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Structure-based optimization of GRP78-binding peptides that enhances efficacy in cancer imaging and therapy

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A B S T R A C T

It is more challenging to design peptide drugs than small molecules through molecular docking and *in silico* analysis. Here, we developed a structure-based approach with various computational and analytical techniques to optimize cancer-targeting peptides for molecular imaging and therapy. We first utilized a peptide-binding protein database to identify GRP78, a specific cancer cell-surface marker, as a target protein for the lead, L-peptide. Subsequently, we used homologous modeling and molecular docking to identify a peptide-binding domain within GRP78 and optimized a series of peptides with a new protein-ligand scoring program, HotLig. Binding of these peptides to GRP78 was confirmed using an oriented immobilization technique for the Biacore system. We further examined the ability of the peptides to target cancer cells through *in vitro* binding studies with cell lines and clinical cancer specimens, and *in vivo* tumor imaging and targeted chemotherapeutic studies. MicroSPECT/CT imaging revealed significantly greater uptake of ^{188}Re-liposomes linked to these peptides as compared with non-targeting ^{188}Re-liposomes. Conjugation with these peptides also significantly increased the therapeutic efficacy of Lipo-Dox. Notably, peptide-conjugated Lipo-Dox significantly reduced stem-cell subpopulation in xenografts of breast cancer. The structure-based optimization strategy for peptides described here may be useful for developing peptide drugs for cancer imaging and therapy.

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1. Introduction

Peptide ligands are commonly discovered by selection from a random peptide library using display technologies or from the binding sequences of the binding proteins [1–5]. However, it remains a great challenge to delineate and optimize their molecular interactions with targeted proteins. Peptide drugs are usually more difficult to design through molecular docking than small molecules, because peptide structures are more flexible, possess intricate molecular conformations, and undergo complex interactions. We have recently developed a protein-ligand scoring program called HotLig [6], which predicts binding mode of protein-ligand complexes with a success rate of 85.6–91.0% [6]. HotLig is a molecular surface-directed scoring function, which utilizes the Connolly surface of a protein to calculate hydrophobic interactions and paired pharmacophore interactions with ligands [6]. Compared with similar programs, such as PLP [7], F-Score [8], LigScore [9], and DrugScore [10], HotLig is relatively effective in predicting both hydrophilic and hydrophobic interactions, and useful for analyzing diverse ligands [6]. Here we describe a strategy of structure-based optimization with the use of HotLig to design peptides targeting surface markers of cancer cells.

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A cancer-targeting peptide, L-peptide (RLLDTNRPLLPY), was discovered through screening phage-display peptide libraries on nasopharyngeal carcinoma (NPC) cells [11]. This peptide binds to NPC cell surfaces both *in vitro* and *in vivo*. In addition, L-peptide-linked liposomes carrying doxorubicin exhibited higher efficacy for tumor suppression than liposomal doxorubicin (Lipo-Dox) alone, without causing overt side effects *in vivo* [11]. However, it remains unclear which cancer cell surface protein is the primary target of L-peptide.

Recently, cell-surface GRP78 (glucose-regulated protein 78) was reported to be a specific cancer surface marker and a promising target for selective cytotoxicity of cancer cells [12–14]. GRP78, which belongs to the HSP70 protein family, is predominantly expressed in the lumen of the endoplasmic reticulum (ER). In addition to serving as molecular chaperone for protein folding [15], GRP78 may play an anti-apoptotic role in cells [16,17]. GRP78 is expressed on the cell surface of cancer cells or certain cell types under stress conditions, such as ER stress or hypoxia [18–20]. GRP78 on the cell surface forms complexes with diverse ligands, and may mediate both pro-survival [21–23] and pro-apoptotic signaling pathways [24,25]. Certain peptide ligands of GRP78 were reported to have the potential to serve as drug carriers for cancer targeting [12,26,27]. However, the structure of human GRP78 protein and how it recognizes peptides are still unknown.

Conjugating peptides to a liposomal drug holds promise as a targeting strategy in cancer therapy and imaging. Such peptide-functionalized liposome not only improves the targeting towards cancer cells [28–30] and their therapeutic efficacy, but also decreases the extent of their nonspecific toxicities [28,29,31,32]. Binding affinity of a ligand is determined by its rates of association and dissociation. A peptide of long tumor-retention requires optimal molecular interactions which form stable complexes with targets and result in slow rate of dissociation. In order to increase drug uptake, peptides that exhibit long retention in tumor tissue are good choice to conjugate with liposomal drugs for cancer targeting therapy and imaging.

