Biomaterials 33 (2012) 2570–2582

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Targeting human epidermal growth factor receptor 2 by a cell-penetrating peptide–affibody bioconjugate

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ARTICLE INFO

Article history:
Received 28 October 2011
Accepted 2 December 2011
Available online 20 December 2011

Keywords:
Affibody
Epidermal growth factor receptor 2
Cell targeting
Nucleic acid delivery
Gene transfer
CPPs

ABSTRACT

Cell-penetrating peptide (CPP)-based delivery systems represent a strategy that facilitates DNA import efficiently and non-specifically into cells. To introduce specificity, we devised an approach that combines a cell-penetrating peptide, TAT-Mu (**TM**) and a targeting ligand, an HER2 antibody mimetic-affibody (**AF**), designated as **TMAF** to deliver nucleic acids into the cells. In this study, we synthesized **TMAF** protein and its truncated versions, i.e. **MAF** and **AF**, by expressing the corresponding plasmids in *Escherichia coli* BL21(DE3)pLysS cells. Purified **TMAF** binds DNA efficiently and protects plasmid DNA from DNaseI action. Transfection of HER2+ breast cancer cell lines MDA-MB-453, SK-OV-3, SK-BR-3 and an ovarian cancer cell line with plasmid DNA pCMVβ-gal, resulted in enhanced β-galactosidase activity when compared to control MDA-MB-231 cells. Maximal activity observed in MDA-MB-453 cells at DNA:**TMAF**:Protamine sulphate (PS) corresponding to 1:8:2 charge ratios. Further the observed gene transfection was resistant to serum, sensitive to the presence of free AF and non-toxic. Variants of **TMAF** although non-toxic, were far less efficient indicating the effective role of the TAT and Mu domains. The observed DNA uptake and reporter gene activity mediated by **TMAF** *in vitro* could be linked with the cell-surface density of tyrosine kinase receptor HER2 (ErbB2) levels estimated by Western blot. Further, we confirmed the efficacy of DNA transfer by **TMAF** protein in xenograft mouse models using MDA-MB-453 cells. Expression of β-galactosidase as the reporter gene, upon intratumoral injection of DNA, in complex with **TMAF**, lends credence to specific DNA import and distribution within the tumor tissue that was attributed to high HER2 receptor overexpression in MDA-MB-453 cells. Through delivery of anti-TF hshRNA: **TMAF**: PS complex, we demonstrate specific knockdown of tissue factor (TF) in MDA-MB-453 cells *in vitro*. Most importantly, in a xenograft mouse model, we observe significant ($P < 0.05$) and specific reduction of tumor volume when anti-TF hshRNA: **TMAF**: PS complex was injected intratumorally. Collectively our data indicate that AF-based chimeric peptides with nucleic acid binding properties may provide an effective tumor specific strategy to deliver therapeutic nucleic acids.

1. Introduction

Efficient gene delivery facilitated by non-viral vectors, in the context of gene therapy is a comparatively safer proposition than viral carriers. Current approaches to gene delivery largely rely on methods that overcome cellular and tissue barriers impeding efficient DNA import [1–3]. Multifunctional peptides as carriers impart specific abilities in assisting the passage of nucleic acids into cells. Domains that aid in cell uptake (CPPs), endosomolytic peptides (HA, His-rich peptides), nuclear localization sequences (NLSs) and cell-specific motifs (RGD, integrin binding, secretin, neurotensin, etc.), have been extensively used either singly or in combination [4–7]. Cell-penetrating peptides (CPPs) were recently harnessed for their unique properties of efficient transduction, DNA-binding and nuclear localization into adherent cells with minimal or no toxicity [8]. Recombinant CPP TAT-Mu (**TM**) includes a stretch of 11 amino acids of sequence 'YGRKKRRQRRR' of HIV-TAT domain and 19 amino acids of the Mu domain, 'MRRAHHRRRRASHRRMRGG' that permit DNA binding and rapid translocation across the plasma membrane, respectively [9]. Prior to this study, cationic viral peptide Mu, poly-L-lysine and protamine sulphate (PS) were demonstrated to enhance liposome-mediated transfection in differentiated neuronal ND7 cell line [10]. The proposed mechanism by which CPPs function is widely discussed [11] for the effects

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doi:10.1016/j.biomaterials.2011.12.003

of generating fusions to basic peptides. However, CPPs generated through many of these strategies are limited by their inability to deliver nucleic acids selectively due to lack of specificity as they deliver to both normal and cancer cells. The combined findings in the last decade have substantially contributed to the growing field of non-viral gene delivery and therefore a compelling prerequisite to the discovery of useful, diverse therapeutic formulations for cell-specific gene delivery.

