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Original Article

Pre-clinical Exploratory Evaluation of Mab806-derived Immunotoxin in Targeting Solid Tumors with High Potency and Selectivity Towards $EGFR_{vIII}$

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Abstract

Background and objectives: The overexpression and mutation of EGFRs are associated with various types of human cancers. Epidermal growth factor receptor variant III ($EGFR_{vIII}$) is one of the most notable EGFR mutations, and is exclusively present in cancer cells, making it a potential therapeutic target. The present study aims to determine whether recombinant immunotoxin 86 (rIT86) can effectively kill $EGFR_{vIII}$-expressing cancer cells, while maintaining adequate levels of tolerance.

Methods: rIT86 consists of an antibody bivalent scFv obtained from $EGFR_{vIII}$-targeting antibody mab806, combined with protein cytotoxin DT390. The *in vitro* and *in vivo* effects were evaluated in $EGFR_{vIII}$-expressing tumor cell lines in culture and xenografts.

Results: rIT86 inhibited the proliferation of several cancer cell lines, including U87, A549, Du145, MDA-MB231 and A431, with high selectivity towards the mutant $EGFR_{vIII}$ over wild-type EGFR. The *in vivo* pre-clinical studies revealed the better survival and greater inhibition of xenograft tumors, when compared to $EGFR_{vIII}$-expressing tumor cells, and that these were achieved in mice treated with prolonged administration of low-dose rIT86, when compared to the control group.

Conclusions: The results demonstrated that rIT86 is very potent and highly selective for killing $EGFR_{vIII}$-expressing cancer cells. However, its potential clinical application warrants further investigation.

Keywords: EGFR; $EGFR_{vIII}$; rIT86; Glioma; Immunotoxin; Diphtheria toxin (DT).

Abbreviations: ACUC, Animal Care and Use Committee; ADPR, adenosine diphosphate ribosyl; ATCC, American Type Culture Collection; B.W., body weight; CMV, cytomegalovirus; DG44 CHO, dihydrofolate reductase negative (dhfr-) in Chinese Hamster Ovary (cells); DT390, diphtheria toxin; DTP, diphtheria-tetanus-pertussis; EGFR, epidermal growth factor receptor; $EGFR_{vIII}$, epidermal growth factor receptor variant III; EMS, ethyl methane sulfonate; FBS, fetal bovine serum; H&E, hematoxylin and eosin; HPLC, high-performance liquid chromatography; i.p., intraperitoneal; i.v., intravenous; MLD, minimal lethal doses; NAD, nicotinamide dinucleotide; NSCLC, non-small cell lung cancer; OMML, Otsuka Maryland Medicinal Laboratories; rIT86, recombinant immunotoxin 86; RT-PCR, reverse transcriptase-polymerase chain reaction; S.C., subcutaneous; SCID, severe combined immunodeficiency disease; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tdap, tetanus diphtheria pertussis; VL-VH, variable (domain) light (chain)-variable (domain) heavy (chain); wt, wild type.

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Introduction

Targeted cancer therapy uses specific cancer molecules as recognition targets to selectively identify and eliminate cancer cells without damaging normal cells. Immunotoxin is a fusion recombinant protein that consists of a targeting moiety (typically an antibody or fragment, or a ligand for a specific receptor on the cell surface) and an effector cytolethal moiety. The truncated form of diphtheria toxin (DT; DT390, with only catalytic and transmembrane domains) is one of the most frequently used cytolethal toxins in pre-clinical investigation.[1,2] For DT-based immunotoxins, when the ligand or antibody fragment recognizes the cancer cells, the transmembrane domain helps to translocate the catalytic domain into the cytosol,

where the latter acts by transferring the adenosine diphosphate ribosyl (ADPR) moiety from nicotinamide dinucleotide (NAD) to the post-transcriptionally modified histidine residue at 715 of elongation factor 2 (EF2), resulting in the irreversible inactivation of EF2, and the subsequent inhibition of protein synthesis of the targeted cancer cells and cell death. Ontak® (denileukin diftitox),[3,4] and more recently, Elzonris® (Tagraxofusp)[5] and LUMOXITI® (moxetumomab pasudotox)[6] have gained FDA approval for clinical application. To date, no immunotoxins against solid tumors have been approved for wide clinical use. Enhanced epidermal growth factor receptor (EGFR) expression is one of the critical signaling pathways involved in tumor development, such as tumor cell proliferation, metastasis and survival. Epidermal growth factor receptor variant III (EGFRvIII), which is a deletion of EGFR exons 2-7, resulting in a truncated extracellular domain, is only present in cancer cells, making it an ideal target for the development of both safe and effective immunotoxins.[7,8] That is, the use of a target-specific antibody that targets $EGFR_{vIII}$ against a tumor would be very safe and effectual. Although the development of DT-based immunotoxins against $EGFR_{vIII}$ (DT390-BiscFv806) has been reported,[8] DT390-BiscFv806 also binds to wild-type (wt) EGFR with high affinity, which is similar to inducing non-specific cytotoxicity. Considering that all newborns have received immunization diphtheria-tetanus-pertussis (DTP) against *Corynebacterium diphtheria* infection,[9] most patients have pre-existing antibodies against DT. Thus, cancer patients will rapidly respond to any DT-based medicine after administration due to the re-activation of memory immune cells. However, due to this, merely humanizing the scFv from monoclonal antibody mab806, such as DT390-BiscFv806,[8] is not sufficient. Therefore, the investigators chose to use the original murine mab806 sequence[10,11] in the recombinant immunotoxin named, rIT86, together with different peptide linkers among the functional domains, in order to minimize the off-target toxicity by enhancing the selectivity towards $EGFR_{vIII}$. In order to keep its post-translation modification, such as glycosylation patterns, if any, similar to the original antibody, the investigators developed and used DT-resistant DG44 CHO cells for the first time to produce rIT86. As a result, in both *in vitro* and *in vivo* studies, rIT86 was more potent than the previously reported DT390-BiscFv806 on cytotoxicity against several $EGRF_{vIII}$-expressing cancer cells, but there was no effect to wt EGFR at the sub-nM range, or even to wt EGFR overexpressing cells. The month-long application in mice revealed that rIT86 is tolerable, and the blood hematology and biochemistry tests demonstrated minimal side-effects. The subsequent xenograft studies mainly focused on exploring its feasibility for potential clinical applications.

