8, 9]. Notably, a short elimination phase was observed for this molecular mixture, and the concentration of the hybrid peptide decreased rapidly in mouse blood after intravenous (IV) administration, as measured by a fluorescent dye coupled to the hybrid peptide [8].

To develop an anti-cancer drug from the EGFR2R-lytic hybrid peptide, it is therefore fundamentally important to know its toxicity, pharmacokinetics, and pharmacodynamics *in vivo*.

Here, toxicity testing of the EGFR2R-lytic hybrid peptide was tested in mice after IV and oral administration. Also, the potential for using the hybrid peptide as an oral formulation is described and discussed.

Materials and methods

Peptide synthesis

The EGFR2R-lytic hybrid peptide (YRWYGYTPQNVIGGGKL**L**LK**L**L**KK**LLK**L**LKKK [bold and underlined letters correspond to D-amino acids]) was synthesized by the American Peptide Company (Sunnyvale, CA) as described previously [5]. The EGFR2R-lytic hybrid peptide was dissolved in 0.9% sterile saline and buffered to pH 7.4 as described previously [4]. The peptide stock was freshly dissolved just before the experiments.

Body weight changes

All animal experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee at Kyoto University (Kyoto, Japan). Six-week-old male BALB/c mice were purchased from Japan SLC (Fukuoka, Japan). Animals were observed daily for clinical signs for 7 days. The body weight of the animals was measured on the day of administration and at 1, 3, and 7 days after injection of the EGFR2R-lytic hybrid peptide.

Hematological and biochemical examinations of blood samples

The EGFR2R-lytic hybrid peptide was injected via the tail vein of male BALB/c mice. The mice were anesthetized with 50 mg/kg pentobarbital via a disposable syringe using a 26-gauge needle, and approximately 1 mL blood was collected from the inferior vena cava. Leucocytes, erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, reticulocytes, neutrophils, eosinophils, basophils, lymphocytes, and monocytes were evaluated as hematological parameters. Clinical biochemistry analysis involved the following parameters: aspartate aminotransferase, alanine aminotransferase, alkaline phosphate, γ-glutamyl transpeptidase, leucine aminopeptidase, lactate dehydrogenase, creatinine phosphokinase, total bilirubin, total protein, triglyceride, total cholesterol, blood urine nitrogen, creatinine, Na, Cl, K, Ca, and inorganic phosphates. Biochemical plasma analyses were carried out by Japan Clinical Laboratories, Inc. (Kyoto, Japan).

Gross necropsy and histopathology

Animals were euthanized at 1, 3, or 7 days after the administration of the EGFR2R-lytic hybrid peptide. Heart, lung, liver, and kidney were harvested and tissues were fixed and preserved in 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan): Histopathological examinations were conducted on all tissues and organ samples from the saline control group and from the 5, 10, and 15 mg/kg EGFR2R-lytic hybrid peptide groups. Fixed tissue and organ samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Toxicity testing after oral administration

Female 7-week-old ICR mice were purchased from SLC Japan (Shizuoka, Japan). Single or repeated oral administration of saline or the EGFR2R-lytic hybrid peptide was performed, and body weight was monitored as described previously [10]. In addition to blood samples, the heart, liver, lung, kidney, spleen, stomach, and intestines were collected for hematological analysis and histological assessments.

Statistical analysis

Values are given as the mean ± standard deviation (SD), and differences were analyzed using one-way analysis of variance with Dunnett's test for multivalent comparisons. Differences were considered to be statistically significant at $P < 0.05$. All statistical analysis was performed using JMP Pro Statistics software (ver. 14.0.0, SAS Institute Inc.).

Results

Clinical observations, body weight, hematology, and clinical biochemistry

A toxicity test for the EGFR2R-lytic hybrid peptide was first performed after IV administration. To examine the solvent effect of the peptide,

the zeta potential of the EGFR2R-lytic hybrid peptide dissolved was determined in saline or a 5% glucose solution (Supplemental materials and methods), which are often used as solvents for anti-cancer drugs [11]. However, no remarkable difference between the vehicle solutions was observed. Nanoparticles of the hybrid peptide were positively charged and the zeta potential was 13.2 ± 10.56 mV in saline and 2.67 ± 10.56 mV in 5% glucose solution (Figure S1). The hemolytic activity of the EGFR2R-lytic hybrid peptide was slightly higher in 5% glucose solution than in saline (Supplemental materials and methods). However, its activity in both solutions was quite low compared with that in the positive control (Figure S2). In this toxicity test, the EGFR2R-lytic hybrid peptide was dissolved in 0.9% sterile saline and administered IV to mice. No deaths occurred in any group throughout the study period. However, black faces were observed in the 15 mg/kg group. When we monitored the body weight of mice after IV administration of the EGFR2R-lytic hybrid peptide, no significant differences were observed among the doses of the EGFR2R-lytic hybrid peptide used compared with the saline group (**Figure 1A**). Several significant differences were noted in the blood tests at day 1 after administration. An increased number of neutrophils and a decreased number of lymphocytes were observed in the 15 mg/kg group (**Table 1**). However, there was no significant difference in leucocytes compared with the control group. Moreover, compared with the saline control group, significant increases in aspartate aminotransferase and chloride were observed in the 15 mg/kg group. No other treatment-related effects on any other clinical or biochemical parameters were observed (**Table 2**). In addition, no remarkable changes were observed among these groups at 3 and 7 days after IV administration of the EGFR2R-lytic hybrid peptide (Tables S1, S2, S3, S4).

Histopathology

Histopathological observations were next made on several organs after IV administration of the EGFR2R-lytic hybrid peptide to the mice. Tissues from heart, lung, liver, and kidney for all experimental and control animals were histologically examined. The histopathological findings in the heart, kidney, and liver were not considered to be of toxicological significance at 7 days after administration and at each concentration used, since no remarkable changes were observed between the saline and EGFR2R-lytic hybrid peptide groups (**Figure 1B**). In contrast, the alveolar space in the lung was observed to be decreased even at 1 and 3 days after administration (Figure S3A and S3B).

Toxicity testing after oral administration

We previously reported that the EGFR2R-lytic hybrid peptide exerts anti-tumor activity after oral administration, and that bile acid is an effective absorption enhancer for improving the oral bioavailability and bioactivity of the hybrid peptide [10]. These results prompted us to examine the toxicity of the EGFR2R-lytic hybrid peptide after oral administration to test its possible future use as an oral formulation. When the body weight of the mice was monitored after a single oral administration of the EGFR2R-lytic hybrid peptide, a significant change in body weight was not observed until a dose of 300 mg/kg (**Figure 2**). In addition, the decrease of the normal alveolar space in the lung was observed for the single oral administration of 750 mg/kg EGFR2R-lytic hybrid peptide by histological observations (data not shown). When the EGFR2R-lytic hybrid peptide was repeatedly administered through the oral route, a decrease in body weight was observed at doses of 75 and 150 mg/kg during the administration period; however, the tendency toward increased body weight was similar between the saline and hybrid peptide groups after completion of the administration period (**Figure 3**). No remarkable differences were observed between the saline and EGFR2R-lytic hybrid peptide groups in these experiments by histopathological observations (data not shown).

