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Potential antitumor therapeutic application of *Grimontia hollisae* thermostable direct hemolysin mutants

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We report on the preparation of a new type of immunotoxin by conjugation of an epidermal growth factor receptor (EGFR)-binding peptide and an R46E mutation of thermostable direct hemolysin from *Grimontia hollisae*, (Gh-TDH^{R46E}/EB). The hybrid immunotoxin was purified to homogeneity and showed a single band with slight slower mobility than that of Gh-TDH^{R46E}. Cytotoxicity assay of Gh-TDH^{R46E}/EB on EGFR highly, moderately, low, and non-expressed cells, A431, MDA-MB-231, HeLa, and HEK293 cells, respectively, showed apparent cytotoxicity on A431 and MDA-MB-231 cells but not on HeLa or HEK293 cells. In contrast, no cytotoxicity was observed for these cells treated with either Gh-TDH^{R46E} or EB alone, indicating enhanced cytotoxic efficacy of Gh-TDH^{R46E} by the EGFR binding moiety. Further antitumor activity assay of Gh-TDH^{R46E}/EB in a xenograft model of athymic nude mice showed obvious shrinkage of tumor size and degeneration, necrosis, and lesions of tumor tissues compared to the normal tissues. Therefore, the combination of Gh-TDH^{R46E} with target affinity agents opens new possibilities for pharmacological treatment of cancers and potentiates the anticancer drug's effect.

Immunotoxins (IT), protein toxins conjugated with cell-specific mAbs or ligands, have been extensively investigated for their potential as therapeutic agents against diseases, including various infections and cancers. Immunotoxins specifically bind to and internalize target cells and kill them. Once internalized into the cytosol, one molecule of IT is sufficient to kill the cell, making it the most potent strategy for anticancer therapy. Numerous plant, bacterial, fungal, and animal toxins, such as ricin, abrin, gelonin, saporin, Pseudomonas exotoxin A, diphtheria toxin, restrictocin, and sticholysins have been evaluated as potential anticancer or anti-AIDS agents.(1–9) Recently, an IT containing human interleukin-2 and truncated diphtheria toxin has been approved for use in cutaneous T-cell lymphoma.(10–14) Another with an anti-CD22 antibody variable domain (Fv) and truncated Pseudomonas exotoxin has also been used to induce complete remission in cases of hairy-cell leukemia.(15,16)

Thermostable direct hemolysin (TDH) is a bacterial pore-forming toxin that lyses blood cells and biological membranes. It is a major virulence factor produced by the pathogenic Vibrio species, including *V. cholerae* non-O1 and O139, *V. parahaemolyticus*, *V. mimicus*, *V. alginolyticus*, and *Grimontia hollisae*.(17–21) *Grimontia hollisae* TDH is composed of 165 amino acid residues and exhibits a variety of biological activities, including hemolysis of various species of erythrocytes, cytotoxicity, enterotoxicity, and cardiotoxicity.(22–29) We previously characterized the individual or collective mutational effect of Tyr^{53}, Thr^{59}, and Ser^{63} of Gh-TDH on hemolytic activity, the Arrhenius effect, and biophysical properties, and identified its hepatotoxicity.(30–32) In addition, Tyr^{53} and Phe^{159} residues of Gh–TDH were found to be involved in directing dimer-based protein quaternary structure formation, whereas Arg^{46} affected monomer to tetramer formation. Structural determination of Gh-TDH revealed that the guanidium side-chain of Arg^{46} forms ion-pair networks with the carboxyl groups of Glu^{138} and Gln^{164} from the adjacent protomer. In addition, the monomeric form of the Arg^{46} mutation lost almost complete hemolytic activity.

In parallel, when human and mouse liver cells as well as HeLa or AGS cells were treated with the Gh-TDH, a clear cell morphological change and decline of viability was directly observed by microscopy and detected by MTT assay, respectively.(32) The cytotoxicity of Gh-TDH in cancer cells has provoked us to investigate its potential as an anticancer agent.

Epidermal growth factor receptor (EGFR), a ligand-stimulated tyrosine kinase, is ubiquitously expressed in all normal epithelial cells and plays diverse functions, including organ morphogenesis, maintenance, and repair. Increased levels of EGFR expression has been observed in cancer cells and implicated in numerous tumorigenic processes, including cell proliferation, angiogenesis, and metastasis, and decreased apoptosis.(33–35) Furthermore, elevated EGFR expression is associated with poor prognosis in head and neck, ovarian, bladder, and esophageal cancer.(36) The pathogenesis of EGFR in various cancers makes it an important target in cancer therapy. In order to target EGFR for cancer therapy, however, an EGFR-specific mAb or binding peptide sequence is prerequisite for targeting highly expressed EGFR cells.

In this study, we describe the construction of an IT using an EGFR-binding peptide (YHWYGYTPQNIVIGG) (EB) and the Arg^{46} to Glu mutation of Gh-TDH, Gh-TDH^{R46E}/EB, and evaluation of its anticancer efficacy. The EGFR-binding peptide, originally screened from a phage display peptide library, has been shown to internalize preferentially into EGFR highly expressing cells and accumulate in EGFR overexpressing tumor xenografts after i.v. delivery *in vivo*.(37–39) The results showed significant decline of cytoviability and remarkable shrinkage of tumor size when human epithelial carcinoma cell line A431 and a mouse tumor model were treated with Gh-TDH^{R46E}/EB, respectively.

Materials and Methods

Bacterial strains and materials. The *G. hollisae* strain ATCC 33564 was obtained in a freeze-dried form from the Culture Collection and Research Center of the Food Industry Research and Development Institute (Hsin-Chu, Taiwan). Phenyl Sepharose 6 Fast Flow and protein molecular weight standards were purchased from GE Healthcare (Piscataway, NJ, USA). The protein assay kit was obtained from Bio-Rad (Hercules, CA, USA). Protein purification chemicals were obtained from Calbiochem (La Jolla, CA, USA).

Molecular cloning, expression, and purification of Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB proteins. The cloning of EGFR-binding sequence (*eb*) into the WT or R46E mutated *Gh-tdh* gene was carried out according to previous publications with the following primers: TDH-EGFR_N1 (5′-ATgAAATACAgACATCT-3′) and TDH-EGFR_C1 (5′-gAATTCgCCCTTTTATCCACCTCCTATAATATTCTgCggTgTgATCggTACCAATgATATTgTTgAgATTC-3′).(30,31) The recombinant pTOPO-*Gh-tdh-eb* and pTOPO-*Gh-tdh^{R46E}-eb* expression plasmids were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA) to ensure its fidelity in the subsequent protein expression and purification experiments. The recombinant plasmids were transformed into *Escherichia coli* BL21(DE3)(pLysS) cells, and selected for decrease or loss of hemolytic activity for growth on 5% sheep blood agar plates. Next, the Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB fusion proteins were produced and purified as previously reported.(30,31) The protein identities of the SDS-PAGE bands corresponding to Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB fusion proteins were confirmed by MALDI-TOF/TOF spectrometry.