Materials and methods

Design and generation of rIT86

The rIT86 was designed to have a linear sequence arrangement of DT390-VL-VH-VL-VH, where VL/VH is the light and heavy chain variable domain sequence from the murine mab806. PW is the two amino acid linker between DT390 and VL, while G4SG and (G4S) x3 are the peptide linkers between VL and VH, and between VH and VL, respectively. In order to remove the N-glycosylation sites in DT390, mutations (Ser18Ala and Asn235Ala) were introduced. The whole coding DNA sequence was chemically synthesized and assembled by Genscript (Piscataway, NJ, USA), and inserted in a pUC19-backbone plasmid, following the CMV promoter. This expression vector contains a DHFR selection marker suitable for DG44 CHO cell stable integration, amplification, and single high-yield clone selection.

DG44 CHO cells were a kind gift from Dr. Lawrence Chasin of Columbia University. These cells were cultured in Eagle's minimum essential medium, supplemented with double concentrations of vitamins and amino acids with 10% fetal calf serum, in a humidified atmosphere that contains 5% CO_2 at 37°C. The doubling time of these cells was approximately 12 hours. The clones of DT-resistant DG44 CHO cells were obtained by treating these cells with 300 pg/ml of ethyl methane sulfonate (EMS, Eastman Kodak), according to the procedure described by Baker *et al*.,[12,13] and selected in the presence of wt DT (Sigma, St. Louise) by increasing the concentration of DT from 0.001–10.000 guinea pig minimal lethal doses (MLD) per ml, followed by a series of limiting dilutions, in order to obtain the single DT-resistant clones.

The linearized expressing vector that contained the cDNA for rIT86 was transformed to DT-resistant DG44 cells by Lipofectamin (Invitrogen, CA). The production clones were selected using culture medium that contained 10% dialyzed fetal calf serum. The culture supernatants were analyzed under a non-reducing condition with SDS-PAGE (Life Technologies, Grand Island, NY, USA). The optimal clone was selected based on the gel electrophoresis, followed by the adaptation to chemically-defined serum-free medium in term of growth rate, and maximum cell density and yield in batch-fed 1,000-mL shaking flasks for 10–15 days. Next, the rIT86 in the harvested supernatants was purified based on a 4-step scheme, according to established protocols: diafiltration, capture by hydrophobic chromatography, borate anion exchange chromatography, and anion exchange chromatography. The recombinant product was characterized with 4–12% SDS-PAGE and HPLC, conforming to the same level of purity and solubility previously reported.[8]

Cell culture and cell proliferation assay

The human glioblastoma cell line U87, human lung adenocarcinoma cell line A549, human breast adenocarcinoma cell line MDA-MB231, human prostate carcinoma cell line Du145, and human epidermoid carcinoma cell line A431 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The single clones of the EGFR- and $EGFR_{vIII}$-expressing sublines were established by the stable transfection of EGFR or $EGFR_{vIII}$ to cells using the lentivirus, followed by puromycin selection and two rounds of limiting dilutions. The EGFR/$EGFR_{vIII}$ expression in the transfected cells were confirmed by RT-PCR and western blot with the EGFR Ab-5 antibody (Thermo Fisher Scientific, Cambridge, MA, USA). All cells were grown in DMEM medium (ATCC, Rockville, MD, USA) with 10% FBS and 100 unit/ml of penicillin-streptomycin, and incubated in a 37°C/5% CO_2 humidified incubator. At one day before the rIT86 treatment, the cells were seeded in 96-well plates at 5,000 cells/well (100 µl). On the day of treatment, the culture medium was removed, and the cells were treated in triplicate with a series of concentrations of rIT86 in fresh medium. Following the additional incubation of 48 hours at 37°C/5% CO_2, cell proliferation assay was performed with the CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega, San Diego, CA, USA), with the addition of 100 µl of reagent to each well of cells. After 15 minutes of incubation at room temperature, the luminescence was measured using a 96-well plate reader (BioTek Gen5). The data analyses were performed using ANOVA (two-tailed), and plotted as mean luminescent ± standard error of the means relative to controls.

Hollow fiber assay

In order to optimize the amount of rIT86 to be administered for *in vivo* studies, hollow fibers that contained the same amount of alive $EGFR_{vIII}$-expressing tumor cells, with micro-pores that only allowed the drug to come in, while preventing cells from moving out, were used at the beginning of the tumor model studies.[14] Briefly, C57BL6 mice, (male, 8–10 weeks old, obtained from the Jackson Laboratory, Bar Harbor, ME, USA) were subjected to general anesthesia using an oxygen/isoflurane chamber (Pitman-Moore, Inc., Mundelein, IL, USA). For the intraperitoneal (i.p.) implants, a small incision was made through the skin and musculature of the dorsal abdominal wall. Then, fibers that contained the same amount of freshly trypsinized $EGFR_{vIII}$-expressing tumor cells were inserted into the peritoneal cavity in the craniocaudal direction, and the incision was closed with skin staples. For subcutaneous (s.c.) implants, a small skin incision was performed at the nape of the neck, in order to allow for the insertion of the 11-gauge tumor implant trocar (Popper & Sons, Inc., New Hyde Park, NY, USA). The trocar, which contained the hollow fiber samples, was caudally inserted through the s.c. tissues, and the fibers were deposited during the withdrawal of the trocar. In general, each mouse hosted up to six hollow fiber samples, in two physiologic compartments (i.p. and s.c.). The drug at a dose of up to 100 µg/kg of body weight (b.w.) was administered intravenously (i.v.) through the tail vein, once daily (q.d.), for three consecutive days, while 100 µl of saline was injected i.v. as the control. At three days after the drug administration, the hollow fibers were removed from the animal, and the cells collected from the hollow fibers were measured using the CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega).

All animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Otsuka Maryland Medicinal Laboratories Animal Care and Use Committee (ACUC; OMML Protocol Number: 09-0014). Mice were housed in the OMML animal facility, and kept in an environment with a controlled temperature and humidity, and a light/dark cycle of 12 hours, under veterinary surveillance for animal health and comfort. All injections (s.c. and i.v.) were performed under oxygen/isofurane chamber anesthesia, in order to minimize animal suffering and distress, and eliminate the extemporaneous injury to mice.

Toxicity study

Severe combined immunodeficiency disease (SCID) hairless mice (male, 8–10 weeks old) were obtained from Charles River Laboratories (Frederick, MD, USA). These test mice were randomly divided into two groups: control (n=15) and rIT86 treatment (n=17) groups. Each mouse in the treatment group was injected i.v. with rIT86 (50 µg/kg b.w.) via the tail vein once daily for 28 days. Each mouse in the control group received the same volume of saline. During the study period, the b.w. was measured every other day, and blood was drawn and collected at day 28 of the trial ending by cardiac puncture. Then, hematology and biochemistry testing were performed on the blood samples obtained from each mouse. For the pathological examination, the brain, heart, liver, kidneys, spleen and lungs were collected at the end, and the tissue sections were examined by an experienced pathologist by H&E staining.

Efficacy of rIT86 in tumor cell xenografts

$EGFR_{vIII}$-expressing tumor cells (2×10^6) in 0.1 ml of cell culture medium were injected into the s.c. flank of SCID hairless mice. The tumor size was measured using a digital caliper, and calculated using the following formula: $mm^3 = L/2 \times W/2 \times H/2 \times 4/3 \times 3.14159$. Then, the obtained data was plotted as the relative mean tumor volume ± standard deviation of the mean.

The initial starting point and time course for drug treatment were either together with the tumor cell inoculations, or when the drug administration started after 2–3 weeks of tumor cell inoculations (when the tumor size reached the planned certain size). The drug dose ranged within 50–180 µg/kg of b.w.. This was injected i.v. through the tail, once daily, for five consecutive days or so, or injected every day or every other day, for some cases, until day 28 (refer to the details in the individual experimental information provided in the Results section and Figure Legends). The control group was injected i.v. with 100 µl of saline. Mice were euthanized either when the tumor reached a maximum diameter of 2 cm, or when it began to ulcerate. Any mice that became hunched, lethargic, or dehydrated, or lost >20% of b.w. were euthanized. At the end of the trial, these mice were euthanized using anesthetic overdose, followed by decapitation.

Results

rIT86 treatment caused loss of viability in $EGFR_{vIII}$-expressing tumor cells with higher affinity, but not wt EGFR-expressing cells

The inhibitory effect of rIT86 on the proliferation of $EGFR_{vIII}$-expressing tumor cell lines (human glioma cell U87, non-small cell lung cancer [NSCLC] cell A549, breast cancer cell MDA-MB231, prostate cancer cell Du145, and epidermoid carcinoma cell A431) was very potent, with little effect to the original control cells up to 1 nM (Fig. 1 and Supplementary Fig. 1). Although there were some differences among these cell lines, all IC_{50}s were within the sub-pM range (within approximately 0.02–0.89 pM, Table 1). The effect of rIT86 on wt EGFR was also determined using EGFR overexpressing U87 stable cell lines. As shown in Supplementary Figure 2a, the EGFR expression was detected and confirmed by western blot (shown on the right side of the figure). rIT86 had no inhibitory effect on the cell proliferation, and there was no difference, when compared to the original cells (non-transfected). It was noteworthy that rIT86 did not exhibit any effect with concentrations of up to 1 nM on the proliferation of U87 and A431, in which both are known to express wt EGFR (Figs. 1a and b, and Supplementary Fig. 2a).[15] The difference in sensitivity to immunotoxin may be due to the cell originality (proliferation rate), and this more likely relied on the $EGFR_{vIII}$-expression level. As shown in Supplementary Figure 2b, three Jurkat cell clones expressing $EGFR_{vIII}$ were subjected for the estimation of heterogeneous receptor expression levels using western blot on the cell lysates, and the IC_{50}s were subsequently evaluated. The results revealed that the IC_{50}s are reversely associated with the $EGFR_{vIII}$ expression levels. Therefore, low $EGFR_{vIII}$-expressing cell clones were used for the subsequent *in vivo* xenograft experiments to ensure clinical relevance.