Discussion

In this study, toxicity testing of the EGFR2R-lytic hybrid peptide using mice showed no remarkable toxicity after IV injection until a dose of 15 mg/kg, although a decrease in the alveolar space of lung sections was observed in histological observations at 1, 3, and 7 days after administration.

Various kinds of pulmonary injuries have been reported with molecular-targeted anti-cancer drug therapy [12]. In particular, interstitial lung

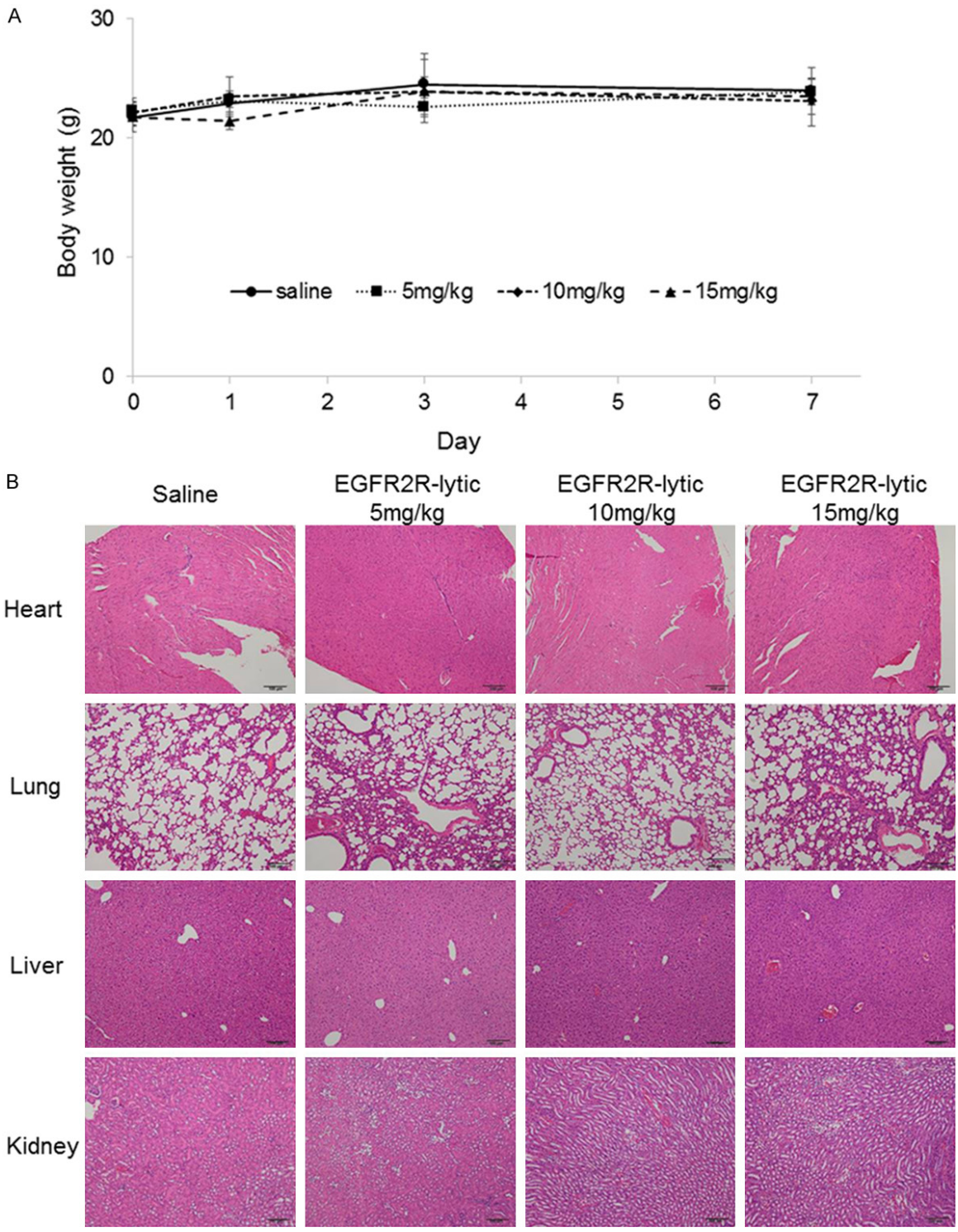

Figure 1. Evaluation of toxicity after IV injection of mice with the EGFR2R-lytic hybrid peptide. A. Mean body weight of mice during the 7-day toxicity study. B. Histological sections stained with hematoxylin-eosin of major organs (heart, lung, liver, and kidney) from mice at 7 days after IV injection of saline or EGFR2R-lytic hybrid peptide (at 5, 10, or 15 mg/kg). All scale bars indicate 100 μm.

disease (ILD) is a severe adverse effect of gefitinib, which targets EGFR similarly to our hybrid peptide. [13]. The incidence of ILD in gefitinib-treated Japanese patients with non-small cell

Table 1. Hematology parameters following IV injection of the EGFR2R-lytic hybrid peptide for 1 day

		0 mg/kg	5 mg/kg	10 mg/kg	15 mg/kg
Leucocytes	1000/μl	3.5 ± 1.6	3.5 ± 2.8	2.2 ± 1.3	1.6 ± 1.0
Erythrocytes	10000/μl	886.0 ± 42.2	833.0 ± 144.8	850.0 ± 69.2	837.5 ± 45.2
Hemoglobin	g/dl	14.1 ± 0.9	13.8 ± 1.2	13.9 ± 0.5	13.4 ± 0.7
Hematocrit	%	46.8 ± 2.8	44.0 ± 7.8	45.4 ± 3.9	43.9 ± 2.2
MCV[a]	fl	52.3 ± 0.5	52.2 ± 0.8	52.8 ± 0.8	52.0 ± 0.0
MCH[b]	pg	15.8 ± 0.3	16.7 ± 1.8	16.3 ± 1.3	16.0 ± 0.1
MCHC[c]	%	30.0 ± 0.3	31.7 ± 3.1	30.6 ± 2.5	30.5 ± 0.2
Platelets	10000/μl	57.7 ± 29.8	64.3 ± 23.7	66.5 ± 18.0	82.3 ± 4.9
Reticulocytes	%	44.3 ± 6.0	45.5 ± 7.1	42.6 ± 1.7	38.0 ± 2.6
Neutrophils	%	14.8 ± 3.5	15.6 ± 7.7	15.8 ± 4.7	29.3** ± 7.3
Eosinophils	%	0.0 ± 0.0	0.2 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
Basophils	%	0.0 ± 0.0	0.2 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes	%	83.8 ± 3.8	82.2 ± 7.6	83.0 ± 4.7	68.8** ± 7.2
Monocytes	%	1.5 ± 0.6	1.8 ± 0.5	1.2 ± 0.5	2.0 ± 0.8

[a]Mean corpuscular volume, [b]mean corpuscular hemoglobin, [c]mean corpuscular hemoglobin concentration. Data are expressed as the mean ± SD; n = 4-5 mice/group. **$p < 0.01$ compared with the control group (0 mg/kg).