Hemolytic activity assay of Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB proteins. Hemolytic activity was assayed according to a previously described method using rabbit erythrocytes that were washed three times with the 100 mM PBS (pH 7.6) and resuspended at a concentration of 4% (v/v).(31) One hundred percent hemolysis was defined as the A_{540} of hemoglobin released from erythrocytes treated with 0.1% Triton X-100.

Cell culture and cytoviability assay. Four different cancer cell lines were used: human epithelial carcinoma cell line (A431), human cervical cancer cells (HeLa), human embryonic kidney 293 (HEK293), and human breast carcinoma cell line (MDA-MB-231). All cell lines were purchased from the Culture Collection and Research Center of the Food Industry Research and Development Institute. All cells were grown in a humidified atmosphere with 5% CO_2 at 37°C, subcultured with a 0.1% trypsin, 2 mM EDTA solution, and maintained in either DMEM (Invitrogen, Carlsbad, CA, USA) or RPMI-1640 (Invitrogen) supplemented with 10% heat-inactivated FBS (Bio-West, Miami, FL, USA) and 1% penicillin/streptomycin (Bio-West). The cytoviability of these cells after treatment with Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB was assessed with an MTT assay kit.(40) The cells were treated with serial dilutions of protein and maintained in a humidified atmosphere consisting of 5% CO_2 in air at 37°C for 24 h. The viability data were compared with the numbers of cells in the untreated cultures and expressed as means and standard deviations from three independent experiments.

Localization of Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB in cells. To detect the TDH binding interaction with cell surface, the Gh-TDH^{WT}/EB or Gh-TDH^{R46E}/EB fusion protein, conjugated FITC fluorescent probes was used. FITC-conjugated Gh-TDH^{WT}/EB or Gh-TDH^{R46E}/EB reactions were carried out using the FluoReporter FITC Protein Labeling Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. To detect the Gh-TDH^{WT}/EB or Gh-TDH^{R46E}/EB binding, 150 μg/mL Gh-TDH^{WT}/EB or Gh-TDH^{R46E}/EB conjugates was exposed to A431 cells and incubated for 1 h, then washed three times with PBS buffer. The plates were visualized by fluorescent microscopy.

Flow cytometry analysis. A431 cells with 4% in PBS-dextran (PBS with 10 mM dextran-10; molecular weight, 10 kDa; a colloidal inhibitor added to prevent the lysis of cell) were incubated with FITC-conjugated Gh-TDH^{WT}/EB or Gh-TDH^{R46E}/EB at a final concentration of 150 μg/mL at 37°C for 1 h. After washing with 1 mL PBS-dextran three times by centrifugation (500 *g*), the TDH-bound cells were suspended in 1 mL PBS-dextran and loaded for flow cytometry (Cytomics FC500; Beckman Coulter Bera, CA, USA).(41,42) The fluorescent intensity of FITC was recorded at 525 nm with excitation at 488 nm, according to the manufacturer's protocol.

Clonogenic assay. The *in vitro* cytotoxicity of Gh-TDH^{R46E}/EB against A431 cells was determined by a colony forming assay using the standard protocol.(43) The cells were plated in triplicate in 6-cm Petri dishes with 3 mL MEM containing 10% FBS (v/v) and were allowed to attach for 24 h. A431 cells were treated with Gh-TDH^{R46E}/EB and the live cells were collected. A431 cells were cultured for 7 days in medium containing various concentrations (0–100 μg/mL) of Gh-TDH^{R46E}/EB. Colonies consisting of at least 50 cells were scored after staining with crystal violet, and the results were expressed as a percentage relative to untreated cells, based on the number of colonies. The cells were washed, fixed, and stained with crystal violet (0.25% w/v in 25% alcohol v/v). Colonies consisting of >50 cells were scored. The percentage of colony survival was determined from the number of colonies formed in the control and treatment groups.

Antitumor activity of Gh-TDH^{WT}/EB or Gh-rTDH^{R46E}/EB in tumor xenografts *in vivo*. Mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Female nude mice (4–6 weeks of age) were inoculated s.c. with 1×10^6 A431 cells in 100 μL PBS.(44,45) Once a tumor mass of 500 mm^3 was established, the animals were treated with Gh-TDH^{WT}/EB, Gh-TDH^{R46E}/EB, or EB peptide once every 4 days. Subsequently, the tumor volumes were measured using a caliper every 4 days, and the volume was calculated using the formula: volume (mm^3) = length × width × height.

Histological examination and quantitative image analyses. The tissue samples were fixed in formalin, decalcified (if necessary), and embedded in paraffin. Paraffin-embedded tissue specimens were cut to 3–5 μm in thickness and stained with hematoxylin–eosin–saffron using routine methods. Serial sections of each tissue specimen were also obtained for special staining. Each set of observations were chosen from surfaces of 10 randomly chosen areas.

Results

Identification and hemolytic activity assay of Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB toxins. The recombinant pTOPO-*Gh-tdh*, pTOPO-*Gh-tdh-eb*, and pTOPO-*Gh-tdh*^{R46E}-*eb* expression plasmids were transformed into *E. coli* BL21(DE3) (pLysS) competent cells for protein overexpression. The produced Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB proteins were collected, extracted, and subjected to protein purification repeatedly using Phenyl Sepharose 6 Fast Flow columns. Electrophoresis of the purified Gh-TDH^{WT}, Gh-TDH^{WT}/EB, and Gh-TDH^{R46E}/EB revealed homogeneous bands, as determined by SDS-PAGE. A single band with slightly slower mobility than that of Gh-TDH^{WT} was observed for Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB, indicating that the toxins were fused with the EB fragment and purified to homogeneity (Fig. 1a). The hemolytic activities of Gh-TDH^{WT}, Gh-TDH^{WT}/EB, and Gh-TDH^{R46E}/EB were detected on sheep blood agar (Fig. 1b). Following the purification of Gh-TDH^{WT}/EB and Gh-TDH^{R46E}/EB, we assessed the hemolytic activity of the purified proteins on human erythrocytes. The median lethal doses (LD$_{50}$) of Gh-TDH^{WT}, Gh-TDH^{WT}/EB, and Gh-TDH^{R46E}/EB proteins were determined to be 2, 35 μg/mL, and negligible, respectively (Fig. 1c). The Gh-TDH^{WT}/EB fusion protein lost 50% of its hemolytic activity when compared to Gh-TDH^{WT}. Furthermore, only negligible hemolytic activity could be detected from the mutated Gh-TDH^{R46E}/EB fusion protein. The partial hemolytic activity of the Gh-TDH^{WT}/EB fusion protein on human erythrocytes excluded itself as a candidate for further investigation, as it may lyse erythrocytes before reaching the target cells. In contrast, the negligible hemolytic activity of the Gh-TDH^{R46E}/EB fusion protein prevented the truncated abolition of human erythrocytes and side effect before reaching the target cells.