The addition of *de novo* protein ligands to CPPs has been considered a promising approach to target DNA specifically and was successfully demonstrated through a fusion of the adenoviral capsid protein, the penton base and ten lysine residues to the EGF-like domain of Heregulin-α1 isoform, [12] in breast cancer cells overexpressing HER2 receptors. In a similar approach, Jeyarajan et al. fused three NLS epitopes to heregulin-α to generate Her-NLS, 34.7 kDa fusion protein. The cloned epitopes retained DNA-binding and efficient cell targeting properties [13]. HER2 receptors were identified nearly two decades ago as an 185 kDa transmembrane glycoprotein receptor tyrosine kinase [14]. Keeping cell-specificity in view, a search for stable and smaller protein ligands, to generate engineered cell-targeting fusions, led to well-characterized small molecules, the HER2-binding affibody. Originally described by Nord et al. [15] the advantages of the affibody and its variants were exploited particularly for diagnostics [16,17] and simultaneous use during therapy regimens with anticancer drugs that were incorporated into targeted nanoparticles [18]. In a study by Nygren, ZHER2, an engineered 58 amino acid variant with a 3-helix bundle, was demonstrated to recognize and bind HER2 receptors [19]. ZHER2 is also capable of rapid and independent folding; possessing a single high-affinity binding site on HER2/ErbB2 receptors expressed in SK-BR-3 and SK-OV-3 cancer cells [20]. The structure of the extracellular domain of HER2 (HER2ecd) complexed with ZHER2 was recently determined by both NMR and X-ray crystallography [21]. It is clear from their study that ZHER2-binding site on the extracellular domain of HER2 is distinct from both Trastuzumab and Pertuzumab [21], to which Heregulin-α binds, making it suitable for use in molecular imaging, diagnostics and other biotechnological applications [22,16]. In this study, ZHER2 affibody was used to generate AF-based chimeric peptides to examine DNA delivery and cell-targeting potential *in vitro* and *in vivo*.

HER2-binding affibody has several merits as a targeting ligand owing to a) its small size (~5.8 kDa), b) ease of conjugation of functional domains away from the active site c) ability to promote receptor-mediated endocytosis, d) high stability *in vitro* and *in vivo* and importantly, non-toxic to cells. The structure of designed **TMAF** fusion protein, harbouring the cloned affibody domain was predicted and validated recently using the I-TASSER structure-prediction program [23]. In the modelled structure, the TAT and Mu epitopes are in α-helical conformation separated from the targeting ligand by a α-helical linker which folds as a 3-helix bundle. The authors hypothesized that **TMAF** would recognize HER2 receptors [23] via the affibody thereby permitting DNA import. This was verified in the present investigation in order to examine the functional attributes of cloned epitopes of the delivery system that would determine gene delivery efficiency. Firstly, we examined the cell-targeting potential of designed recombinant fusion protein **TMAF**, 21.9 kDa and a deletion variant designated as **MAF** that is devoid of the TAT epitope *in vitro* through reporter gene expression assays. MDA-MB-453 and SK-BR-3 [24,25] and the ovarian cancer cells SK-OV-3 [26], which were earlier investigated to understand the molecular mechanisms of HER2 receptors, were used to perform the *in vitro* studies. Having ascertained receptor-specific targeting *in vitro*, we then investigated the efficiency of **TMAF**-based formulations to deliver plasmid DNA *in vivo* using MDA-MB-453 xenograft mouse models. We discuss the attributes and therapeutic potential of designed molecules for optimal cell-specific DNA targeting.