Table 2. Clinical biochemistry parameters following IV injection of the EGFR2R-lytic hybrid peptide for 1 day

		0 mg/kg	5 mg/kg	10 mg/kg	15 mg/kg
AST[a]	U/L	81.7 ± 5.8	82.4 ± 9.9	86.0 ± 11.5	113.0** ± 10.8
ALT[b]	U/L	56.7 ± 12.6	55.4 ± 30.7	37.0 ± 6.9	75.0 ± 21.5
ALP[c]	U/L	355.0 ± 39.1	333.6 ± 23.7	334.0 ± 35.6	310.3 ± 30.1
γ-GT[d]	U/L	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0
LAP[e]	U/L	46.7 ± 5.8	47.6 ± 10.3	48.7 ± 1.5	49.0 ± 3.6
LDH[f]	U/L	2201.7 ± 672.0	2497.0 ± 445.6	2848.3 ± 743.4	2206.7 ± 407.0
CPK[g]	U/L	601.7 ± 172.8	919.0 ± 294.9	846.7 ± 287.2	594.3 ± 112.5
Total bilirubin	mg/dL	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total protein	g/dL	4.5 ± 0.0	4.5 ± 0.5	4.9 ± 0.2	4.6 ± 0.3
Triglyceride	mg/dL	168.3 ± 32.2	165.0 ± 46.9	103.3 ± 41.0	91.3 ± 30.8
Total cholesterol	mg/dL	120.0 ± 10.0	126.0 ± 16.7	130.3 ± 16.2	126.3 ± 9.5
BUN[h]	mg/dL	23.3 ± 2.9	23.8 ± 2.5	25.3 ± 4.2	28.3 ± 2.5
CRE[i]	mg/dL	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
Na[j]	mEq/L	157.5 ± 3.5	159.0 ± 5.5	160.0 ± 13.1	155.3 ± 0.6
Cl[k]	mEq/L	95.0 ± 0.0	96.8 ± 5.4	102.3 ± 3.2	106.7* ± 1.5
K[l]	mEq/L	6.2 ± 1.0	7.1 ± 1.7	5.9 ± 0.7	5.8 ± 0.6
Ca[m]	mEq/L	6.7 ± 0.3	6.6 ± 1.1	7.7 ± 0.6	7.7 ± 0.3
Inorganic P	mg/dl	16.8 ± 2.5	17.4 ± 2.7	13.0 ± 3.5	14.5 ± 1.7

[a]Aspartate aminotransferase, [b]alanine aminotransferase, [c]alkaline phosphate, [d]γ-glutamyltranspeptidase, [e]leucine aminopeptidase, [f]lactate dehydrogenase, [g]creatinine phosphokinase, [h]blood urine nitrogen, [i]creatinine, [j]sodium, [k]chloride, [l]potassium, [m]calcium. The peptide was bolus injected via the tail vein of BALB/c mice. Data are expressed as the mean ± SD; n = 2-5 mice/group. **$P < 0.01$, *$P < 0.05$, compared with the control group (0 mg/kg).

lung cancer is approximately 3.5% [14]. Several studies have reported that pre-existing pulmonary fibrosis is significantly associated with the development of ILD [15, 16]. These reports are directly relevant to the study by Suzuki et al. in that decreased EGFR phosphorylation and regenerative epithelial proliferation augment pulmonary fibrosis [17]. EGFR deficiency induces the overproduction of pulmonary surfactant, which is a lipid-protein complex [18] that can

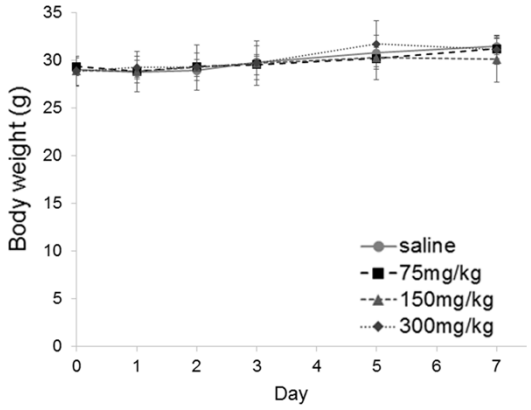

Figure 2. Change in body weight after single oral administration of the EGFR2R-lytic hybrid peptide. High doses (up to a maximum of 300 mg/kg) of the EGFR2R-lytic hybrid peptide were administrated orally to 7-week-old female ICR mice, and body weight was monitored as shown in the graph. The saline administered group was used as a negative control. Data are expressed as the mean ± SD, and each group included 4-5 mice.

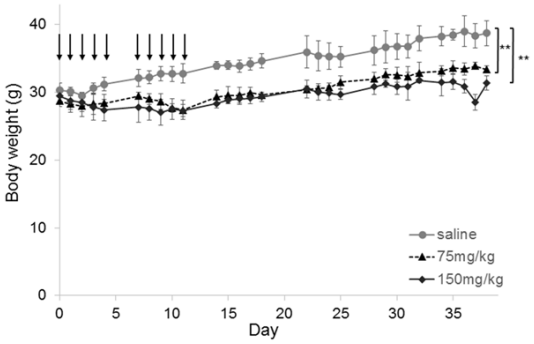

Figure 3. Changes in body weight after repeated oral administration of the EGFR2R-lytic hybrid peptide. The EGFR2R-lytic hybrid peptide (75 or 150 mg/kg) was administrated orally 5 days a week for 2 weeks (a total of 10 times) to 7-week-old female ICR mice, and body weight was monitored as shown in the graph. The saline administered group was used as a negative control. Arrows indicate the day of administration. Data are expressed as mean ± SD, and each group included 4-6 mice. $^{**}P < 0.01$ compared with the saline-treated group.

lead to lung collapse, respiratory distress syndrome [19], and fatal apnea. Previous studies have reported that EGF signaling regulates surfactant expression in alveolar type II cells, and ILD treated with an EGFR-tyrosine kinase inhibitor might reduce the expression of surfactant protein [20, 21]. It should be pointed that serum surfactant protein A and D may predict overall pulmonary function [22]. Although these studies could not confirm the parameters necessary to evaluate ILD, high-dose IV administration of the EGFR2R-lytic hybrid peptide might cause damage to mouse lung cells and lead to the observed decrease in alveolar space, with a similar mechanism similar to that of ILD. Further investigation is required to elucidate this issue.