We next investigated the cytoviability of the Gh-TDH^{R46E}/EB fusion protein on A431, MDA-MB-231, HeLa, and HEK293 cells, which show different EGFR expression

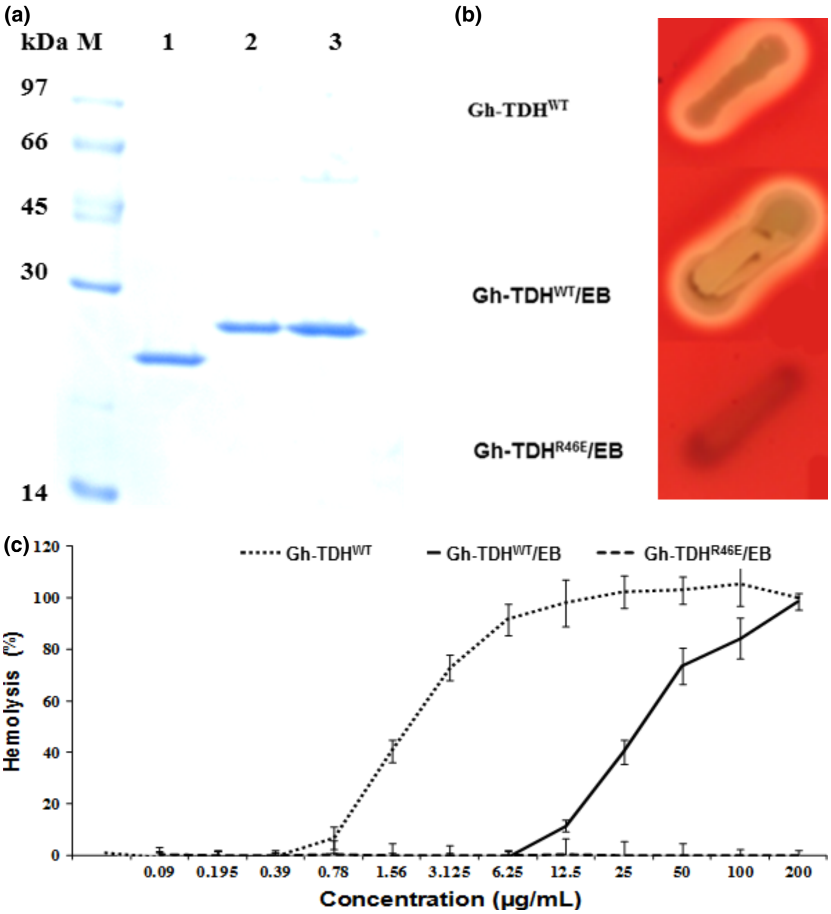

Fig. 1. Purification and characterization of hemolytic activity of G. *hollisae* thermostable direct hemolysin (Gh-TDH) conjugated with WT (Gh-TDH^{WT}), WT and epidermal growth factor receptor-binding peptide (Gh-TDH^{WT}/EB), and mutated R46E /EB (Gh-TDH^{R46E}/EB) proteins expressed in *Escherichia coli* pLysS strain. (a) The overexpressed protein was passed through a Phenyl Sepharose 6 Fast Flow column to obtain a homogeneous protein. M, molecular markers; Lane 1, Gh-TDH^{WT}; Lane 2, Gh-TDH^{WT}/EB; Lane 3, Gh-TDH^{R46E}/EB. (b) Hemolytic activity detected when Gh-TDH^{WT}, Gh-TDH^{WT}/EB, and Gh-TDH^{R46E}/EB containing *E. coli* pLysS cells were grown on a sheep blood agar plate. (c) Comparison of hemolytic activity of Gh-TDH^{WT}, Gh-TDH^{WT}/EB, and Gh-TDH^{R46E}/EB on human erythrocytes. The data are the means and SD from at least three independent experiments.

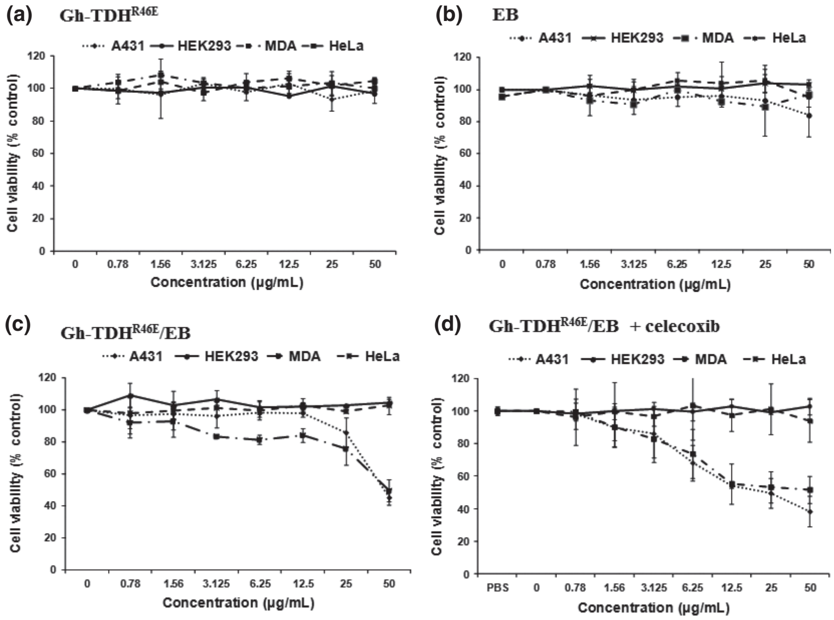

Fig. 2. Effect of immunotoxins on cellular viability. (a) *Grimontia hollisae* thermostable direct hemolysin (Gh-TDH) conjugated with mutated R46E Gh-TDH^{R46E} (a), epidermal growth factor receptor-binding peptide (EB) (b), Gh-TDH^{R46E}/EB (c), and Gh-TDH^{R46E}/EB + celecoxib (10 μM, 0.38 μg/mL) (d). Different concentrations of Gh-TDH^{R46E} (0–50 μg/mL), EB, Gh-TDH^{R46E}/EB, or Gh-TDH^{R46E}/EB + celecoxib (10 μM) were incubated with A431, MDA-MB-231, HeLa, and HEK293 cells in growth media for 24 h at 37°C. After incubation, viable cell numbers were quantified by the MTT assay. The PBS control was referenced as 100% viable. Results are expressed as percentages of PBS treated cells. Results are the mean ± SD of three independent experiments carried out in triplicate.

