Potential antitumor therapeutic application of Grimontia hollisae thermostable direct hemolysin mutants

Sheng-Cih Huang,¹ Yu-Kuo Wang,¹ Wan-Ting Huang,¹ Tsam-Ming Kuo,¹² Bak-Sau Yip,¹ Tien-Hsiung Thomas Li³ and Tung-Kung Wu¹

¹Department of Biological Science and Technology, National Chiao Tung University, Hsin-Chu; ²Department of Neurology, National Taiwan University Hospital, Hsin-Chu; ³Graduate Institute of Biochemistry, National Chung Hsing University, Taichung, Taiwan

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Correspondence
Tung-Kung Wu, Department of Biological Science and Technology, National Chiao Tung University, 30068 Hsin-Chu, Taiwan.
Tel: +886-3-5729287; Fax: +886-3-5725700; E-mail: tkwu@mail.nctu.edu.tw
and
Tien-Hsiung Thomas Li, Institute of Biochemistry, National Chung Hsing University, 40227 Taichung, Taiwan.
Tel: +886-4-22840468; Fax: +886-4-22853487; E-mail: lithomas@dragon.nchc.edu.tw
and
Bak-Sau Yip, Department of Neurology, National Taiwan University Hospital, Hsin-Chu 30059, Taiwan.
Tel: +886-3-5326151; Fax: +886-3-5322140; E-mail: neuron.nctu@gmail.com

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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.¹³–⁹⁹ Recently, an IT containing human interleukin-2 and truncated diphtheria toxin has been approved for use in cutaneous T-cell lymphoma.¹⁰⁰–¹⁰⁴ 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.¹¹⁵–¹¹⁶

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.¹¹⁷–²¹¹ 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.¹²²–²²⁹ We previously characterized the individual or collective mutational effect of Tyr¹⁵³, Thr¹⁵⁹, and Ser¹⁶⁵ of Gh-TDH on hemolytic activity, the Arrhenius effect, and biophysical properties, and identified its hepatotoxicity.²³⁰–²³⁹ In addition, Tyr¹⁵³ and Phe¹⁵⁹ residues of Gh–TDH were found to be involved in directing dimer-based protein quaternary structure formation, whereas Arg⁴⁶ affected monomer to tetramer formation. Structural determination of Gh-TDH revealed that the guanidium side-chain of Arg⁴⁶ forms ion-pair networks with the carboxyl groups of Glu¹⁸⁸ and Gln¹⁶⁴ from the adjacent protomer. In addition, the monomeric form of the Arg⁴⁶ mutation lost almost complete hemolytic activity.

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-TDH⁴⁶⁶/EB). The hybrid immunotoxin was purified to homogeneity and showed a single band with slight slower mobility than that of Gh-TDH⁴⁶⁶. Cytotoxicity assay of Gh-TDH⁴⁶⁶/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-TDH⁴⁶⁶ or EB alone, indicating enhanced cytotoxic efficacy of Gh-TDH⁴⁶⁶ by the EGFR binding moiety. Further antitumor activity assay of Gh-TDH⁴⁶⁶/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-TDH⁴⁶⁶ with target affinity agents opens new possibilities for pharmacological treatment of cancers and potentiates the anticancer drug’s effect.
were produced and purified as previously reported. 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, metastasis, and decreased apoptosis. Furthermore, elevated EGFR expression is associated with poor prognosis in head and neck, ovarian, bladder, and esophageal cancer. 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 (YHWGYGTPQNVIGG) (EB) and the Arg46EB 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. 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-TDHWT/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'-ATGAATATTACAgACATCT-3') and TDH-EGFR_C1 (5'-CCAGATCCgCgCCTTATACCACTTATAATATgCCgTgCgATgCCgTgACCCACgTgATgTTgAgACATCT-3'). 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-TDHWT/EB and Gh-TDH(R46E)/EB fusion proteins were produced and purified as previously reported. The protein identities of the SDS-PAGE bands corresponding to Gh-TDHWT/EB and Gh-TDH(R46E)/EB fusion proteins were confirmed by MALDI-TOF/TOF spectrometry.

**Hemolytic activity assay of Gh-TDHWT/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). One hundred percent hemolysis was defined as the A590 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% CO2 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-TDHWT/EB and Gh-TDH(R46E)/EB was assessed with an MTT assay kit. The cells were treated with serial dilutions of protein and maintained in a humidified atmosphere consisting of 5% CO2 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-TDHWT/EB and Gh-TDH(R46E)/EB in cells.** To detect the TDH binding interaction with cell surface, the Gh-TDHWT/EB or Gh-TDH(R46E)/EB fusion protein, conjugated FITC fluorescent probes was used. FITC-conjugated Gh-TDHWT/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-TDHWT/EB or Gh-TDH(R46E)/EB binding, 150 μg/mL Gh-TDHWT/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-TDHWT/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, Brea, CA, USA). The fluorescent intensity of FITC was recorded at 525 nm with excitation at 488 nm, according to the manufacturer’s protocol.