A significant increase in aspartate aminotransferase was observed at 1 day after the administration of 15 mg/kg EGFR2R-lytic hybrid peptide. This increase was not observed at 3 and 7 days after administration, suggesting that hepatic damage was resolved during this period. This might be associated with rapid clearance of the peptide from the body.

When the toxicity of the EGFR2R-lytic hybrid peptide was tested using rats after IV administration, several rats died, even at a dose of 10 mg/kg (data not shown); however, remarkable toxicity was not observed in mice until a dose of 15 mg/kg. A similar response has also been observed for several compounds, the lethal doses of which were different among different animals [23, 24]. A detailed configuration of the maximum tolerated dose for the IV injection of the EGFR2R-lytic hybrid peptide, together with the route and frequency of administration, will be necessary.

In this study, the solvent effect of the EGFR2R-lytic hybrid peptide in saline or 5% glucose solution was also tested, and no dramatic changes were observed except for a faint increase of hemolytic activity in 5% glucose solution. This suggests that the vehicle solvent might be another factor to consider in further development of this hybrid peptide as an anti-cancer drug.

The toxicity of the EGFR2R-lytic hybrid peptide was also tested after oral administration to consider the possibility of using it as an oral formulation in the future. A significant change in body weight was not observed until a single dose of 300 mg/kg, but a decrease in body weight was observed at repeated doses of 75 and 150 mg/kg. However, the trend of body weight increase was similar between the saline and hybrid peptide groups after the completion of the administration period. This suggested that the decrease in body weight was transient during the period of oral administration. Thus, oral administration of the EGFR2R-lytic hybrid

peptide might be able to reduce the toxicity observed following IV administration, and that it would be possible to increase the dose. In this study, ICR mice were used for toxicity tests after oral administration of the EGFR2R-lytic hybrid peptide because these mice are a general-purpose model used to test the safety of peptide drugs and anti-cancer drugs [25, 26]. Thus, general toxicity can be observed by administering the hybrid peptide as an oral formulation and must be taken into consideration. Toxicity tests using BALB/c mice with oral administration of the hybrid peptide were also performed, and similar results were observed as with the ICR mice without remarkable toxicity (data not shown).

The therapeutic effect of orally administered EGFR2R-lytic hybrid peptide at a higher dosage was examined using a human gastric carcinoma xenograft model (Supplemental materials and methods). The EGFR2R-lytic hybrid peptide exerted a therapeutic anti-tumor effect in this model, in which treatment was started at an average tumor size of 150 mm^3 and dosage was increased up to 15 mg/kg (Figure S4). Thus, taken together with our previous reports [10], the present study suggests that the EGFR2R-lytic hybrid peptide could be developed as an oral formulation of a novel type of peptide-based anti-cancer drug, although further examinations are necessary to confirm this hypothesis.

An analytical method for the EGFR2R-lytic hybrid peptide in rat blood after IV administration was attempted, and pharmacokinetics of the peptide were determined by liquid chromatography-tandem mass spectrometry as a pilot study. The highest plasma concentration of the EGFR2R-lytic hybrid peptide was observed within 5 min, and it declined quickly within 30 min after IV injection in rats at 1 mg/kg (data not shown). Although further studies are necessary to confirm the results of this pilot study, its findings are consistent with our previous study in which the hybrid peptide was labelled with a fluorescent dye and injected into mice [8]. In that system, the concentration of the fluorescence-labeled hybrid peptide decreased rapidly in blood, and a high fluorescence intensity was observed in the liver and kidney at 48 hours after IV administration of the peptide, as confirmed by ex vivo imaging [8]. This suggests the rapid transport of the EGFR2R-lytic hybrid peptide, which has high water solubility, to the liver and kidney after IV administration. It is also suggested that the stability of the hybrid peptide in blood is relatively high and is thus metabolized in the liver and kidney. The transportation and circulation of the hybrid peptide will be the target of future studies.

Combined with our previous analyses describing the efficacy of the EGFR2R-lytic hybrid peptide as a drug delivery system [8, 9], the results from the present study suggest that the route and frequency of administration for the hybrid peptide are significant and indispensable factors to perform further preclinical studies and to develop this peptide as an anti-cancer drug.

In summary, the toxicity testing of the EGFR2R-lytic hybrid peptide after IV and oral administration was reported here. The feasibility of administering this hybrid peptide as an oral formulation was also discussed. Taken together with our previous studies, the observations reported here are useful for the design of further preclinical studies and for the development of novel types of peptide-based anti-cancer drugs from this peptide.

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Disclosure of conflict of interest

None.

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References

[1] Redmond KL, Papafili A, Lawler M and Van Schaeybroeck S. Overcoming resistance to targeted therapies in cancer. Semin Oncol 2015; 42: 896-908.

[2] Cree IA and Charlton P. Molecular chess? Hallmarks of anti-cancer drug resistance. BMC Cancer 2017; 17: 10.

[3] Kohno M, Horibe T, Haramoto M, Yano Y, Ohara K, Nakajima O, Matsuzaki K and Kawakami K. A novel hybrid peptide targeting EGFR-expressing cancers. Eur J Cancer 2011; 47: 773-783.

[4] Tada N, Horibe T, Haramoto M, Ohara K, Kohno M and Kawakami K. A single replacement of histidine to arginine in EGFR-lytic hybrid peptide demonstrates the improved anticancer activity. Biochem Biophys Res Commun 2011; 407: 383-388.

[5] Kikuchi O, Ohashi S, Horibe T, Kohno M, Nakai Y, Miyamoto S, Chiba T, Muto M and Kawakami K. Novel EGFR-targeted strategy with hybrid peptide against oesophageal squamous cell carcinoma. Sci Rep 2016; 6: 22452.

[6] Linardou H, Dahabreh IJ, Kanaloupiti D, Siannis F, Bafaloukos D, Kosmidis P, Papadimitriou CA and Murray S. Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet Oncol 2008; 9: 962-972.

[7] Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ and Zalcberg JR. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008; 359: 1757-1765.