levels, using the MTT assay. The A431, MDA-MB-231, HeLa, and HEK293 cells express EGFR at high, moderate, low, and non-expression levels, respectively. Cells were treated with different concentrations of EB, Gh-TDH^{R46E}, or Gh-TDH^{R46E}/EB fusion protein and then incubated at 37°C for 24 h. As shown in Figure 2(a), no cytotoxicity was observed with the addition of the mutated Gh-TDH^{R46E} protein alone for up to 50 μg/mL concentration. In addition, only slight cytotoxicity was detected for cell lines that highly express EGFR, such as A431, when treated with EB alone, as shown in Figure 2(b). In contrast, apparent cytotoxicity was detected when cells expressing EGFR moderately (MDA-MB-231) or highly (A431) were treated with the Gh-TDH^{R46E}/EB fusion protein (Fig. 2c). These results indicate that conjugation of EGFR binding moiety to protein enhanced the IC$_{50}$ for cancer cells, suggesting that cancer cells are more susceptible to the EGFR–lytic protein (Gh-TDH^{R46E}/EB) than to the lytic protein (Gh-TDH^{R46E}) alone. Furthermore, the targeting to EGFR by the combination of Gh-TDH^{R46E} with EGFR binding moiety increased the cytotoxic activity to cancer cells with EGFR expression. Thus, Gh-TDH^{R46E}/EB induces cytotoxic activity in cancer cells rather than normal cells, and has superior cytotoxic activity to EGFR-expressing cancer cells. Perhaps the EGFR binding moiety enhances the cytotoxic efficacy of Gh-TDH^{R46E}, which is dependent on the expression level of EGFR on the cell surface.

We also determined the potential cooperative effect of treatment with celecoxib and Gh-TDH^{R46E}/EB. A431, MDA-MB-231, HeLa, and HEK293 cells were treated with different concentrations of Gh-TDH^{R46E}/EB alone or in combination with 10 μM celecoxib (Fig. 2d). As shown in Figure 2(d), celecoxib combined with Gh-TDH^{R46E}/EB enhanced the IC$_{50}$ for A431 cells approximately fourfold than that of Gh-TDH^{R46E}/EB, when compared with Gh-TDH^{R46E}/EB alone. In contrast, celecoxib alone did not induce killing of A431 or MDA-MB-231 cells. These findings suggest that the pore-forming activity of Gh-TDH^{R46E}/EB enhances the internalization of celecoxib into the cells and kills the cells.

Inhibition of A431 cell growth by Gh-rTDH^{R46E}/EB. To study the inhibitory effect of the Gh-TDH^{R46E}/EB fusion protein on the proliferation and differentiation of A431 cells, a colony forming assay was carried out.[46,47] The A431 cells were cultured for 7 days in medium containing various concentrations (0–100 μg/mL) of Gh-TDH^{R46E}/EB and the grown colonies consisting of at least 50 cells were scored and expressed as a percentage relative to untreated cells, after staining with crystal violet. The results showed that Gh-TDH^{R46E}/EB attenuated the *in vitro* proliferation of A431 cell and inhibited the cell growth in a concentration-dependent manner (data not shown).

Internalization assay flow cytometric analysis of Gh-TDH^{R46E}/EB to A431 cells. To evaluate the effect of EGFR-binding peptide on enhancing toxins on cell membrane binding and morphological change, A431 cells were treated with FITC-conjugated Gh-TDH^{R46E}/EB or Gh-TDH^{R46E} for 1 h at 37°C and visualized by fluorescent microscopy. As shown in Figure 3(a), both the Gh-TDH^{R46E}/EB and Gh-TDH^{R46E} treated A431 cells showed morphological changes, including cell detachment and fluorescent signals, but with differential effect. The Gh-TDH^{R46E}/EB treated A431 cells showed stronger fluorescent signals than that of the Gh-TDH^{R46E} treated cells, indicating specific binding of TDH protein to targeted cells can be enhanced by a functional peptide at the C-terminus.

In parallel, the binding abilities of Gh-TDH^{R46E}/EB to the cell surface of A431 cells were analyzed using flow cytometry and the results were compared with that of Gh-TDH^{R46E}. A431 cells were incubated with FITC-conjugated Gh-TDH^{R46E}/EB or Gh-TDH^{R46E} at 37°C for 1 h and then analyzed by flow cytometry with fluorescence intensity detection of FITC at 525 nm. Cells treated with BSA, which showed an inability to bind to the cell membrane, were analyzed in the same way as the negative control. The fluorescence intensity increased when the A431 cells were treated with Gh-TDH^{R46E}/EB and Gh-TDH^{R46E}, but remained constant for BSA treated A431 cells (Fig. 3b). In addition, higher fluorescence intensity was observed for Gh-TDH^{R46E}/EB or Gh-TDH^{R46E} than for BSA. These results suggest that the EGFR-binding peptide can enhance the binding of Gh-TDH^{R46E} to the targeted cells.

Antitumor activity of Gh-rTDH^{R46E}/EB *in vivo*. To assess the antitumor effect of the Gh-TDH^{R46E}/EB in a xenograft model of human cancer, the A431 cells were implanted s.c. into athy-