**Cloning analysis.** The in vitro cytotoxicity of Gh-TDH(R46E)/EB against A431 cells was determined by a colony forming assay using the standard protocol. 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<sup>WT</sup>/EB or Gh-TDH<sup>R46E</sup>/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<sup>6</sup> A431 cells in 100 µL PBS.<sup>(44,45)</sup> Once a tumor mass of 500 mm<sup>3</sup> was established, the animals were treated with Gh-TDH<sup>WT</sup>/EB, Gh-TDH<sup>R46E</sup>/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<sup>3</sup>) = 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<sup>WT</sup>/EB and Gh-TDH<sup>R46E</sup>/EB toxins. The recombinant pTOPO-Gh-tdh, pTOPO-Gh-tdh-eb, and pTOPO-Gh-tdh<sup>R46E</sup>-eb expression plasmids were transformed into E. coli BL21(DE3) (pLysS) competent cells for protein overexpression. The produced Gh-TDH<sup>WT</sup>/EB and Gh-TDH<sup>R46E</sup>/EB proteins were collected, extracted, and subjected to protein purification repeatedly using Phenyl Sepharose 6 Fast Flow columns. Electrophoresis of the purified Gh-TDH<sup>WT</sup>, Gh-TDH<sup>WT</sup>/EB, and Gh-TDH<sup>R46E</sup>/EB revealed homogeneous bands, as determined by SDS-PAGE. A single band with slightly slower mobility than that of Gh-TDH<sup>WT</sup> was observed for Gh-TDH<sup>WT</sup>/EB and Gh-TDH<sup>R46E</sup>/EB, indicating that the toxins were fused with the EB fragment and purified to homogeneity (Fig. 1a). The hemolytic activities of Gh-TDH<sup>WT</sup>, Gh-TDH<sup>WT</sup>/EB, and Gh-TDH<sup>R46E</sup>/EB were detected on sheep blood agar (Fig. 1b). Following the purification of Gh-TDH<sup>WT</sup>/EB and Gh-TDH<sup>R46E</sup>/EB, we assessed the hemolytic activity of the purified proteins on human erythrocytes. The median lethal doses (LD<sub>50</sub>) of Gh-TDH<sup>WT</sup>, Gh-TDH<sup>WT</sup>/EB, and Gh-TDH<sup>R46E</sup>/EB proteins were determined to be 2, 35 µg/mL, and negligible, respectively (Fig. 1c). The Gh-TDH<sup>WT</sup>/EB fusion protein lost 50% of its hemolytic activity when compared to Gh-TDH<sup>WT</sup>. Furthermore, only negligible hemolytic activity could be detected from the mutated Gh-TDH<sup>R46E</sup>/EB fusion protein. The partial hemolytic activity of the Gh-TDH<sup>WT</sup>/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<sup>R46E</sup>/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<sup>R46E</sup>/EB fusion protein on A431, MDA-MB-231, HeLa, and HEK293 cells, which show different EGFR expression levels.

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**Fig. 1.** Purification and characterization of hemolytic activity of *G. hollisae* thermostable direct hemolysin (Gh-TDH) conjugated with WT (Gh-TDH<sup>WT</sup>), WT and epidermal growth factor receptor-binding peptide (Gh-TDH<sup>WT</sup>/EB), and mutated R46E/EB (Gh-TDH<sup>R46E</sup>/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<sup>WT</sup>; Lane 2, Gh-TDH<sup>WT</sup>/EB; Lane 3, Gh-TDH<sup>R46E</sup>/EB. (b) Hemolytic activity detected when Gh-TDH<sup>WT</sup>, Gh-TDH<sup>WT</sup>/EB, and Gh-TDH<sup>R46E</sup>/EB containing *E. coli* pLysS cells were grown on a sheep blood agar plate. (c) Comparison of hemolytic activity of Gh-TDH<sup>WT</sup>, Gh-TDH<sup>WT</sup>/EB, and Gh-TDH<sup>R46E</sup>/EB on human erythrocytes. The data are the means and SD from at least three independent experiments.
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-TDHR46E, or Gh-TDHR46E/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-TDHR46E 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-TDHR46E/EB fusion protein (Fig. 2c). These results indicate that conjugation of EGFR binding moiety to protein enhanced the IC50 for cancer cells, suggesting that cancer cells are more susceptible to the EGFR–lytic protein (Gh-TDHR46E/EB) than to the lytic protein (Gh-TDHR46E) alone. Furthermore, the targeting to EGFR by the combination of Gh-TDHR46E with EGFR binding moiety increased the cytotoxic activity to cancer cells with EGFR expression. Thus, Gh-TDHR46E/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-TDHR46E, 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-TDHR46E/EB. A431, MDA-MB-231, HeLa, and HEK293 cells were treated with different concentrations of Gh-TDHR46E/EB alone or in combination with 10 µM celecoxib (Fig. 2d). As shown in Figure 2(d), celecoxib combined with Gh-TDHR46E/EB enhanced the IC50 for A431 cells approximately fourfold than that of Gh-TDHR46E/EB, when compared with Gh-TDHR46E/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-TDHR46E/EB enhances the internalization of celecoxib into the cells and kills the cells.