[8] Gaowa A, Horibe T, Kohno M, Sato K, Harada H, Hiraoka M, Tabata Y and Kawakami K. Combination of hybrid peptide with biodegradable gelatin hydrogel for controlled release and enhancement of anti-tumor activity in vivo. J Control Release 2014; 176: 1-7.

[9] Gaowa A, Horibe T, Kohno M, Tabata Y, Harada H, Hiraoka M and Kawakami K. Enhancement of anti-tumor activity of hybrid peptide in conjugation with carboxymethyl dextran via disulfide linkers. Eur J Pharm Biopharm 2015; 92: 228-236.

[10] Gaowa A, Horibe T, Kohno M and Kawakami K. Bile acid as an effective absorption enhancer for oral delivery of epidermal growth factor receptor-targeted hybrid peptide. J Pharm Sci 2018; 107: 1322-1329.

[11] Chevrier R, Sautou V, Pinon V, Demeocq F and Chopineau J. Stability and compatibility of a mixture of the anti-cancer drugs etoposide, cytarabine and daunorubicine for infusion. Pharm Acta Helv 1995; 70: 141-148.

[12] Barber NA and Ganti AK. Pulmonary toxicities from targeted therapies: a review. Target Oncol 2011; 6: 235-243.

[13] Inoue A, Saijo Y, Maemondo M, Gomi K, Tokue Y, Kimura Y, Ebina M, Kikuchi T, Moriya T and Nukiwa T. Severe acute interstitial pneumonia and gefitinib. Lancet 2003; 361: 137-139.

[14] Kudoh S, Kato H, Nishiwaki Y, Fukuoka M, Nakata K, Ichinose Y, Tsuboi M, Yokota S, Nakagawa K, Suga M, Japan Thoracic Radiology G, Jiang H, Itoh Y, Armour A, Watkins C, Higenbottam T and Nyberg F. Interstitial lung disease in Japanese patients with lung cancer: a cohort and nested case-control study. Am J Respir Crit Care Med 2008; 177: 1348-1357.

[15] Takano T, Ohe Y, Kusumoto M, Tateishi U, Yamamoto S, Nokihara H, Yamamoto N, Sekine I, Kunitoh H, Tamura T, Kodama T and Saijo N. Risk factors for interstitial lung disease and predictive factors for tumor response in patients with advanced non-small cell lung cancer treated with gefitinib. Lung Cancer 2004; 45: 93-104.

[16] Hotta K, Kiura K, Tabata M, Harita S, Gemba K, Yonei T, Bessho A, Maeda T, Moritaka T, Shibayama T, Matsuo K, Kato K, Kanehiro A, Tanimoto Y, Matsuo K, Ueoka H and Tanimoto M. Interstitial lung disease in Japanese patients with non-small cell lung cancer receiving gefitinib: an analysis of risk factors and treatment outcomes in Okayama Lung Cancer Study Group. Cancer J 2005; 11: 417-424.

[17] Suzuki H, Aoshiba K, Yokohori N and Nagai A. Epidermal growth factor receptor tyrosine kinase inhibition augments a murine model of pulmonary fibrosis. Cancer Res 2003; 63: 5054-5059.

[18] Miettinen PJ, Berger JE, Meneses J, Phung Y, Pedersen RA, Werb Z and Derynck R. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature 1995; 376: 337-341.

[19] Ware LB and Matthay MA. The acute respiratory distress syndrome. N Engl J Med 2000; 342: 1334-1349.

[20] Whitsett JA, Weaver TE, Lieberman MA, Clark JC and Daugherty C. Differential effects of epidermal growth factor and transforming growth factor-beta on synthesis of Mr = 35,000 surfactant-associated protein in fetal lung. J Biol Chem 1987; 262: 7908-7913.

[21] Inoue A, Xin H, Suzuki T, Kanehira M, Kuroki Y, Fukuhara T, Kikuchi T, Maemondo M, Nukiwa T

and Saijo Y. Suppression of surfactant protein a by an epidermal growth factor receptor tyrosine kinase inhibitor exacerbates lung inflammation. Cancer Sci 2008; 99: 1679-1684.

[22] Takahashi H, Fujishima T, Koba H, Murakami S, Kurokawa K, Shibuya Y, Shiratori M, Kuroki Y and Abe S. Serum surfactant proteins A and D as prognostic factors in idiopathic pulmonary fibrosis and their relationship to disease extent. Am J Respir Crit Care Med 2000; 162: 1109-1114.

[23] Vogel G, Trost W, Braatz R, Odenthal KP, Brusewitz G, Antweiler H and Seeger R. Pharmacodynamics, site and mechanism of action of silymarin, the antihepatoxic principle from Silybum mar. (L) Gaertn. 1. Acute toxicology or tolerance, general and specific (liver-) pharmacology. Arzneimittelforschung 1975; 25: 82-89.

[24] Mereish KA, Bunner DL, Ragland DR and Creasia DA. Protection against microcystin-LR-induced hepatotoxicity by Silymarin: biochemistry, histopathology, and lethality. Pharm Res 1991; 8: 273-277.

[25] Wu G, Deng X, Wu P, Shen Z and Xu H. Subacute toxicity of antimicrobial peptide S-thanatin in ICR mice. Peptides 2012; 36: 109-113.

[26] Kim SH, Kim KJ, Kim JH, Kwak JH, Song H, Cho JY, Hwang DY, Kim KS and Jung YS. Erratum: Comparison of doxorubicin-induced cardiotoxicity in the ICR mice of different sources. Lab Anim Res 2017; 33: 319.

Supplemental materials and methods

Materials

Triton-X 100 was purchased from Nacalai Tesque (Kyoto, Japan).

Zeta potential

Zeta potential values of the EGFR2R-lytic hybrid peptide in solution (saline or 5% glucose) were obtained with a Malvern Zetasizer Nano ZS (Westborough, MA) as described previously [1].

Hemolytic activity

The hemolytic activity of the EGFR2R-lytic hybrid peptide was assessed using murine erythrocytes as described previously [2].

In vivo tumor growth assay

Female 7-week-old BALB/c nu/nu mice (body weight, 17-20 g) were purchased from SLC Japan (Shizuoka, Japan) and maintained under specific pathogen-free conditions. Antitumor activity was evaluated with a mouse xenograft model as described previously [3]. Briefly, 5.0×10^6 MKN45 cells were suspended in 150 µL phosphate-buffered saline and transplanted subcutaneously into the right flank region of nude mice (day 0). The mice were randomized into 3 groups: saline (negative control), S-1 (positive control as an oral formulation), and EGFR2R-lytic hybrid peptide (15 mg/kg daily) on day 7, in which average tumor size was 150 mm^3. All agents were administered orally once daily for 5 days a week (Monday through Friday) for 4 weeks. Tumors were measured with a caliper, and tumor volume was calculated as follows: $width^2 \times length \times 0.5$. At the end of the observation period, the mice were killed and main tissues were removed. Histological examinations were performed by light microscopy after hematoxylin and eosin staining.