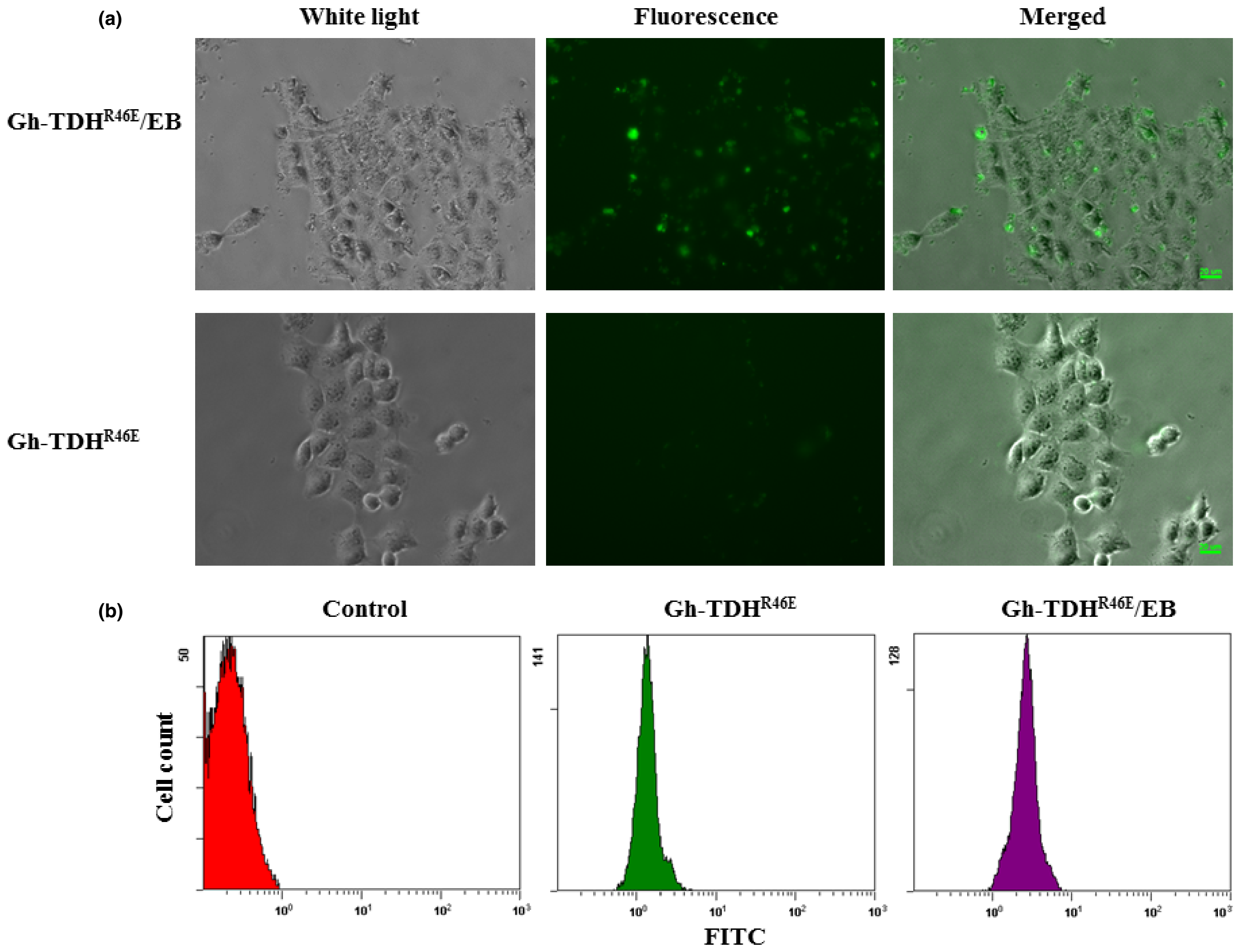

Fig. 3. Characterization of *Grimontia hollisae* thermostable direct hemolysin (Gh-TDH) conjugated with mutated R46E and epidermal growth factor receptor-binding peptide (EB) (Gh-TDH^{R46E}/EB)-induced A431 cell death. (a) Fluorescence image analysis of Gh-TDH^{R46E}/EB binding to A431 cells. Left panel, white field of cell image; middle panel, fluorescence images; right panel, merged diagrams. Scale bar = 20 μm. (b) Flow cytometric analysis of Gh-TDH^{R46E}/EB binding to A431 cells. Left panel, control peak; middle panel, Gh-TDH^{R46E}; right panel, Gh-TDH^{R46E}/EB.

mic nude mice. Gh-TDH^{R46E}/EB was injected i.v. at a dose of 6 mg/kg, two times a week for a total of five doses. In parallel, PBS was injected as a negative control. Tumor volume was measured every 2 days after treatment. As shown in Figure 4(a), significant tumor size difference was observed for Gh-TDH^{R46E}/EB injected mice, as compared with that of PBS, EB, and Gh-TDH^{R46E} injected mice. The tumor volume of the A431 cells in the dosage group was inhibited in growth (1000 mm^3), relative to the control group with saline (3000 mm^3) (Fig. 4c). There were no differences in body weights or blood chemistries between the saline and toxin treated groups.

Histopathological findings of mouse. To investigate the virulence of IT *in vivo*, nude mice were i.v. injected with PBS, EB, or Gh-TDH^{R46E}/EB protein. Histological examination of various organs (liver, kidney, and spleen) after treatment with PBS, EB, or Gh-TDH^{R46E}/EB was carried out by staining with H&E. As shown in Figure 5, no significant lesions in spleen were observed when mice were i.v. injected with PBS, EB, or Gh-TDH^{R46E}/EB (kidney and liver results not shown). In contrast, degeneration, necrosis, and lesions were found in the tumor region in Gh-TDH^{R46E}/EB injected mice but not in those injected with PBS or EB. These results indicated the specific binding affinity and lytic activity of Gh-TDH^{R46E}/EB to tumor tissues compared with normal tissues.

Discussion

Immunotoxins have been postulated by Paul Ehrlich for more than a century and envisaged as a "magic bullet" in cancer therapy.(48) Bacterial or plant toxins such as ricin A chain gelonin, saporin, and Pseudomonas exotoxin A have been used to prepare anti-HER-2 immunotoxins.(49–51) Denileukin diftitox (Ontak; Eisai Corp., Woodcliff Lake, NJ, USA), an engineered protein combining interleukin-2 and diphtheria toxin, has been approved by the US FDA for treatment of cutaneous T-cell lymphoma.(52) The relationships between increased levels of EGFR expression in various cancers and high levels of EGFR signaling associated with high degrees of invasiveness, metastasis, and drug