Inhibition of A431 cell growth by Gh-TDHR46E/EB. To study the inhibitory effect of the Gh-TDHR46E/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-TDHR46E/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-TDHR46E/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-TDHR46E/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-TDHR46E/EB or Gh-TDHR46E for 1 h at 37°C and visualized by fluorescent microscopy. As shown in Figure 3(a), both the Gh-TDHR46E/EB and Gh-TDHR46E treated A431 cells showed morphological changes, including cell detachment and fluorescent signals, but with differential effect. The Gh-TDHR46E/EB treated A431 cells showed stronger fluorescent signals than that of the Gh-TDHR46E 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-TDHR46E/EB to the cell surface of A431 cells were analyzed using flow cytometry and the results were compared with that of Gh-TDHR46E. A431 cells were incubated with FITC-conjugated Gh-TDHR46E/EB or Gh-TDHR46E 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-TDHR46E/EB and Gh-TDHR46E, but remained constant for BSA treated A431 cells (Fig. 3b). In addition, higher fluorescence intensity was observed for Gh-TDHR46E/EB or Gh-TDHR46E than for BSA. These results suggest that the EGFR-binding peptide can enhance the binding of Gh-TDHR46E to the targeted cells.

Antitumor activity of Gh-TDHR46E/EB in vivo. To assess the antitumor effect of the Gh-TDHR46E/EB in a xenograft model of human cancer, the A431 cells were implanted s.c. into athymic

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mic nude mice. Gh-TDHR46E/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-TDHR46E/EB injected mice, as compared with that of PBS, EB, and Gh-TDHR46E injected mice. The tumor volume of the A431 cells in the dosage group was inhibited in growth (1000 mm³), relative to the control group with saline (3000 mm³) (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-TDHR46E/EB protein. Histological examination of various organs (liver, kidney, and spleen) after treatment with PBS, EB, or Gh-TDHR46E/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-TDHR46E/EB (kidney and liver results not shown). In contrast, degeneration, necrosis, and lesions were found in the tumor region in Gh-TDHR46E/EB injected mice but not in those injected with PBS or EB. These results indicated the specific binding affinity and lytic activity of Gh-TDHR46E/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. Bacterial or plant toxins such as ricin A chain gelonin, saporin, and Pseudomonas exotoxin A have been used to prepare anti-HER-2 immunotoxins. Demileukin 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. 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
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 cytolyisins. 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-TDHR46E/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-TDHR46E. Consistent with the observation is the enhanced cytotoxic activity of treatment with celecoxib and Gh-TDHR46E/EB in A431 cells. Perhaps the conjugation of EGFR binding moiety to Gh-TDHR46E might synergistically enhance the cytotoxic efficacy of Gh-TDHR46E to the targeted cells. Furthermore, the inherent toxicity of protein aggregates identified in disease caused by β-strand-rich proteins, such as Alzheimer’s disease, type II diabetes, and Creutzfeldt-Jakob disease, was also observed in Gh-TDHR protein, which exhibits a phenomenon known as the Arrhenius effect and shows paradoxic responses to heat treatment and reversible fibrillar aggregation. Therefore, the agglutinative property of Gh-TDHR 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-TDHR, to anticancer drug development by the construction of an IT containing an EGFR-binding peptide and the C-terminus of the Gh-TDHR46E 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-TDHR46E/EB. Therefore, the combination of Gh-TDHR with target affinity agents opens new possibilities for pharmacological treatment of cancers and potentiates the anticancer drug’s effect. In future, Gh-TDHR could also be used in therapies conjugated to target specific peptides or antibodies or small molecules. The original cytotoxicity of Gh-TDHR could be hidden as a pro-toxin by conjugating a target-specific protease sequence to the N-terminus of Gh-TDHR. 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-TDHR by the protease. Further studies to increase the specificity and efficacy of the Gh-TDHR-conjugated IT are underway.

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Disclosure Statement

The authors have no conflict of interest.

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