Cytotoxic effect in vivo

In order to determine the dose of immunotoxin rIT86 for the *in vivo* evaluation of therapeutic efficacy, hollow fiber encapsulation/implantation assay was applied to provide the quantitative indices for rIT86 efficacy at the beginning of the animal model study. As

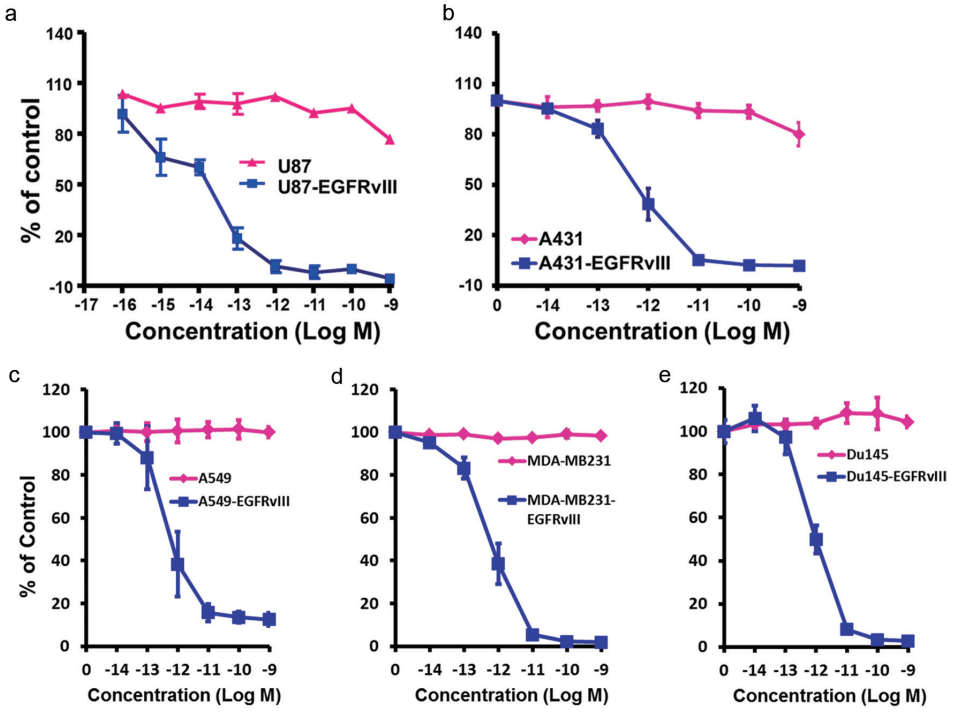

Fig. 1. The effect of rIT86 on cell proliferation: U87 (a), A431 (b), A549 (c), MDA-MB231 (d), and Du145 (e). Both original and $EGFR_{vIII}$-expressing (single clone) cells were prepared at the same time, and a series of diluted rIT86 was applied in triplicate wells. The measured values from wells without any drug were taken as 100% (control). All means ± SD were obtained from three separate experiments.

shown in Supplementary Figure 3, the i.v. injection (once a day, for three consecutive days) of rIT86 at 50 μg/kg of b.w. or higher was sufficient to demonstrate the *in vivo* cytotoxic effect towards $EGFR_{vIII}$-expressing U87 cells.

The general toxicity of rIT86 was determined by monitoring the b.w. of the tested animals (n = 10, each group) for 18 days after the i.v. administration of 60 μg/kg of b.w., once daily, for five consecutive days, and these mice well-tolerated this when the b.w. dropped within 5% of the initial b.w. (Supplementary Fig. 4a). In three separate subsequent experiments, evaluations were also performed at 80, 100 and 120 μg/kg of b.w. (four mice in each group). Although the 80 μg/kg dose was well-tolerated, the maximum average b.w. decreased to 87.0 ± 4.8% of the initial b.w. at one time point, but this was rapidly fully recovered (Supplementary Fig. 4b). Furthermore, one mouse (25%) and two mice (50%) died within the two-week observation period for 100 and 120 μg/kg of b.w., respectively, in the treatment group (data not shown). Therefore, 80 μg/kg of b.w. daily or a lower dose was used for most of the subsequent xenograft experiments, except otherwise specified for the rIT86 efficacy evaluation.

The blood hematology and biochemistry were also examined at day 28 after the administration of 50 μg/kg of b.w., once daily, for 27 days (Supplementary Tables 1 and 2). No significant changes in biochemistry were observed between the treatment group (n = 17) and control group (n = 11), except for ALT, ACT and glucose levels. Furthermore, no sign of disease and no death were observed for rIT86-treated animals. Moreover, the pathological examination of multiple organ tissue sections after H&E staining revealed no clinical abnormalities (data not shown).

rIT86 inhibited tumor growth in heterogeneously $EGFR_{vIII}$-expressing tumor xenografts

The xenografts were established by subcutaneously inoculating 8–10 week-old immunodeficiency mice (SCID hairless) with

Table 1. Estimated IC_{50}s of rIT86 on $EGFR_{vIII}$-expressing cell lines.

$EGFR_{vIII}$ cell line	Original cell line	Cancer type	IC_{50}
Jurkat (27)	Jurkat	T-cell leukemia	0.02 pM
U87 (4)	U87MG	Brain tumor	0.02 pM
A431 (2)	A431	Human epidermoid	0.30 pM
A549 (8)	A549	Lung cancer	0.05 pM
MDA-MB231 (4)	MDA-MB231	Breast cancer	0.61 pM
Du145 (9)	Du145	Prostate cancer	0.89 pM

Notes: The $EGFR_{vIII}$-expressing cell clones in numbers (enclosed in brackets) are noted next to the name of the cell line. IC_{50}s were presented as the means of at least three estimates.