Supplemental References

[1] Gaowa A, Horibe T, Kohno M, Sato K, Harada H, Hiraoka M, Tabata Y and Kawakami, K. Combination of hybrid peptide with biodegradable gelatin hydrogel for controlled-release and improvement of anti-tumor activity in vivo. J Control Release 2014; 176: 1-7.

[2] Kawamoto M, Kohno M, Horibe T and Kawakami K. Immunogenicity and toxicity of transferring receptor-targeted hybrid peptide as a potent anticancer agent. Cancer Chemother Pharmacol 2013; 71: 799-807.

[3] Gaowa A, Horibe T, Kohno M and Kawakami K. Bile acid as an effective absorption enhancer for oral delivery of epidermal growth factor receptor-targeted hybrid peptide. J Pharm Sci 2018; 107: 1322-1329.

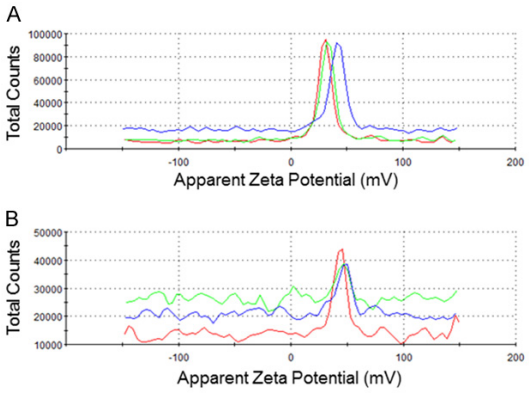


Figure S1. Zeta potential of the EGFR2R-lytic hybrid peptide in solution. The EGFR2R-lytic hybrid peptide was dissolved in saline (A) or 5% glucose solution (B), and the zeta potential values were obtained as described in the Supplemental Materials and Methods section.

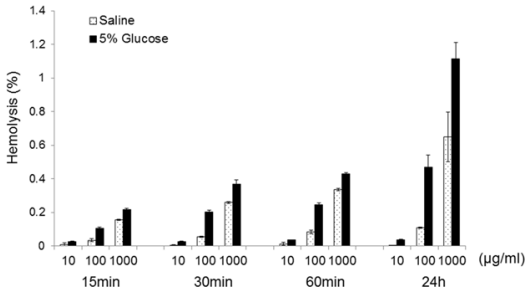


Figure S2. Hemolytic activity of isolated erythrocytes after incubation with the EGFR2R-lytic hybrid peptide in different solvents. Erythrocytes were incubated with different concentrations of the EGFR2R-lytic hybrid peptide (10-1000 μg/mL) for different periods of time (15, 30, and 60 min and 24 h). Hemolytic activity was measured as described in the Supplemental Materials and Methods section. Erythrocytes were treated with 0.1% Triton-X 100 as a positive control, and activity was set to 100% hemolytic activity. The values are mean ± S.D.

Table S1. Hematology parameters following i.v. injection of the EGFR2R-lytic hybrid peptide for 3 days

		0 mg/kg	5 mg/kg	10 mg/kg	15 mg/kg
Leucocytes	1000/μl	2.1 ± 0.8	3.0 ± 1.7	2.6 ± 1.4	3.4 ± 0.7
Erythrocytes	10000/μl	792.0 ± 70.7	888.0* ± 30.8	812.0 ± 43.1	840.6 ± 49.2
Hemoglibun	g/dl	12.8 ± 1.3	13.9 ± 0.4	12.8 ± 0.8	13.1 ± 0.5
Hematocrit	%	42.3 ± 4.3	47.3* ± 1.3	43.0 ± 2.3	45.1 ± 2.9
MCV	fl	53.3 ± 1.7	52.6 ± 0.9	52.2 ± 0.5	53.2 ± 0.5
MCH	pg	16.1 ± 0.4	15.7 ± 0.3	15.7 ± 0.3	15.5 ± 0.5
MCHC	%	30.2 ± 1.6	29.5 ± 0.9	29.6 ± 0.6	21.0 ± 10.9
Platelets	10000/μl	83.5 ± 15.1	75.0 ± 10.0	77.4 ± 4.0	85.3 ± 11.1
Reticulocytes	%	50.0 ± 7.7	48.4 ± 2.7	50.4 ± 4.7	42.2 ± 9.9
Neutrophils	%	14.3 ± 4.0	21.8 ± 9.7	16.2 ± 6.8	11.8 ± 3.8
Eosinophils	%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Basophils	%	0.3 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes	%	83.5 ± 3.9	75.8 ± 9.7	81.6 ± 7.8	85.8 ± 4.0
Monocytes	%	2.0 ± 0.8	2.4 ± 1.7	2.2 ± 1.3	2.4 ± 0.6

*$p < 0.05$ compared with the control group (0 mg/kg).

Table S2. Clinical biochemistry parameters following i.v. injection of the EGFR2R-lytic hybrid peptide for 3 days

		0 mg/kg	5 mg/kg	10 mg/kg	15 mg/kg
AST	U/L	559.0 ± 841.1	79.6 ± 15.3	124.8 ± 59.6	85.0 ± 25.0
ALT	U/L	56.0 ± 46.9	33.2 ± 6.4	140.0 ± 196.0	35.0 ± 9.1
ALP	U/L	312.7 ± 55.2	343.2 ± 16.2	288.2 ± 26.0	258.0* ± 15.3
γ-GT	U/L	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0
LAP	U/L	47.7 ± 10.7	44.0 ± 2.7	39.4 ± 4.3	36.2* ± 5.1
LDH	U/L	3035.7 ± 2764.2	1464.6 ± 326.4	2024.2 ± 826.3	1461.8 ± 301.9
CPK	U/L	3885.7 ± 6335.3	329.4 ± 60.3	423.6 ± 221.1	309.0 ± 285.5
Total bilirubin	mg/dL	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total protein	g/dL	5.0 ± 0.8	4.6 ± 0.4	4.1* ± 0.4	4.3 ± 0.4
Triglyceride	mg/dL	118.3 ± 88.2	65.2 ± 49.6	67.2 ± 29.4	61.8 ± 23.1
Total cholesterol	mg/dL	125.7 ± 38.7	110.0 ± 15.6	103.6 ± 14.5	104.0 ± 12.1
BUN	mg/dL	33.7 ± 7.1	26.8 ± 3.8	26.2 ± 4.4	31.2 ± 6.0
CRE	mg/dL	0.3 ± 0.3	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
Na	mEq/L	176.7 ± 28.9	164.4 ± 7.1	163.0 ± 7.2	159.6 ± 3.4
Cl	mEq/L	108.3 ± 2.9	96.8 ± 9.7	97.6 ± 8.8	106.2 ± 5.5
K	mEq/L	6.6 ± 3.0	5.3 ± 0.7	5.2 ± 0.6	5.4 ± 0.4
Ca	mEq/L	10.6 ± 2.8	6.9* ± 0.8	7.1 ± 0.8	7.0* ± 1.4
Inorganic P	mg/dl	21.7 ± 4.7	19.6 ± 4.2	17.0 ± 4.4	19.0 ± 3.7

*$p < 0.05$ compared with the control group (0 mg/kg).

