EGF ligand fused to truncated *Pseudomonas aeruginosa* exotoxin A specifically targets and inhibits EGFR-positive cancer cells

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**Abstract.** Cancer cells have been known to overexpress the epidermal growth factor receptor (EGFR) and hence relevant multiple-targeted therapies have been developed, with a recent clinical application of the antibody-mediated inhibition of the EGFR. However, this strategy is not useful in cancer cells with mutations in KRAS; a GTPase downstream of EGFR which constitutively activates the pathway without EGF stimulation. Furthermore, mutations in EGFR also reduce the binding of monoclonal antibodies and thereby render them ineffective. In the present study, we designed a chimeric EGF protein fused to the truncated N-terminal domain fragment of *Pseudomonas aeruginosa* exotoxin A (EGF-ETA), which has ADP-ribosylation activity and induces apoptosis. The EGF-ETA protein was expressed in *E. coli* as a His-tagged fusion. Our results showed that EGF-ETA significantly inhibited the proliferation of EGFR-positive A431 epidermoid carcinoma (IC50 27 ng/ml) and HN5 head and neck squamous cell carcinoma (IC50 36 ng/ml) cells. However, its effect on cancer cells with little or no EGFR expression was limited (A549-IC50 1,000 ng/ml; MCF-7-IC50 >10,000 ng/ml). Compared to cetuximab, EGF-ETA was highly potent in its killing capacity of HN5 cancer cells at 1,000 ng/ml, while cetuximab had little effect at 1,000 ng/ml. Furthermore, EGF-ETA was just as potent in HCT116 (KRAS G13D) and SW480 (KRAS G12V) colon cancer cell lines harbouring KRAS hyperactivating mutations when compared to KRAS wild-type HT29 colon cancer cells. Finally, co-incubation of EGF-ETA with an anti-EGF antibody abrogated its effect on the EGFR-positive A431 cells. Our results show that the chimeric EGF-ETA toxin is extremely effective against EGFR-positive cancers and raises the potential to further develop this chimera for use in targeting EGFR-positive tumours resistant to monoclonal antibodies.

**Introduction**

Cancer is a deadly disease in humans that is characterised by the dysregulation of the cell cycle leading to uncontrolled cell division and continuing growth. Unfortunately, current chemo/radiation therapies are not sufficiently effective, but instead are mostly palliative. Thus, the patient survival rate is very poor for most diagnosed cancers. Therefore, there is an urgent need for a new line of therapeutics which offer more selective, effective and curative prospects such as molecular-targeted therapies that have shown promise in both *in vitro* and *in vivo* models (1).

The epidermal growth factor receptor (EGFR) is part of the receptor tyrosine kinase family ErbB. The EGFR is important in the signalling pathway for the control of fundamental cellular functions including cell growth and survival (2). The receptor, when activated by its ligand (EGF), leads to autophosphorylation of a number of tyrosine residues, leading to the activation of downstream Ras/MAPK and PI3K/AKT proteins which promote cell survival and proliferation. The deregulation and overexpression of the EGFR has been shown to be a hallmark of several neoplastic malignancies (3). Thus, generating new anticancer agents that selectively interfere with specific signalling pathways critical to a malignant phenotype, metastasis and tumour progression such as EGFR would block or slow the growth of EGFR-positive cancers, while minimising harm to other normal cells. Current leading agents are monoclonal antibodies (mAbs) and small chemical inhibitors (SCIs) that target EGFR (3).

There are two EGFR-directed monoclonal antibodies (cetuximab and panitumumab) currently in clinical use for cancer patients. These monoclonal antibodies function extracellularly to block the EGFR and potential receptor activation (4) However, mutations in the EGFR and the...
EGF-ETA is an extremely potent exotoxin released from *Pseudomonas aeruginosa*, a common Gram-negative, rod-shaped bacterium, which is comprised of three domains: a receptor binding domain (domain I), translocation domain (domain II) and catalysis domain (domain III). ETA inhibits ADP-ribosylation of eEF2 (eukaryotic elongation factor 2), arresting protein synthesis and leading to cell death (7,8). In a previous research, a truncated fragment of *Pseudomonas aeruginosa* exotoxin A (ETA) lacking the receptor binding domain was fused with the variable domain of a single chain antibody fragment (425scFv) specific to EGFR and was found to be effective in human Hodgkin's lymphoma in a SCID-mouse model (9). Furthermore, we have previously shown that CLDN-4 targeted ETA (CPE-ETA) specifically inhibited the growth of cancer cells overexpressing the CLDN-4 receptor (10).