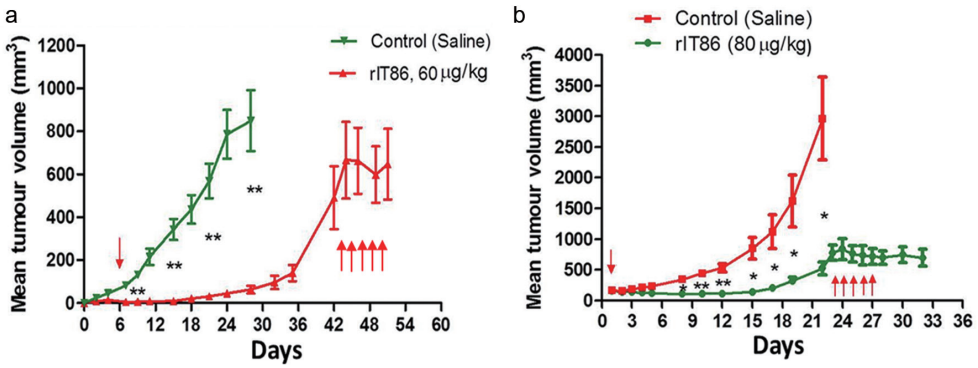

Fig. 2. Effect of rIT86 on the U87-$EGFR_{vIII}$ xenograft. (a) The effect of rIT86 on the U87-$EGFR_{vIII}$ xenograft when rIT86 was first administered, and when the cells were inoculated in mice. rIT86, 60 μg/kg of b.w. in 100 μL saline was injected i.v., once daily, for five consecutive days (n = 12), and the same volume of saline was used as control (n = 11). The last day of injection is indicated by the red arrow. The re-injection was initiated on day 44, 50 μg/kg of b.w., every other day, for total five times; **indicates p<0.01, when compared to controls (student's t-test). (b) The effect of rIT86 on the U87-$EGFR_{vIII}$ xenograft when rIT86 was initially administered, and when the xenografts reached an average size of 167 mm^3. rIT86, 80 μg/kg of b.w. in 100 μL saline was injected i.v., once daily, for five consecutive days (n = 6), and the same volume of saline was used for the controls (n = 6), as indicated by the red arrow. The same treatment was repeated on day 23, that is, 80 μg/kg of b.w., once daily, for a total of five times, as indicated by the arrows; *indicates p<0.05; **indicates p<0.01, when compared to controls (student's t-test). The control groups were ended early on day 28 (a) or day 23 (b), respectively, according to the experimental protocols.

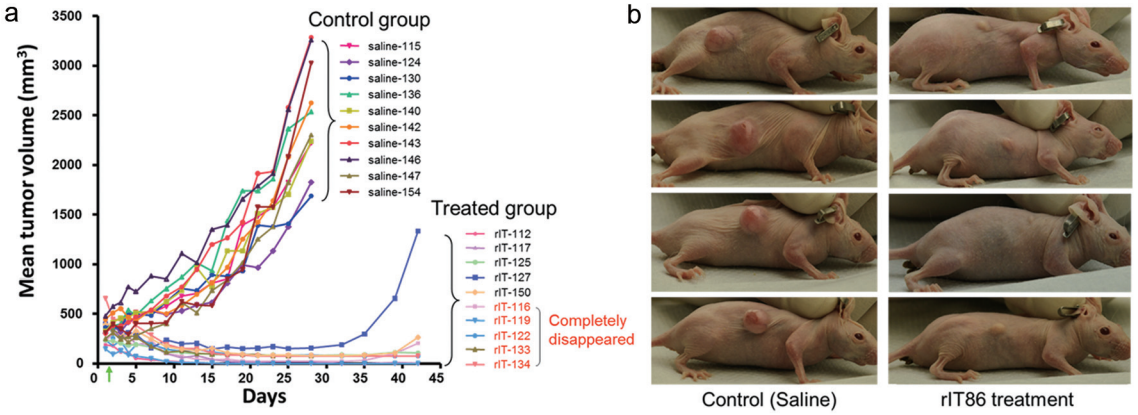

Fig. 3. Efficacy of rIT86 on individual U87-$EGFR_{vIII}$ xenografts. rIT86 was initially administered when the xenografts reached an average size of 269 mm^3, 70 μg/kg of b.w. in 100 μL saline, i.v., once daily, for five consecutive days (n = 10), and the same volume of saline was used for the controls (n = 10), as indicated by the green arrow. Afterwards, the same treatment was repeated every other day until day 28. (a) The tumor volume changes in individual mice (the ID# is shown on the right side) were plotted over time. (b) Representative images of the xenografts at day 11, with controls on the left, four each.

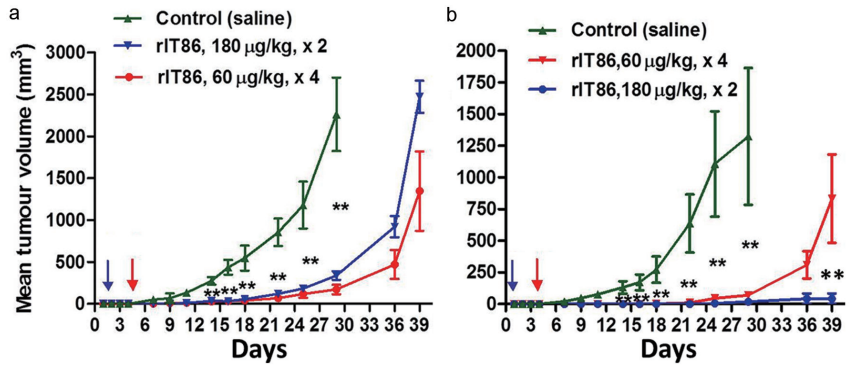

Fig. 4. Comparison of the rIT86 effect on two regimens on the U87-$EGFR_{vIII}$ (a) and A549-$EGFR_{vIII}$ (b) xenografts. rIT86 was initially administered, and the cells were inoculated in mice. rIT86, 60 μg/kg of b.w. or 180 μg/kg of b.w., in 100 μL of saline, was injected i.v., once daily, for four or two consecutive days (as indicated by the blue and red arrows), respectively (n = 6, each), and the same volume of saline was used for the controls (n = 6) for both xenografts; **indicates p<0.01, when compared to controls (student's t-test).