Table S3. Hematology parameters following i.v. injection of the EGFR2R-lytic hybrid peptide for 7 days

		0 mg/kg	5 mg/kg	10 mg/kg	15 mg/kg
Leucocytes	1000/μl	2.4 ± 1.7	2.1 ± 0.9	3.1 ± 2.2	3.0 ± 0.6
Erythrocytes	10000/μl	815.8 ± 50.2	855.4 ± 29.9	853.6 ± 48.9	840.6 ± 26.2
Hemoglibun	g/dl	13.3 ± 0.9	13.9 ± 0.5	14.1 ± 0.8	13.8 ± 0.7
Hematocrit	%	43.9 ± 2.6	45.5 ± 1.5	45.6 ± 2.6	45.6 ± 1.8
MCV	fl	53.0 ± 0.0	52.6 ± 0.6	53.0 ± 1.2	53.8 ± 0.8
MCH	pg	16.2 ± 0.2	16.2 ± 0.2	16.4 ± 0.4	16.4 ± 0.4
MCHC	%	30.1 ± 0.4	30.6 ± 0.4	30.8 ± 1.2	30.3 ± 0.6
Platelets	10000/μl	98.8 ± 6.6	97.3 ± 5.3	91.7 ± 12.0	97.6 ± 7.2
Reticulocytes	%	56.0 ± 5.4	43.2* ± 1.3	44.8 ± 2.6	48.8 ± 13.1
Neutrophils	%	12.8 ± 5.7	17.6 ± 14.3	18.6 ± 8.7	17.0 ± 2.2
Eosinophils	%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Basophils	%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes	%	85.8 ± 6.1	79.4 ± 15.4	80.4 ± 8.7	81.0 ± 2.9
Monocytes	%	1.5 ± 0.6	3.0 ± 1.9	1.0 ± 0.0	2.0 ± 0.7

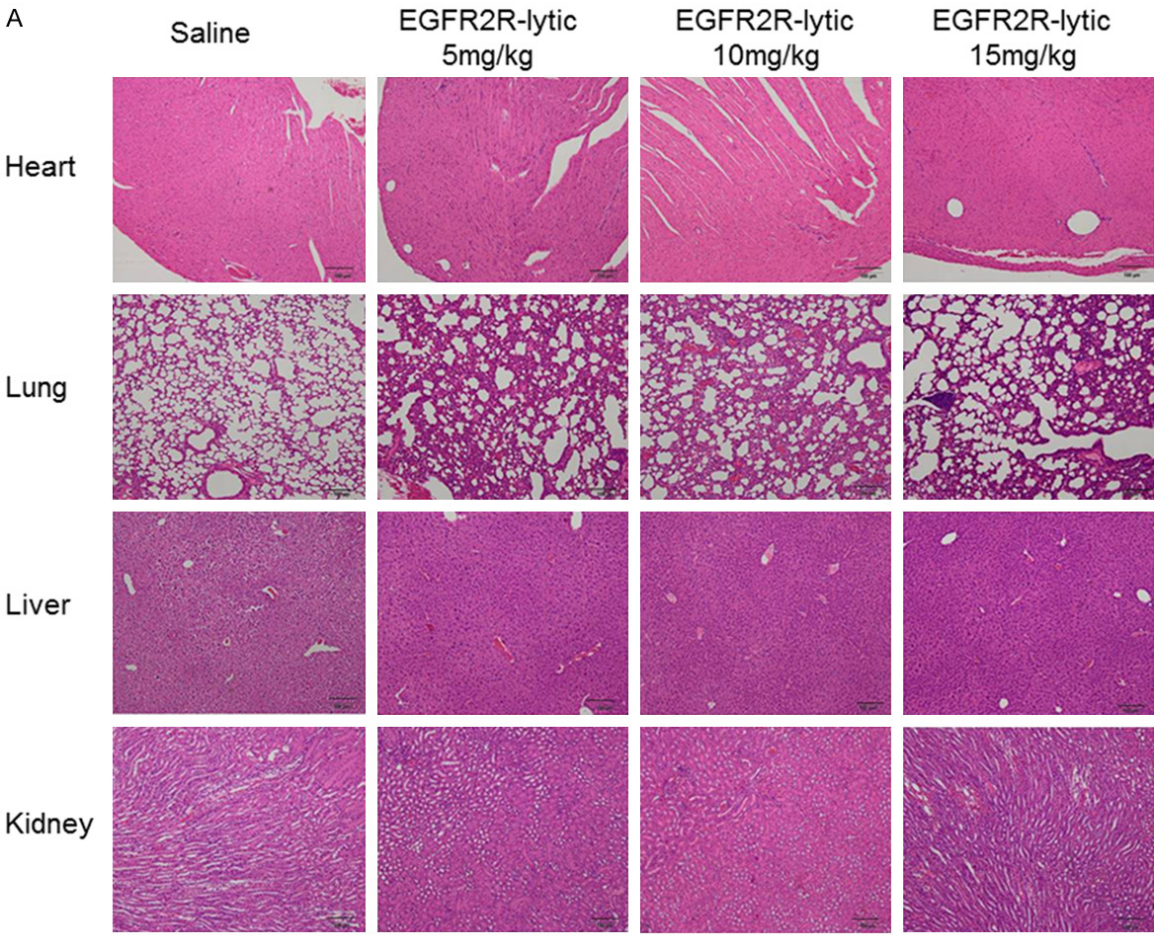
*$p < 0.05$ compared with the control group (0 mg/kg).