In this study, a chimeric molecule was constructed by fusing EGF and ETA. Its N-terminal domain has ADP-ribosylation activity and induces apoptosis predominantly (11). We then characterised the ability of EGF-ETA to specifically bind EGFR-expressing cancer cells and subsequently induce cell-specific death in vitro. This receptor-facilitated molecular-directed therapy warrants additional research in order to ascertain its therapeutic potential for a variety of EGFR-expressing cancers.

Materials and methods

Cell culture conditions. The following human cell lines were used in the present study: head and neck squamous carcinoma HN5 cells, breast ductal carcinoma MCF-7 cells, non-small lung cancer A549 cells, colorectal cancer cell lines HCT116, HT29, SW480 and epidermoid carcinoma A431 cells. All cells were cultured in a complete medium containing: 500 ml Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 12.5 ml HEPES buffer solution (1 M), 10% fetal bovine serum (FBS) and penicillin (5,000 U)/streptomycin (5,000 µg) (all are from Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured in a humidified atmosphere at 37°C and 5% CO₂. Routine methods were used for the culturing of cell lines (12). The cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), except for HN5, which was provided by Dr. Hong-Jian Zhu, University of Melbourne, Australia.

Construction of EGF-ETA. To isolate the EGF gene, mRNA was isolated from human FHC cells and subsequently converted to cDNA using the Invitrogen™ SuperScript III cDNA synthesis kit (Thermo Fisher Scientific, Inc.). Primers incorporating NdeI (EGF forward: CCCATATGAAATGTA ACTCTGATATTGACTTCTGATGTCCCTCTGTCC) and NolI (EGF reverse: GGGGCGGCGGCGGCGGCGGCGTGTCCCTCCACCACCTTTAC) were designed to isolate the mature EGF coding sequence. After amplification, the EGF PCR product was cloned into the pET-32a vector (13) to give pET-32A-EGF. This cloning step removes the ScFv and replaces it with EGF, resulting in the 10His-EGF-ETA in-frame fusion protein which lacks the native ETA receptor binding domain (9). The plasmid was sequenced to verify the correct insertion and sequence of EGF-ETA.

Expression and purification of EGF-ETA. After p10His- EGF-ETA was transferred to E. coli BL21 Al by heat shock, cells were cultured in LB media containing 50 µg/ml kanamycin. For purification, 15 ml of starter culture was used to inoculate a 300 ml LB liquid medium with 50 µg/ml kanamycin. After the OD₆₀₀ reached 0.4, 3 ml of 20% L-arabinose and 1 ml isopropyl thiogalactoside (IPTG) (100 mM) (Sigma-Aldrich, St. Louis, MO, USA) were used to induce protein expression in the cells. The cells were further cultured for another 2 h at 30°C before harvesting. Centrifugation was used to pellet the cells and they were stored at -80°C until further use.

Before purification, the cell pellet was resuspended in 15 ml lysis buffer (Qiagen GmbH, Hilden, Germany) with 75 µl PMSF (200 mM) and 300 µl protease inhibitor cocktail. The cells were lysed by sonication 7 times at 30-sec bursts each time on ice. The mixture was then centrifuged at 20,000 x g for 25 min to remove insoluble proteins. The supernatant containing the recombinant protein was processed using the Ni-NTA Fast Start kit (Qiagen) according to the protocols provided. Purified EGF-ETA was dialysed in phosphate-buffered saline (PBS) using ultrafiltration (Amicon Ultra-15 30 kDa cut-off) (Merck Millipore, Billerica, MA, USA). The purified protein was stored at -80°C in PBS buffer containing 20% glycerol, and protein concentrations were determined by DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

MTT cell proliferation assays. MTT cell proliferation assays measure the metabolic activity of a cell via the conversion of the tetrazolium dye, MTT, to the insoluble purple dye formazan. This insoluble dye can be detected colorimetrically and used to determine the effects of drugs on cells. To perform MTT assays, cells were seeded in 96-well plates at a density of 10⁴ cells/well and incubated for 24 h. EGF-ETA (1-10,000 ng/ml) or cetuximab (100 or 1,000 ng/ml) was introduced into the wells and the cells were incubated for a further 48 h. Cell proliferation was measured by MTT assays as described previously (14). The absorbance of the samples was quantified by using a spectrophotometer (POLARstar Omega; BMG Labtech, Ortenberg, Germany). Absorbance readings were transformed into percentage of proliferation relative to the control PBS group.