$EGFR_{vIII}$-expressing tumor cells. The treatment started during the inoculation or when the tumors reached a certain size. Then, mice with the xenografts were randomly assigned to the rIT86 treatment group or control group (saline, with the same volume). As shown in Figure 2, mice were treated with rIT86 at 60 μg/kg of b.w. during the inoculation of $EGFR_{vIII}$-expressing U87 cells, once daily, for five days (2A), or with 80 μg/kg of b.w. when the tumors reached an average size of 167 mm^3, once daily, for five days (2B). rIT86 significantly inhibited the tumor growth in treated animals, when compared to the controls (*p<0.05, **p<0.01, two-tailed student t-test), in both experimental designs. On average, the tumor size decreased by 80–90% in the treatment group, when compared to the control group. However, regardless of the dose used, the inhibition was temporary. The repeated use of immunotoxin could inhibit the tumor growth, without any loss of sensitivity to the drug.

The following experiment was performed to determine the drug effect at longer treatment periods. Figure 3a presents the experiment, in which the U87-$EGFR_{vIII}$ xenograft reached an average size of 269 mm^3, and the treatment group (n = 10) received 70 μg/kg of b.w., once daily, for five days, followed by the same dose every other day, until day 28. Compared to the controls, a significant inhibition in tumor growth (on average) was observed starting on day three, after the administration of rIT86 (p<0.05, student's t-test, p<0.005 starting on day five, and p<0.001 remained for the remaining period of observation, starting on day seven). At the end of the experiment (a total 45 days), five mice were free of tumors (50%), while for the other five mice in the treatment group, tumors reappeared (Fig. 3b). The U87 cells were further isolated from the xenografts that reappeared in the treatment group, and the cells continued to respond to the immunotoxin treatment in culture (data not shown). Therefore, the tumor regrowth after the treatment was not due to the loss in expression of $EGFR_{vIII}$ in the xenografts. The histological examination of tumor tissue sections revealed that the groups of regrown tumor cells were embedded at the center of the large area of scar tissues. This may suggest that the cytotoxic effect of the immunotoxin on tumor cells was rapidly triggered and aggressively induced scar formation, which in turn, restricted the penetration of immunotoxins to the remaining tumor cells at the center, and prevented the complete elimination of residual tumor cells.

Similarly, prolonged treatment regimens and different doses were applied to the other three $EGFR_{vIII}$-expressing tumor cell xenografts for optimization. At a tumor size of 158 mm^3 for $EGFR_{vIII}$ expressing A549 cells for NSCLC, rIT86 at 70 μg/kg of b.w. once daily, for five days, followed by 7 μg/kg of b.w. every other day, until day 28, appeared to be insufficient to sustain the tumor suppression effect over time (Supplementary Fig. 5a). In another experiment with an average tumor size of 358 mm^3, the administration of immunotoxins at 50 μg/kg of b.w., once daily, for the first 12 days, followed by 30 μg/kg of b.w., twice per day, until day 21, kept the tumor growth under control (the tumor size continued to decrease, even after treatment; Supplementary Fig. 5b). For the inoculated $EGFR_{vIII}$-expressing MDA-MB231 breast cancer cell xenografts, 50 μg/kg of b.w., once daily, for five days, followed by 25 μg/kg of b.w., once daily, for an additional 23 days, failed to prevent the tumor (from 229 mm^3) from growing (Supplementary Fig. 5c). A daily dose of 50 μg/kg of b.w. for 27 days was required for tumors with a starting size of 138 mm^3 (Supplementary Fig. 5d). For $EGFR_{vIII}$-expressing Du145-inoculated prostate cancer xenografts (size: 200–500 mm^3), both regimens above were effective, but the latter resulted in tumor disappearance during the treatment period (Supplementary Figs. 5e and f). Therefore, different responses to the rIT86 treatment and the dose requirement were observed for the four different types of cancer xenografts.

Finally, regimens with higher doses, but had shorter periods for comparison, were evaluated. Increasing the dose of immunotoxins to 180 μg/kg of b.w., once daily, for two days, presented no better outcome, when compared to 60 μg/kg of b.w., once daily, for four days, in the U87-$EGFR_{vIII}$ xenografts (Fig. 4a). However, in the $EGFR_{vIII}$-expressing A549 cell-inoculated xenografts, immunotoxins at 180 μg/kg of b.w., once daily, for two days, significantly delayed the reappearance of tumors, when compared to 60 μg/kg of b.w., once daily, for four days (**p<0.01, Fig. 4b). The difference in efficacy at higher doses between the two xenograft models may be due not only to the different cell proliferation rates and different $EGFR_{vIII}$ expression levels, but also to the possible easier penetration of rIT86 into NSCLC tissues (sponge-like texture).

Discussions

DT is one of the most potent cytotoxic agents used among immunotoxins, and it is known that one molecule of DT is lethal to cells once it goes into the cytosol.[16] Therefore, DT is ideal for use as part of immunotoxins, considering its easy production and purification as one molecule of fusion protein, and *in vivo* safety profile, since the catalytic domain itself cannot diffuse into cytosol. Hence, a broken-down fragment of immunotoxin outside the cell imposes little toxicity. In addition, DT-based immunotoxins possess efficient translocation into the cytosol. Cancer-specific marker $EGFR_{vIII}$ is not expressed in healthy tissues, indicating that it is an ideal target for therapeutic applications, and that any off-target toxic concern could be minimized. Compared to previously reported immunotoxins, such as DT390-BiscFv806,[17–19] the immunotoxin rIT86 presented in the present study, with the original murine sequence obtained from monoclonal antibody mab806, is more potent and selective towards $EGFR_{vIII}$, sparing wt EGFR at the therapeutic range. Indeed, mab806, which was originally raised in mice, has been shown to bind to $EGFR_{vIII}$ and wt EGFR when overexpressed.[17,18] Therefore, it was speculated that the specificity of the immunotoxin presented in the present study towards only $EGFR_{vIII}$ was likely due to the short link PW and only two aa, when compared to other previously reported ones,[8] between the DT390 and scFv fragment, which may limit its binding to wt EGFR.