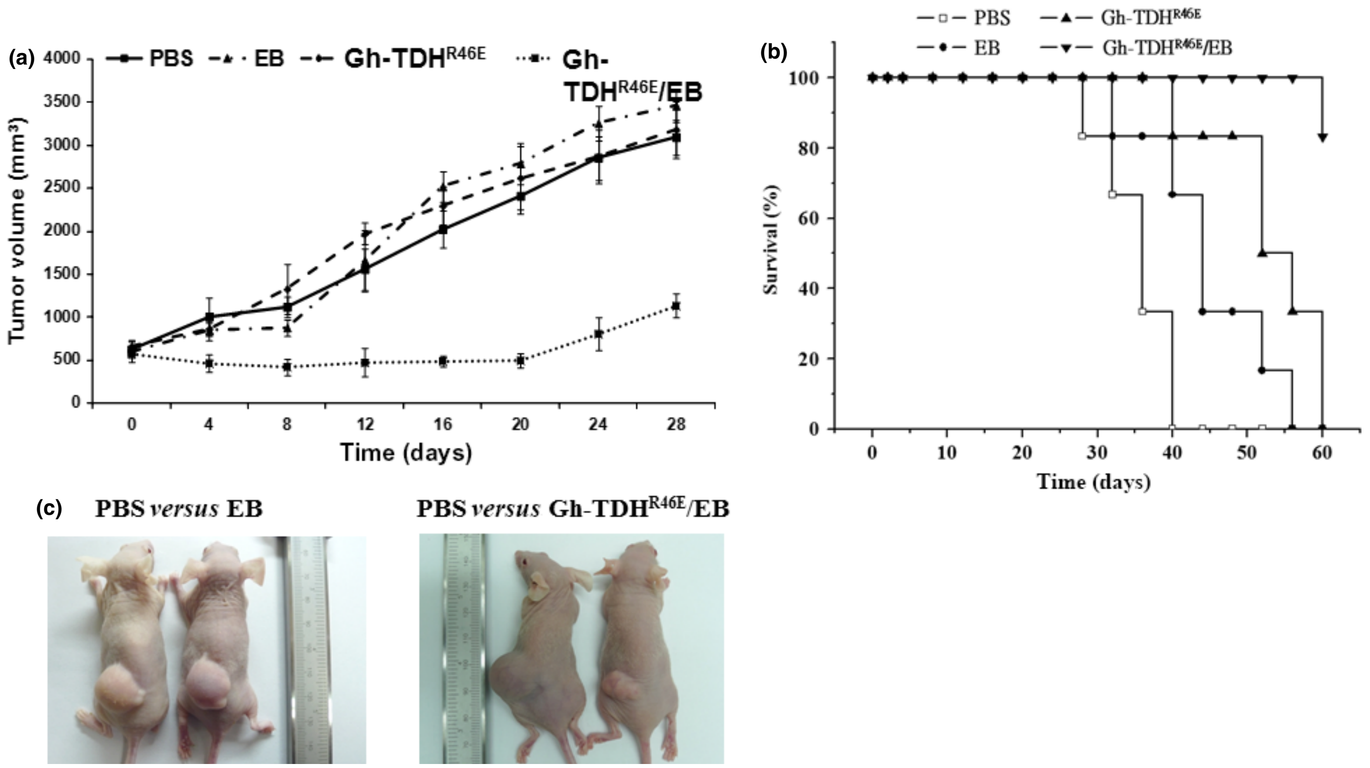

Fig. 4. Antitumor activity of *Grimontia hollisae* thermostable direct hemolysin (Gh-TDH) conjugated with mutated R46E and epidermal growth factor receptor-binding peptide (EB) (Gh-TDH^{R46E}/EB) *in vivo*. (a) Antitumor activity of PBS, EB, Gh-TDH^{R46E}, or Gh-TDH^{R46E}/EB treatment on nude mice bearing A431 cells. Nude mice bearing A431 cells (n = 3/group) were treated with PBS, EB, Gh-TDH^{R46E}, or Gh-TDH^{R46E}/EB (6 mg/kg) by i.v. injection twice per week for a total of five doses. Tumor volume was measured every 2 days after treatment. (b) Significant differences after treatment were found for the treated group compared with the negative control group in terms of tumor size. (c) Kaplan–Meier survival curve of PBS, EB, Gh-TDH^{R46E}, or Gh-TDH^{R46E}/EB treatment in nude mice bearing A431 cells. Mice (n = 6/group) were treated with PBS, EB, Gh-TDH^{R46E}, or Gh-TDH^{R46E}/EB (6 mg/kg) by i.v. injection twice per week for a total of five doses. The survival curve was measured every 3 days after treatment.

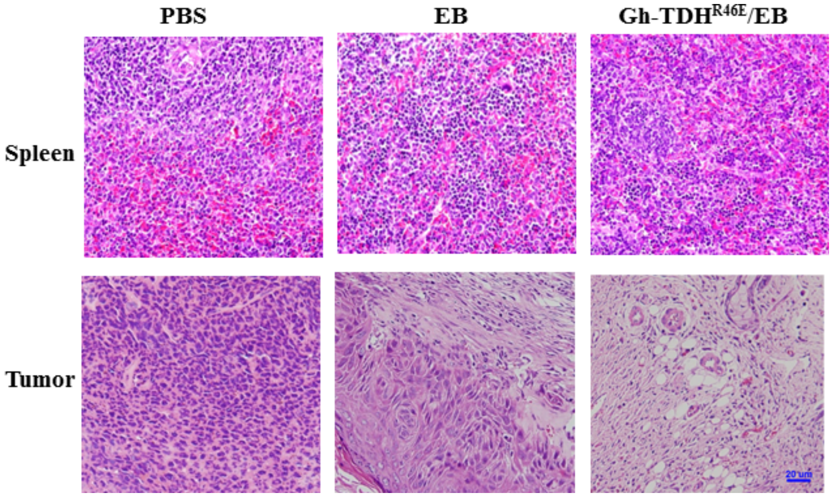

Fig. 5. Histological examination of spleen and tumor after treatment with PBS, epidermal growth factor receptor-binding peptide (EB), or *Grimontia hollisae* thermostable direct hemolysin (Gh-TDH) conjugated with mutated R46E and and EB (Gh-TDH^{R46E}/EB). Images (magnification, ×400) of spleen and region of tumor from A431-bearing nude mice treated with PBS, EB, or Gh-TDH^{R46E}/EB (6 mg/kg) seven times were obtained by staining with H&E.

resistance have been noticed, making it a promising target for novel anticancer agents. The application of pore-forming toxins to generate a novel class of anticancer drugs has not been extensively explored, except for two sea anemone cytolysins.(53) In this study, an EGFR-binding peptide was attached to the C-terminus of the Gh-TDH^{R46E} mutated toxin and its cytotoxicity was investigated in cells with high, moderate, low, and non-expression of EGFR (A431, MDA-MB-231, HeLa, and HEK293 cells, respectively). Significant decline of cytoviability on the A431 cell line highly expressing EGFR was observed when treated with Gh-TDH^{R46E}/EB, whereas no and slight cytotoxicity was observed with the addition of Gh-TDH^{R46E} and EB, respectively. In addition, dose-dependent inhibition was observed for A431 and MDA-MB-231 cells. Furthermore, we investigated the *in vivo* antitumor activity of Gh-TDH^{R46E}/EB in mice bearing A431 cells and showed suppression of tumor growth.

We show here that Gh-TDH^{R46E}/EB can induce cytotoxicity on EGFR-expressing A431 and MDA-MB-231 cells. The mechanism of potentiating cell death can be a direct consequence of initially increased binding affinity of the EGFR-binding peptide to the cell membrane and then penetrating the cell surface by Gh-TDH^{R46E}. Consistent with the observation is the enhanced cytotoxic activity of treatment with celecoxib and Gh-TDH^{R46E}/EB in A431 cells. Perhaps the conjugation of EGFR binding moiety to Gh-TDH^{R46E} might synergistically enhance the cytotoxic efficacy of Gh-TDH^{R46E} to the targeted cells. Furthermore, the inherent toxicity of protein aggregates identified in diseases caused by β-strand-rich proteins, such as Alzheimer's disease, type II diabetes, and Creutzfeldt–Jakob disease, was also observed in Gh-TDH protein, which exhibits a phenomenon known as the Arrhenius effect and shows paradoxical responses to heat treatment and reversible fibrillar aggregation. Therefore, the agglutinative property of Gh-TDH could imply a common mechanism for cell death among β-strand-rich proteins.