Binding specificity assays. To determine the affinity of EGF-ETA towards the EGF receptor, an anti-EGF monoclonal antibody (cat. no. ab10409, 100 ng/ml) (Abcam, Cambridge, MA, USA) was used to block the binding of EGF-ETA to EGFR. Briefly, 10⁴ A431 cells were seeded in a 96-well plate and incubated overnight. The cells were treated according to the following regimes: PBS, 100 ng/ml EGF-ETA, 100 ng/ml anti-EGF mAB, and 100 ng/ml EGF-ETA pre-incubated with 100 ng/ml of anti-EGF mAB for 1 h. Treated cells were incubated for 48 h and MTT assays were used to determine cell proliferation.

Western blot analysis. The total soluble protein was extracted from cells using a RIPA cell extraction buffer (Invitrogen;
Thermo Fisher Scientific, Inc.) following the instructions provided. Equal amounts of proteins (25 µg) were run on 8-16% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad Laboratories, Gladesville, NSW, Australia) and subsequently transferred to polyvinylidene fluoride (PVDF) membranes for immunoblot detection. The primary antibodies used were human anti-EGFR (1:500 dilution; cat. no. ab131498; Abcam) and loading control human anti-GAPDH (1:2,500 dilution; cat. no. ab9485; Abcam). The secondary antibody used was mouse anti-rabbit IgG HRP-linked (1:5,000 dilution; cat. no. ab99697; Abcam). The bands that reacted were detected using the Pierce ECL chemiluminescent detection kit (Thermo Fisher Scientific, Inc.). Imaging and analysis were performed with the VersaDoc MP 4000 imaging system (Bio-Rad Laboratories).

**Real-time quantitative PCR.** Total RNA was extracted from cancer cells using TRIzol reagent according to the instructions provided (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA (1 µg) was converted to cDNA using the Superscript III reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The primers used were human EGFR (NM_005228, HP208404) and human control GAPDH (NM_002046, HP205798) (OriGene Technologies, Inc., Beijing, China). Quantitative PCR reactions were carried out with iQ SYBR Green Supermix according to the instructions provided (Bio-Rad Laboratories). The Bio-Rad iQ5 cycler was used for the quantification and analysis of the PCR reactions.

**Statistical analysis.** ANOVA with Tukey's multiple comparison test was used to determine the significance between the treatment groups. All statistical tests were conducted using the statistical programme GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference between groups. All experiments were in triplicate, and the results are shown as means with standard errors.

**Results**

**Confirmation of EGFR expression in cancer cells.** Western blot analysis showed that head and neck cancer HN5 cells and melanoma A431 cells showed a significantly higher expression of EGFR. Furthermore, the non-small cell lung cancer cell line A549 was found to express a significantly low level of EGFR compared to the high expressing lines, while EGFR expression in the breast cancer cell line MCF-7 was completely absent (Fig. 1A). Real-time PCR analysis of the mRNA expression of EGFR was consistent with the western blot results, except for MCF-7 transcripts which were slightly higher compared to A549 (Fig. 1B).

**Construction and expression of EGF-ETA.** Fig. 2A shows the map of the EGF-ETA expressed in the p425-ScFv-ETA backbone. This plasmid allows for the expression of EGF-ETA with an N-terminal 10xHis-tag for easy protein purification. Therefore, EGF-ETA was purified by the immobilized metal ion affinity chromatography (IMAC) using the 10xHis-tag (Fig. 2A). SDS-PAGE analysis of the purified protein resolved a protein of the predicted size of
47 kDa (Fig. 2B). A total of 5 mg of protein was isolated and subsequently used in the in vitro cell toxicity assays.