Table S4. Clinical biochemistry parameters following i.v. injection of the EGFR2R-lytic hybrid peptide for 7 days

		0 mg/kg	5 mg/kg	10 mg/kg	15 mg/kg
AST	U/L	77.3 ± 19.1	96.4 ± 24.2	78.8 ± 22.8	96.0 ± 29.9
ALT	U/L	40.8 ± 16.3	29.4 ± 4.4	28.4 ± 5.9	34.8 ± 12.9
ALP	U/L	359.3 ± 61.0	337.2 ± 44.2	357.6 ± 25.4	322.0 ± 48.6
γ-GT	U/L	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0

Toxicity testing of hybrid peptide

LAP	U/L	42.5 ± 5.0	47.0 ± 5.7	43.4 ± 2.7	43.0 ± 5.7
LDH	U/L	1291.3 ± 253.8	1617.4 ± 521.8	1204.4 ± 287.5	1716.0 ± 670.4
CPK	U/L	260.3 ± 153.6	387.4 ± 149.6	471.2 ± 323.6	398.8 ± 215.8
Total bilirubin	mg/dL	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total protein	g/dL	4.3 ± 0.7	4.7 ± 0.7	4.7 ± 0.4	4.2 ± 0.3
Triglyceride	mg/dL	78.5 ± 30.9	124.0 ± 44.6	62.3 ± 22.7	71.2 ± 24.4
Total cholesterol	mg/dL	105.8 ± 18.6	113.4 ± 19.9	99.8 ± 12.7	109.2 ± 4.6
BUN	mg/dL	28.8 ± 4.1	28.8 ± 4.2	31.4 ± 5.5	30.8 ± 5.1
CRE	mg/dL	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Na	mEq/L	165.0 ± 5.8	176.2 ± 14.5	168.4 ± 7.9	173.8 ± 14.9
Cl	mEq/L	93.8 ± 10.1	97.6 ± 9.4	94.8 ± 9.0	94.4 ± 8.2
K	mEq/L	5.6 ± 0.3	6.7 ± 0.8	5.7 ± 1.2	5.9 ± 0.9
Ca	mEq/L	7.9 ± 0.7	7.0 ± 0.9	7.5 ± 0.5	7.7 ± 0.8
Inorganic P	mg/dl	19.4 ± 4.8	21.8 ± 3.2	17.6 ± 2.7	21.1 ± 4.0



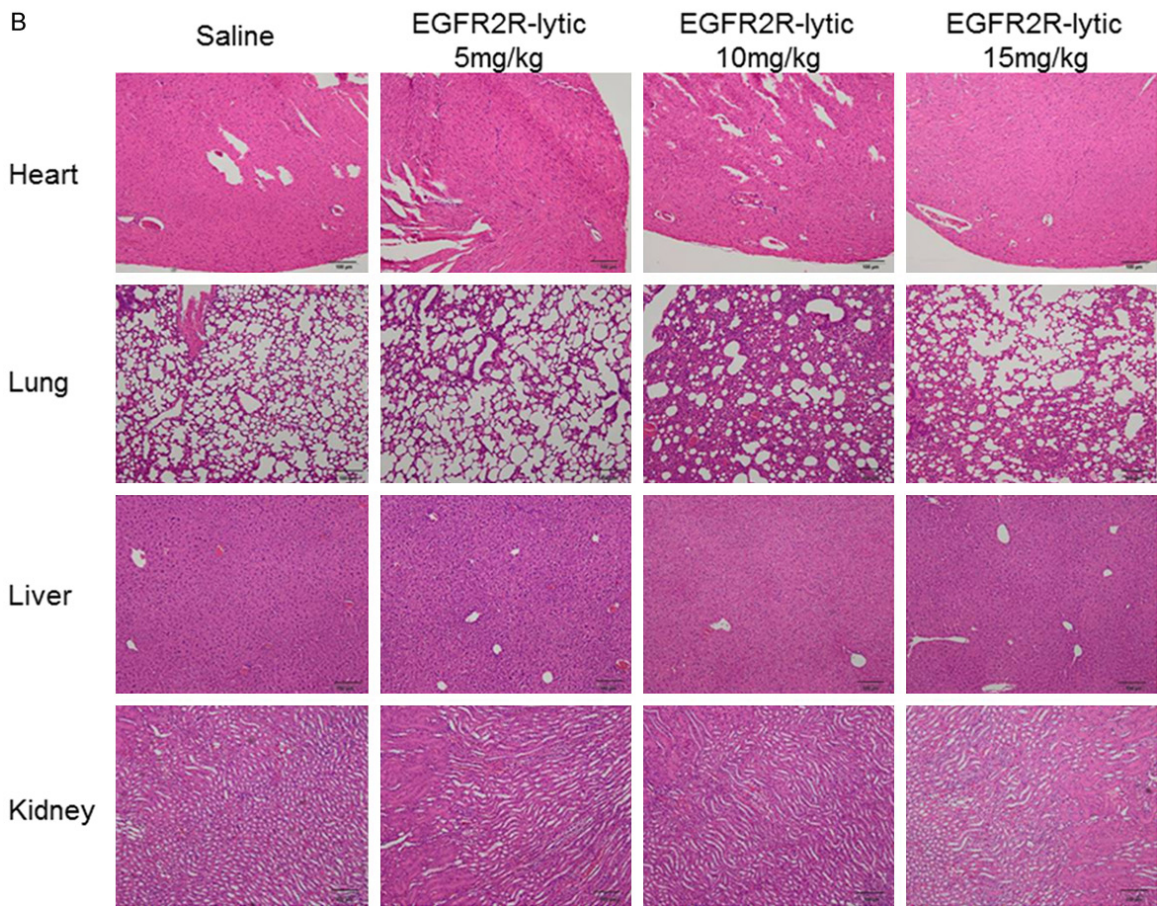


Figure S3. Hematoxylin-eosin-stained histological sections of major organs (heart, lung, liver, and kidney) prepared from the mice at 1 day (A) or 3 days (B) after i.v. injection of saline or EGFR2R-lytic hybrid peptide (5, 10, or 15 mg/kg). All scale bars are 100 μm.

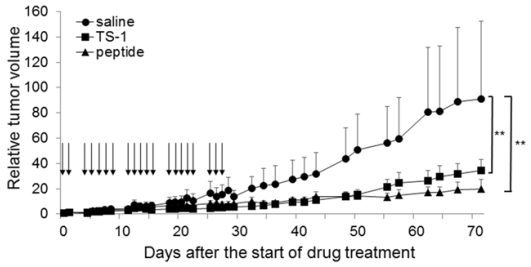


Figure S4. *In vivo* antitumor activity of the EGFR2R-lytic hybrid peptide against human gastric carcinoma xenografts. Therapeutic antitumor effect after oral administration of the EGFR2R-lytic hybrid peptide was evaluated by monitoring tumor growth as relative tumor volume, in which the volume of each tumor on day 1 was set to 1.0, as shown in the graph. Arrows in the graph indicate the days of oral administration. Values are expressed as the mean ± standard error of the mean. Saline and S-1 treatments were used as negative and positive control, respectively (**$P < 0.01$).