2. Materials and methods

2.1. Reagents

Mouse monoclonal antibody anti-erbB2 (intracellular domain) was from Millipore. Mouse monoclonal antibody Ms × GAPDH (glyceraldehydes 3-phosphate dehydrogenase) MAB 374 antibody was from Millipore, 6-x His monoclonal antibody from BD biosciences. Goat anti-mouse IgG-ALP was purchased from Bangalore Genei. Trastuzumab was a kind gift from Reliance Life Sciences, Mumbai, India. Oregon green nucleic acid labelling kit, DNaseI, SYBR Green and TRIzol were obtained from Invitrogen, NiNTA-agarose was from Qiagen. Nitrocellulose membrane was from Amersham. Lab-Tek chambered coverglass was from Nalge Nunc International, Expression plasmid vectors pFRT-H1T-hTFsh (hTFshRNA) and pFRT-H1T-mTFsh (non-targeting vector control) were a gift from Dr. Mohammed Amarzguioui, siRNAsense, University of Oslo, Oslo, Norway. Reporter gene plasmids pCMVβ-gal and pEGFPN$_3$ were from laboratory stocks. Dulbecco's Modified Eagle's Medium (D-MEM/F12) was from Gibco. Plasmids were purified using Nucleobond AX kits supplied by Macherey–Nagel. Cholesterol and protamine sulphate (PS) were purchased from Sigma–Aldrich. Omniscript RT kit was from Qiagen. RNA storage solution was from Ambion. All other reagents were of the highest purity. Synthetic cationic lipid *N*,*N*-di-*n*-hexadecyl-*N*,*N*-dihydroxyethylammonium chloride (DHDEAC) were from laboratory stocks. Primers for qPCR were synthesized at Bioserve Biotechnologies, Hyderabad, India.

2.2. General procedures and production of recombinant fusion peptides

Expression plasmid pTMAF was amplified, expressed in *E. coli* BL21(DE3) pLysS and purified to homogeneity on 12% SDS-PAGE gels along with truncated versions of **MAF** and **AF** using procedures described [13,23] and verified by Western blotting using monoclonal anti-His antibody. Briefly, the deletion of the TAT moiety, from **TMAF**, followed by religation, resulted in **MAF**. The AF construct (137 amino acids), in this study cloned in pET28a, harbours the N-terminal His-tag epitopes followed by linker sequences and the Affibody. The molecular mass of TM is 11.34 kDa [8]. Expression plasmids pFRT-H1T-hTFsh and pFRT-H1T-mTFsh were amplified and purified using Nucleobond AX midi prep kit. Adherent high expression HER2+ breast cancer cell lines MDA-MB-453, SK-BR-3 and SK-OV-3 and low expression MDA-MB-231, were obtained from Medina-Lali Kauwe, University of Southern California Keck School of Medicine, Los Angeles, USA. Cells were maintained and grown in D-MEM/F12 medium plus 10% foetal calf serum at 37 ^{2}C, 5% CO_2.

2.3. Analysis of HER2 receptor expression by Western blotting

Lysates of MDA-MB-453, MDA-MB-231, SK-OV-3 and SK-BR-3 cells were prepared by suspending them in cell lysis buffer (250 mM Tris·HCl (pH 8.0), 0.5% NP40). Protein concentrations were determined using the modified method of Lowry [27]. To determine the HER2 levels in the four cell lines, proteins from equal amounts of cell lysates (45 μg) were resolved on 8% SDS polyacrylamide gels, electrotransferred to nitrocellulose membranes and treated with anti-erbB2 antibody. Immunoreactive bands were detected by alkaline phosphatase labelled secondary antibody and GAPDH was used as a loading control.

2.4. Gene delivery formulations

Binary complex formulations with **TMAF**, **MAF**, and **AF** or **TM** were prepared by addition of the fusion protein to DNA at 1:8 charge ratios of DNA: Protein and incubated for 20′ at RT. This was followed by the addition of 2 nmol of PS for every nmol of DNA to give 1:8:2 charge ratios of DNA: Fusion protein: PS to form ternary complexes and incubated for another 20′ at RT. Charge ratios were varied and used accordingly for *in vitro* and *in vivo* studies. Cationic lipid DHDEAC formulated with cholesterol at 1:1 mol ratio was prepared as described before [28] and added to DNA at 1:1 N/P charge ratio.

2.5. DNA-binding, electrophoretic mobility shift and DNaseI protection assays

Charge neutralization and DNA condensation was monitored by electrophoresis of the ternary complexes of plasmid pEGFPN$_3$ DNA:**TMAF**:PS formulated at varying charge ratios on agarose gels. Following incubation, complexes were treated with DNaseI and analyzed as described [13]. Binding with proteins was observed as retardation in the migration of the complexes on an agarose gel when compared to naked DNA alone visualized under UV by ethidium bromide (EtBr) staining.