In summary, the results of the present study indicate the feasibility of applying the pore-forming toxin, Gh-TDH, to anticancer drug development by the construction of an IT containing an EGFR-binding peptide and the C-terminus of the Gh-TDH^{R46E} mutated toxin. The results showed significant decline of cytoviability and remarkable shrinkage of tumor size in human epithelial carcinoma cell line A431 and a mouse tumor model, respectively, treated with Gh-TDH^{R46E}/EB. Therefore, the combination of Gh-TDH with target affinity agents opens new possibilities for pharmacological treatment of cancers and potentiates the anticancer drug's effect. In future, Gh-TDH could also be used in therapies conjugated to target specific peptides or antibodies or small molecules. The original cytotoxicity of Gh-TDH could be hidden as a pro-toxin by conjugating a target-specific protease sequence to the N-terminus of Gh-TDH. After affinity binding to the target cells, the pro-toxin could be converted into the potent toxin through the cleavage at the N-terminus of Gh-TDH by the protease. Further studies to increase the specificity and efficacy of the Gh-TDH-conjugated IT are underway.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Fitzgerald D, Pastan I. Targeted toxin therapy for the treatment of cancer. *J Natl Cancer Inst* 1989; **81**: 1455–63.
2 Zimmermann S, Wels W, Froesch BA, Gerstmayer B, Stahel RA, Zangemeister-Wittke U. A novel immunotoxin recognising the epithelial glycoprotein-2 has potent antitumoural activity on chemotherapy-resistant lung cancer. *Cancer Immunol Immunother* 1997; **44**: 1–9.
3 Engert A, Diehl V, Schnell R *et al.* A phase-I study of an anti-CD25 ricin A-chain immunotoxin (RFT5-SMPT-dgA) in patients with refractory Hodgkin's lymphoma. *Blood* 1997; **89**: 403–10.
4 Pastan II, Kreitman RJ. Immunotoxins for targeted cancer therapy. *Adv Drug Deliv Rev* 1998; **31**: 53–88.
5 Rathore D, Batra JK. Construction, expression and characterization of chimaeric toxins containing the ribonucleolytic toxin restrictocin: intracellular mechanism of action. *Biochem J* 1997; **324**: 815–22.
6 Schnell R, Borchmann P, Staak JO *et al.* Clinical evaluation of ricin A-chain immunotoxins in patients with Hodgkin's lymphoma. *Ann Oncol* 2003; **14**: 729–36.
7 Frankel AE, Kreitman RJ. CLL immunotoxins. *Leuk Res* 2005; **29**: 985–6.
8 Youn YS, Na DH, Yoo SD, Song SC, Lee KC. Carbohydrate-specifically polyethylene glycol-modified ricin A-chain with improved therapeutic potential. *Int J Biochem Cell Biol* 2005; **37**: 1525–33.
9 Avila AD, Calderón CF, Pérez RM, Pons C, Pereda CM, Ortiz AR. Construction of an immunotoxin by linking a monoclonal antibody against the human epidermal growth factor receptor and a hemolytic toxin. *Biol Res* 2007; **40**: 173–83.
10 Piascik P. FDA approves fusion protein for treatment of lymphoma. *J Am Pharm Assoc* 1999; **39**: 571–2.
11 Foss FM. DAB(389)IL-2 (ONTAK): a novel fusion toxin therapy for lymphoma. *Clin Lymphoma* 2000; **1**: 110–6.
12 Sandvig K, van Deurs B. Membrane traffic exploited by protein toxins. *Annu Rev Cell Dev Biol* 2002; **18**: 1–24.
13 Kawakami K, Nakajima O, Morishita R, Nagai R. Targeted anticancer immunotoxins and cytotoxic agents with direct killing moieties. *ScientificWorldJournal* 2006; **6**: 781–90.
14 Kreitman RJ. Immunotoxins for targeted cancer therapy. *AAPS J* 2006; **8**: E532–51.
15 Kreitman RJ, Hansen HJ, Jones AL, Fitzgerald DJ, Goldenberg DM, Pastan I. Pseudomonas exotoxin-based immunotoxins containing the antibody LL2 or LL2-Fab' induce regression of subcutaneous human B-cell lymphoma in mice. *Cancer Res* 1993; **53**: 819–25.
16 Theuer CP, Kreitman RJ, FitzGerald DJ, Pastan I. Immunotoxins made with a recombinant form of Pseudomonas exotoxin A that do not require proteolysis for activity. *Cancer Res* 1993; **53**: 340–7.
17 Yoh M, Honda T, Miwatani T. Purification and partial characterization of a non-O1 *Vibrio cholerae* hemolysin that cross-reacts with thermostable direct hemolysin of *Vibrio parahaemolyticus*. *Infect Immun* 1986; **52**: 319–22.
18 Nishibuchi M, Janda JM, Ezaki T. The thermostable direct hemolysin gene (tdh) of *Vibrio hollisae* is dissimilar in prevalence to and phylogenetically distant from the tdh genes of other vibrios: implications in the horizontal transfer of the tdh gene. *Microbiol Immunol* 1996; **40**: 59–65.
19 Shinoda S. Protein toxins produced by pathogenic vibrios. *J Nat Toxins* 1999; **8**: 259–69.
20 Shinoda S, Nakagawa T, Shi L *et al.* Distribution of virulence-associated genes in *Vibrio mimicus* isolates from clinical and environmental origins. *Microbiol Immunol* 2004; **48**: 547–51.
21 Cai SH, Wu ZH, Jian JC, Lu YS. Cloning and expression of gene encoding the thermostable direct hemolysin from *Vibrio alginolyticus* strain HY9901, the causative agent of vibriosis of crimson snapper (*Lutjanus erythopterus*). *J Appl Microbiol* 2007; **103**: 289–96.
22 Miyamoto Y, Kato T, Obara Y, Akiyama S, Takizawa K, Yamai S. In vitro hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. *J Bacteriol* 1969; **100**: 1147–9.
23 Sakurai J, Honda T, Junguji Y, Arita M, Miwatani T. Cytotoxic effect of the thermostable direct hemolysin produced by *Vibrio parahaemolyticus* on FL cells. *Infect Immun* 1976; **13**: 876–83.
24 Goshima K, Honda T, Hirata M, Kikuchi K, Takeda Y, Miwatani T. Stopping the spontaneous beating of mouse and rat myocardial cells in vitro by a toxin from *Vibrio parahaemolyticus*. *J Mol Cell Cardiol* 1977; **9**: 191–213.
25 Honda T, Iida T. The pathogenicity of *Vibrio parahaemolyticus* and the role of the thermostable direct haemolysin and related haemolysin. *Rev Med Microbiol* 1993; **4**: 106–13.
26 Tang GQ, Iida T, Yamamoto K, Honda T. Ca(2+)-independent cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin (TDH) on Intestine 407, a cell line derived from human embryonic intestine. *FEMS Microbiol Lett* 1995; **134**: 233–8.
27 Raimondi F, Kao JPY, Fiorentini C, Fabbri A, Donelli G, Gasparini N. Enterotoxicity and cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin in in vitro systems. *Infect Immun* 2000; **68**: 3180–5.
28 Naim R, Yanagihara I, Iida T, Honda T. *Vibrio parahaemolyticus* thermostable direct hemolysin can induce an apoptotic cell death in Rat-1 cells from inside and outside of the cells. *FEMS Microbiol Lett* 2001; **195**: 237–44.