**EGF-ETA inhibits the proliferation of EGFR-expressing cells.** The toxicity of purified EGF-ETA was tested using MTT proliferation assays (14). Living cells convert MTT to formazan, which can be measured spectrophotometrically. EGF-ETA was added to the cultured cells in the range of 0 to 10,000 ng/ml to provide a dose response for calculating the 50% inhibitory concentration (IC50) (Fig. 3). PBS containing 20% glycerol was used as a control and DMEM media served as a blank for the MTT assay. EGF-ETA was found to trigger rapid cell death in HN5 and A431 cancer cells with an IC50 of 26-37 ng/ml (Fig. 3). However, A549 cancer cells exhibited low sensitivity towards EGF-ETA (IC50 >1,000 ng/ml), while the EGFR-negative cell line MCF-7 was the least responsive (IC50 >10,000 ng/ml).

**Anti-EGF antibody abrogates the effects of EGF-ETA.** To test the specific binding of EGF-ETA to the EGFR, the immunotoxin was pre-incubated with an anti-EGF antibody prior to its addition to HN5 cells. MTT analysis was used to determine the effect of the immunotoxin on HN5 cells. It was found that the pre-treated EGF-ETA with the anti-EGF antibody had little effect on cell survival when compared to the cells that were exposed to EGF-ETA alone which had a significant inhibitory effect (Fig. 4A). Furthermore, the EGF
antibody alone did not elicit cell death when compared with the PBS control. Microscopic analysis confirmed the MTT results (Fig. 4B).

EGF-ETA inhibits the growth of cancer cells harbouring KRAS mutations. To ascertain the effect of KRAS-hyperactivating mutations on the efficacy of EGF-ETA, HCT116 (KRAS G13D) and SW480 (KRAS G12V) colorectal cancer cell lines were used. HT29 colorectal cancer cells served as KRAS wild-type controls. Western blot analysis showed that HCT116, SW480 and HT29 cells expressed similar levels of EGFR, but at lower levels than A431 cells (Fig. 5A). EGF-ETA was most effective against SW480 cells (IC$_{50}$ 835 ng/ml), while the proliferation inhibitory effect on HT29 (IC$_{50}$ >10,000) and HCT116 (IC$_{50}$ 7,700) was of a lesser extent (Fig. 5B).

Efficacy of EGF-ETA vs. cetuximab. The efficacy of EGF-ETA was compared to the FDA-approved monoclonal anti-EGFR antibody, cetuximab. Treatment of EGFR-positive cells with EGF-ETA at concentrations 100 and 1,000 ng/ml reduced the viability of the cells to 30-40% in relation to that of the PBS-treated cells (Fig. 6). However, when the cells were exposed to 100 ng/ml of cetuximab, there was no significant change in the viability of the cells. Increasing the dose of cetuximab to 1,000 ng/ml significantly reduced the viability of...
the cells to 90% of that of the PBS controls. Morphologically, cells treated with 1,000 ng/ml of EGF-ETA were rounded and detached, while those exposed to 1,000 ng/ml of cetuximab were mostly attached to the culture plates.

Discussion

Cancerous cells arise from deregulated cellular signalling pathways due to accumulated mutations in the genome. Upregulation of EGFR leads to the activation of multiple branching pathways including, but not limited to, the Ras/Raf/mitogen-activated protein kinase, phosphatidylinositol 3-kinase/Akt and Src kinase pathways, leading to cell proliferation, migration, adhesion, angiogenesis and survival (15). Therefore, targeting the EGFR provides an excellent opportunity to disrupt the survival and progression of cancer cells. In the present study, we synthesised a recombinant targeted chimera of EGF (ligand of EGFR) with a truncated version of the well-known bacterial toxin (ETA) lacking the cell binding domain (16).
The expression of EGFR was analysed in four different cancer cell lines at the protein and gene level. Our data confirmed the overexpression of EGFR in head and neck squamous carcinoma HN5 cells and epidermoid carcinoma A431 cells (17), while non-small cell lung cancer A549 cells had minimal EGFR expression. The breast ductal carcinoma MCF-7 cell line was found to be negative at the protein level, while the transcript level was similar to A549.

Suppression of EGFR activity in cancers can occur through two regimens. Tyrosine kinase inhibitors (TKIs) such as gefitinib or erlotinib inhibit the catalytic domain of EGFR, while monoclonal antibodies (mAb) such as cetuximab target the EGFR extracellular domain, resulting in downregulation (18). These treatment regimens have been found to be functionally restricted from being effective as a result of the mutations developed in the EGFR signalling pathway (19). The uniqueness of ETA is that it ADP-ribosylates the eukaryotic elongation factor 2 (eEF-2) of the host cells and as a consequence abrogates protein synthesis (11). Thus, the targeted delivery of ETA to the tumour microenvironment through EGF would selectively target cancer cells harbouring increased levels of the EGFR. Furthermore, the novelty of using EGF as the targeting moiety is due to its small size (6.2 kDa) and human origin (20). By contrast, single chain variable fragments of monoclonal antibodies are of murine origin and are significantly larger in size (26 kDa) (21).