In this study, we first employed a peptide-binding protein database to predict that L-peptide might bind GRP78 on the surface of cancer cells. We subsequently subjected the human GRP78 protein to homologous modeling to investigate its structural features contributing to its interactions with various binding peptides using HotLig [6], and delineated the GRP78-binding motif of peptides. This study also utilized a novel peptide binding assay, in which GRP78 was covalently linked to a sensor chip with a defined orientation for Biacore analysis. Based on the molecular modeling results, peptides were designed for optimal binding to GRP78, and targeting was confirmed through Biacore analysis. We also examined the capacity of optimized peptides to target cancer cells *in vitro* by examining binding to cell lines and clinical cancer specimens and *in vivo* by tumor imaging and targeted chemotherapy with Lipo-Dox, conjugated to these peptides.

2. Results

The schematic diagram of the workflow for optimizing cancer-targeting peptides is illustrated. #1: The structure of human GRP78 was first modeled by Modeller and energy-minimized by DeepView. Two major structural domains were evident: a peptide-binding (green) and an ATPase (blue) domain (see Fig. 2 in next section). #2: Molecular docking for L-peptide against GRP78 was performed to predict the molecular interactions, and identify the key amino-acid residues on L-peptide for such interactions (as the round in #3–9 described below). #3: Structures of peptides for molecular docking were generated using Buildpep and Modeller programs. #4: Flexible docking with Dock and scoring with PscanMS and HotLig were performed to generate initial models consisting of the peptide-binding domain of GRP78 with the peptides under study. #5: The resulting protein-peptide complexes were further energy-optimized, followed by evaluation of docking energy and interaction analysis using PscanMS and HotLig (#6). Candidate peptides were synthesized (#7) and examined for binding *in vitro*, as determined using Biacore analysis (#8). Hence, the key pharmacophores of the GRP78-targeting motif required for binding were identified according to the verified models; and new peptides were redesigned based on the predicted interactions (#9). After repeated *in silico* screening of the peptide library (#3 to #9), the optimized peptides were validated by *in vitro* binding assays using various cancer cells, and *in vivo* tumor imaging and therapeutic studies in mice (#10). The Chimera and Ligplot packages were used for rendering molecular models. More detailed procedures were described in Methods.

2.1. Development of cancer-targeting peptides

The L-peptide (RLLDTNRPLLPY), discovered through screening of a phage-displayed random peptide library, is reported to bind specifically to NPC cells [11]. To identify potential binding proteins of L-peptide, the “PepBind Predict” [33] program was applied to search for targets in the “Peptide Binding Protein Database” (http://pepbind.bicpu.edu.in). With the “PepBind Predict” software, we identified six proteins which were reported to have known peptide ligands and these ligands possess amino-acid sequence similar to L-peptide. These six proteins were thus considered to be candidates of potential targets for L-peptide (Table S1). Of the human orthologues of these proteins, only GRP78, the DnaK homologue [34], has been reported to reside on the cell surface of cancer [12–14]. We therefore proceeded to interrogate the structure of GRP78 protein and its molecular interactions with potential candidates of binding peptides.

The overall strategy to delineate a peptide-binding site in GRP78 for designing optimized binding peptides is illustrated in the flow diagram in Fig. 1. First, the structure of the GRP78 protein was constructed using homologous modeling (see Methods) (Fig. 1#1). Then, flexible docking and structural energy minimization were applied to assess various possibilities to form the complex structure of GRP78 with peptide ligands like L-peptide (Fig. 1#2–5). The HotLig package [6] was subsequently used to define the detailed molecular interactions and identify the key pharmacophores of the GRP78-targeting motif in the binding ligands (Fig. 1#6). A structural library of peptides derived from this analysis was then built based on these predicted interactions with GRP78 (Fig. 1#7). After *in silico* screening of this peptide library, new peptides were designed and examined for their binding to GRP78 *in vitro*, using Biacore analysis (Fig. 1#8). The results were finally validated by *in vitro* binding to various cancer cells and *in vivo* tumor imaging and therapeutic studies (Fig. 1#10), as described in greater detail in the following sections.

2.2. Structural features of human GRP78

An X-ray-determined structure of substrate-binding domain of DnaK in *E. Coli* is shown in Fig. 2A. This substrate-binding domain is able to bind with an NRLLLTG peptide [34]. When compared to the sequence of human GRP78 (Fig. S1), amino acid residues at many regions are conserved evolutionarily (marked in white in Fig. 2B), while many residues in close proximity to the substrate-binding site of DnaK are disparate (marked in red). Similar distribution of the conserved or varied residues was also observed when viewed from the reverse side of the binding pocket (Fig. 2B). Hence, in order to delineate the structural features involved in peptide

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