There are several $EGFR_{vIII}$-driven cancers, including glioblastoma (50–60% of $EGFR_{vIII}$ positive), late stage NSCLC (36–39% of $EGFR_{vIII}$ positive), breast cancer (29–54% of $EGFR_{vIII}$ positive), and prostate cancer (35% of $EGFR_{vIII}$ positive).[20–22] In order to target solid tumors, and in addition to having high affinity and specificity, it is also necessary for immunotoxins to balance its half-life in the circulation, with efficient penetration to the targeted tumor cells. The present study demonstrated that rIT86 is significantly effective in inhibiting several cancerous $EGFR_{vIII}$-expressing cells in culture and xenografts in mice models. Overall, the ability of rIT86 to treat $EGFR_{vIII}$-expressing solid tumors with high efficacy and tolerable off-target toxicity makes this approach very attractive.

Most patients have pre-existing antibodies against the DT-toxin, because they usually receive five doses of DTP vaccines and a Tdap vaccine booster during their childhood and preteens, and adults would receive one dose of Tdap every 10 years for the booster. Cancer patients respond to any DT-based medicine soon after its administration due to the re-activation of memory immune cells during treatment. Therefore, the initial regimens were designed to use the immunotoxin for up to five days. However, regardless of the dose levels (50–180 μg/kg of b.w., once daily), it was shown that rIT86 is insufficient to eliminate all tumor cells

and prevent relapse, when the treatment period is too short. Since the mice well-tolerated the tested potential therapeutic dose levels for prolonged duration of use, as reflected by both the b.w. and blood counts/biochemistry parameters, it is necessary to consider its prolonged application to achieve clinical efficacy, as demonstrated in the study for several tumor models, leaving out for the time being the concern on immunogenicity and the potential limitation on how to use it in clinic due to previous vaccinations.

Immunotoxins that target both overexpressed wt EGFR and $EGFR_{vIII}$ using the original murine mab806,[19] humanized mab806,[8] or other monoclonal antibodies, such as D2C7 or 40H3, have been reported and evaluated on cultured tumor cells,[8,19,23,24] and *in vivo* experiments, and these have exhibited reduced tumor growth and enhanced survival.[8,19,23] The $EGFR_{vIII}$-specific antibody peptide MK-1 has also been used to develop immunotoxin MR1(Fv)-PE38.[25] Due to the molecular heterogeneity in tumor cells, where the tumor often includes cells with the expression of either $EGFR_{vIII}$ or wt EGFR, presently targeting both $EGFR_{vIII}$ and overexpressed wt EGFR for immunotoxin development is preferred, avoiding wt EGFR in normal tissues.

Future directions

Following the example of PE-based immunotoxins, a systemic approach should be taken to eliminate the immunogenic epitopes in DT, which is necessary for its long-term repetitive use, achieving better clinical efficacy, as demonstrated in the present study. Although the humanization of mab806 has been reported, and that this alone is presently under clinical evaluation for glioblastoma multiforme (GBM),[22] mab806-based immunotoxins with a pharmacological profile, such as rIT86, would be a better candidate to consider. The combination of local immunotoxin-mediated cytotoxicity with immune-check point modulation for promoting anticancer immunity may represent the future direction to eradicate solid tumors.[26] A clinical trial for examining the efficacy of combining immunotoxin D2C7-IT with checkpoint inhibitor Atezolizumab in recurrent GBM patients (ClinicalTrials.gov Identifier: NCT04160494) is presently in phase I, and is being undertaken.

Conclusions

Since wt EGFR is expressed in normal cells, it would be worthwhile to target $EGFR_{vIII}$ that are only expressed in cancer cells with high selectivity. The present study is first to report the production of rIT86 with DG44 CHO cells. Both the *in vitro* and *in vivo* data demonstrated that rIT86 is highly selective for $EGFR_{vIII}$, and is more potent than the previously reported DT390-BiscFv806.[8] This would likely be a potential promising therapy for $EGFR_{vIII}$-driven solid tumors. Due to its superior combined potency and selectivity for targeting cancer cells, rIT86 can offer numerous advantages over other immunotoxins previously reported. This would allow for the use of lower doses of rIT86, which in return would lead to less side effects, combined with higher selectivity to cancer cells only, achieving better clinical outcome, and benefiting patients through lower cost.

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/JERP.2022.00005.

Supplementary Fig. 1. Photoimages of original and $EGFR_{vIII}$-expressing U87 (a) and A431 (b) cells, 48 hours after incubation with 0.1 nM, 0.1 pM or 0.001 pM rIT86, under Nikon inverted microscope (Model 1500), 200 x.

Supplementary Fig. 2. (a) The effect of rIT86 on original U87, U87-EGFR (wt) and U87-$EGFR_{vIII}$ cells in culture and cell proliferation assays. The wt EGFR and $EGFR_{vIII}$ expression levels of the three cell lines by Western blotting are shown on the right. The cells of three sources were treated with a series of rIT86 and the cell viability comparing to non-drug treatment was presented on the left. All the Means +/- SD were from 3 separate experiments of triplicate measurements. The measured values from wells without any drug treatment were taken as 100% (control). (b) The effect of rIT86 on Jurkat and Jurkat-$EGFR_{vIII}$ cell clones in culture and cell proliferation assay. The $EGFR_{vIII}$ expression levels by Western blotting are shown on the right for three individual clones (No. 27, 8 and 17), at high, relatively high and low expressing levels for $EGFR_{vIII}$. The three clones together with non-transfected Jurkat cells were treated with a series of rIT86 concentrations for 2 days and the cell viability comparing to non-drug treatment was presented on the left as well as the corresponding IC_{50}s (the mean of triplicate measurements from one experiment). The measured values from wells without any drug treatment were taken as 100% (control).