29 Lang PA, Kaiser S, Myssina S, Birka C, Weinstock C, Northoff H. Effect of *Vibrio parahaemolyticus* haemolysin on human erythrocytes. *Cell Microbiol* 2004; **6**: 391–400.

30 Wang YK, Huang SC, Wu YF *et al.* Purification, crystallization and preliminary X-ray analysis of a thermostable direct haemolysin from *Grimontia hollisae*. *Acta Crystallogr F Struct Biol Cryst Commun* 2011; **67**: 224–7.

31 Wang YK, Huang SC, Wu YF *et al.* Site-directed mutations of thermostable direct hemolysin from *Grimontia hollisae* alter its Arrhenius effect and biophysical properties. *Int J Biol Sci* 2011; **7**: 333–46.

32 Lin YR, Chen YL, Wang KB *et al.* The thermostable direct hemolysin from *Grimontia hollisae* causes acute hepatotoxicity *in vitro* and *in vivo*. *PLoS ONE* 2013; **8**: e56226.

33 Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995; **19**: 183–232.

34 Raymond E, Faivre S, Armand JP. Epidermal growth factor receptor tyrosine kinase as a target for anticancer therapy. *Drugs* 2000; **60**(Suppl 1): 15–23.

35 Venook AP. Epidermal growth factor receptor-targeted treatment for advanced colorectal carcinoma. *Cancer* 2005; **103**: 2435–46.

36 Nicholson RI, Gee JM, Harper ME. EGFR and cancer prognosis. *Eur J Cancer* 2001; **37**(Suppl 4): S9–15.

37 Li Z, Zhao R, Wu X *et al.* Identification and characterization of a novel peptide ligand of epidermal growth factor receptor for targeted delivery of therapeutics. *FASEB J* 2005; **19**: 1978–85.

38 Song S, Liu D, Peng J *et al.* Peptide ligand-mediated liposome distribution and targeting to EGFR expressing tumor in vivo. *Int J Pharm* 2008; **363**: 155–61.

39 Kohno M, Horibe T, Haramoto M *et al.* A novel hybrid peptide targeting EGFR-expressing cancers. *Eur J Cancer* 2011; **47**: 773–83.

40 Marks DC, Belov L, Davey MW, Davey RA, Kidman AD. The MTT cell viability assay for cytotoxicity testing in multidrug-resistant human leukemic cells. *Leuk Res* 1992; **16**: 1165–73.

41 Harrison A, Liu Z, Makweche S, Maskell K, Qi H, Hale G. Methods to measure the binding of therapeutic monoclonal antibodies to the human Fc receptor FcγRIII (CD16) using real time kinetic analysis and flow cytometry. *J Pharm Biomed Anal* 2012; **63**: 23–8.

42 Du J, Li M, Zhang D *et al.* Flow cytometry analysis of glucocorticoid receptor expression and binding in steroid-sensitive and steroid-resistant patients with systemic lupus erythematosus. *Arthritis Res Ther* 2009; **11**: R108.

43 Bradley EC, Catino JJ, Issell BF *et al.* Cell-mediated inhibition of tumor colony formation in agarose by resting and interleukin 2-stimulated human lymphocytes. *Cancer Res* 1985; **45**: 1464–8.

44 Sparrow S, Jones M, Billington S, Stace B. The in vivo malignant transformation of mouse fibroblasts in the presence of human tumour xenografts. *Br J Cancer* 1986; **53**: 793–7.

45 Fiebig HH, Maier A, Burger AM. Clonogenic assay with established human tumour xenografts: correlation of in vitro to in vivo activity as a basis for anticancer drug discovery. *Eur J Cancer* 2004; **40**: 802–20.

46 Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ. Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haematol* 1999; **107**: 275–81.

47 Sarma NJ, Takeda A, Yaseen NR. Colony forming cell (CFC) assay for human hematopoietic cells. *J Vis Exp* 2010; **46**: 2195–9.

48 Ehrlich P. The colected papers of Paul EEhrlich. In: Himmelweite F, Marquardt M, Doles H, Eds. Oxford: Pergamon Press 1956; pp. 596–6118.

49 Boyer CM, Pusztai L, Wiener JR *et al.* Relative cytotoxic activity of immunotoxins reactive with different epitopes on the extracellular domain of the c-erbB-2 (HER-2/neu) gene product p185. *Int J Cancer* 1999; **82**: 525–31.

50 Rosenblum MG, Shawver LK, Marks JW, Brink J, Cheung L, Langton-Webster B. Recombinant immunotoxins directed against the c-erb-2/HER2/neu oncogene product: in vitro cytotoxicity, pharmacokinetics, and in vivo efficacy studies in xenograft models. *Clin Cancer Res* 1999; **5**: 865–74.

51 von Minckwitz G, Harder S, Hövelmann S *et al.* Phase I clinical study of the recombinant antibody toxin scFv(FRP5)-ETA specific for the ErbB2/HER2 receptor in patients with advanced solid malignomas. *Breast Cancer Res* 2005; **7**: R617–26.

52 Heisler I, Keller J, Tauber R, Sutherland M, Fuchs H. A cleavable adapter to reduce nonspecific cytotoxicity of recombinant immunotoxins. *Int J Cancer* 2003; **103**: 277–82.

53 Soletti RC, de Faria GP, Vernal J *et al.* Potentiation of anticancer-drug cytotoxicity by sea anemone pore-forming proteins in human glioblastoma cells. *Anticancer Drugs* 2008; **19**: 517–25.