Cell proliferation assays were performed to ascertain the inhibitory effects of EGF-ETA on various cancer cells overexpressing the EGFR. The EGFR-positive cells HN5 and A431 were selectively inhibited by the chimeric ETA protein, indicating its binding capacity and potency, while having little effect on EGFR-negative MCF-7 cells. Furthermore, our results showed that EGF-ETA was significantly more effective than cetuximab. Previous studies have shown that when heparin-binding epidermal growth factor (HBEGF) was fused with the plant toxin saporin (SAP), it was effective in killing EGFR-positive cancer cells (22). Yang et al showed that a diphtheria toxin-epidermal growth factor chimera inhibited urinary bladder cancer cells in vitro and in vivo (23). Furthermore, Liu et al showed that diphtheria toxin-epidermal growth factor inhibited glioblastoma multiforme subcutaneous tumours in nude mice (24).

Antibody-mediated inference confirmed the binding capacity of EGF-ETA. EGF-ETA pre-incubated with a monoclonal anti-EGF antibody showed minimal inhibition of EGFR-positive cells, indicating that EGFR binding to the EGFR was critical. Since EGF-ETA contains the translocation domain and ADP-ribosylation domain of Pseudomonas aeruginosa exotoxin A (ETA), it is expected that, upon binding to the EGFR, the chimeric protein will be internalised and translocated to the cytoplasm, cleaved inside the endosomes and transferred by the Golgi apparatus to the endoplasmic reticulum (Fig. 7), subsequently binding to elongation factor-2 (EF-2) and inhibiting protein synthesis (16). By contrast, the monoclonal antibody cetuximab targets the EGFR by competing for EGF ligand binding, subsequent internalisation and downregulation (18). However, recent studies have found that colorectal cancer cells acquire resistance to cetuximab due to mutations of the extracellular domain of EGFR at S492R, R451C, K467T, G465R and S464L (5,6). The presence of mutations in KRAS, a known downstream signalling effector of EGFR, is a predictor for resistance to this antibody. Thus, tumour cells can evade downregulation of EGFR by the anti-EGFR antibody through constitutive activation of the KRAS pathway (5). Notably, Arena et al showed that EGF was not perturbed in binding the cetuximab-resistant EGFR mutants as shown by EGFR phosphorylation upon exposure to EGF (5). This is a significant finding since the mechanism of action for EGF-ETA is different to cetuximab, and binding of the ETA moiety is sufficient for the action of EGF-ETA (Fig. 7).

Tumour cells have been shown to harbour resistance against TKIs as a result of mutations in EGFR and/or its downstream pathway effector, KRAS (25). A study conducted on lung carcinoma cells has reported that KRAS is the main reason for resistance to gefitinib and erlotinib (26). It has been claimed that KRAS mutations which mediate TKI resistance in non-small cell lung carcinoma are not targetable with the current treatment regimens (27). By contrast, the cancer killing capacity of EGF-ETA is not expected to be perturbed by mutations in the KRAS pathway or in TKI-resistant EGFR mutants, as only binding to EGFR is required (Fig. 7). Furthermore, our results show that EGF-ETA is effective in hyperactivating KRAS mutations in HCTT116 (KRAS G13D) and SW480 (KRAS G12V) colorectal cancer cells when compared to HT29 wild-type colorectal cancer cells.

Collectively, the findings of the present study confirm that EGF specifically delivers the ETA toxin to EGFR-positive cancer cells and has the potential to circumvent the mechanisms which cause cetuximab and TKI resistance. Furthermore, in vivo studies are required to ascertain the effective killing capacity of EGF-ETA in the tumour microenvironment and determine its ability to overcome cancer cells resistant to anti-EGFR drugs.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

SMH conceived, designed and performed the experiments. BG, NA and FA performed the experiments and contributed to the writing of the manuscript. SMH and MQW reviewed and edited the manuscript. MQW was also involved in the conception of the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.
Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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