Supplementary Fig. 3. The evaluation of in vivo cytotoxicity of rIT86 on $EGFR_{vIII}$-expressing U87 cells using hollow fiber implantation assay in mice. The mice with implanted hollow fibers (3 s.c. and 3 i.p.) containing the same amount of the U87-$EGFR_{vIII}$ cells were divided into control (saline) and treatment groups (50 μg/kg b.w., once daily i.v. from tail vein for 3 consecutive days). OD values are the reflection of cells survival after 3 days of treatment. * indicates $P < 0.01$ comparing to controls (Student's t test).

Supplementary Fig. 4. The effect of rIT86 on mice body weight. (a) Twenty mice were randomly divided into two groups (n = 10 each). One group received 60 μg/kg of initial body weight rIT86 in 100 μL, tail vein injection, once daily for 5 consecutive days, and the other group received saline as controls. The initial body weight was taken as 100%. (b) Twelve mice were randomly divided into two groups (n = 6 each). One group received 80 μg/kg of initial body weight rIT86 in 100 μL, tail vein injection, once daily for 5 consecutive days, and the other group received saline as controls. The initial body weight was taken as 100%. No significant difference was observed.

Supplementary Fig. 5. The effect of rIT86 on the $EGFR_{vIII}$-expressing xenografts. (a) The effect of rIT86 on the A549-$EGFR_{vIII}$ xenografts when rIT86 was first administered when the xenografts reached the average size of 158 mm^3. rIT86, 70 μg/kg b.w. in 100 μL saline, was injected i.v. once daily for 5 consecutive days (n = 7), and the same volume of saline was used as control (n = 7), as indicated with red arrowheads from Day 1 to 5, followed by treatment at 7 μg/kg b.w., once daily as indicated by green arrowheads until Day 28. * indicates $P < 0.01$; # indicates $P < 0.01$ comparing to controls (Student's t test). (b) The effect of rIT86 on the A549-$EGFR_{vIII}$ xenografts when rIT86 was first administered when the xenografts reached the average size of 358 mm^3. rIT86, 50 μg/kg b.w. in 100 μL saline, was injected i.v. once daily for 12 consecutive days (n = 5), and the same volume of saline was used as control (n = 7), as indicated with blue arrowhead on Day 1, followed by treatment at 30 μg/kg b.w. twice daily as indicated by red arrow on Day 13, until Day 21. (c) The effect of rIT86 on the

MDA-MB231-$EGFR_{vIII}$ xenografts when rIT86 was first administered when the xenografts reached the average size of 229 mm^3. rIT86, 50 μg/kg b.w. in 100 μL saline was injected i.v. once daily for 5 consecutive days (n = 7), and the same volume of saline was used as control (n = 9), as indicated with red arrowheads from Day 1 to 5, followed by treatment at 25 μg/kg b.w. once daily as indicated by blue arrowheads until Day 28. * indicates $P < 0.05$; + indicates $P < 0.01$ comparing to controls (Student's t test). (d) The effect of rIT86 on the MDA-MB231-$EGFR_{vIII}$ xenografts when rIT86 was first administered when the xenografts reached the average size of 138 mm^3. rIT86, 50 μg/kg b.w. in 100 μL saline was injected i.v. once daily from Day 1 for 27 consecutive days (n = 9), and the same volume of saline was used as control (n = 8). * indicates $P < 0.05$; # indicates $P < 0.01$ comparing to controls (Student's t test). (e) The effect of rIT86 on the Du145-$EGFR_{vIII}$ xenografts. rIT86, 50 μg/kg b.w. in 100 μL saline was injected i.v. once daily for 5 consecutive days when the Du145-$EGFR_{vIII}$ xenografts reached the average size of 479 mm^3 (n = 11), and the same volume of saline was used as control (n = 14, average size of 220 mm^3) from Day 1 to 5, followed by treatment at 25 μg/kg b.w. once daily until Day 28. * indicates $P < 0.05$ comparing to controls (Student's t test). (f) The effect of rIT86 on the Du145-$EGFR_{vIII}$ xenografts when rIT86 was first administered when the xenografts reached the average size of 200 mm^3. rIT86, 50 μg/kg b.w. in 100 μL saline was injected i.v. once daily from Day1 for 27 consecutive days (n = 8), and the same volume of saline was used as control (n = 8). * indicates $P < 0.05$ comparing to controls (Student's t test).

Supplementary Table 1. The hematology of the blood collected from mice treated with rIT86, 50 μg/kg b.w., i.v. once daily for 28 days and with saline control. White blood cell (WBC), red blood cell (RBC), platelets (PLT), hemoglobin (HGB), hemotocrit (HCT), mean corpuscular volume (MCV or mean red blood cell volume), mean corpuscular hemoglobin (MCH or mean erythrocyte hemoglobin content), mean corpuscular hemoglobin concentration (MCHC) were measured and data were listed. Significant difference was found in white blood cell count and platelets count.

Supplementary Table 2. The biochemistry of the blood collected from mice treated with rIT86, 50 μg/kg b.w., i.v., once daily for 28 days and with saline as control. Significant differences ($P < 0.05$ or 0.01, Student's t test) are presented in the right column.

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Conflict of interest

Dr. Bing Sun has been an editorial board member of *Journal of Exploratory Research in Pharmacology* since September 2019. The authors have no other conflicts of interest to declare.

Author contributions

YZ, QYQ, JX and HYY carried out the experiments; YZ, QYQ and BS designed the experiments, analyzed the data and wrote the manuscript. All authors have made a significant contribution to the study, and approved the final manuscript.

Data sharing statement

The technical appendix, statistical code and dataset are available from the corresponding author (bs464@georgetown.edu) upon reasonable request.

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