Phage antibody library screening for the selection of novel high-affinity human single-chain variable fragment against gastrin receptor: an in silico and in vitro study

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Abstract

Background As a membrane G protein coupled receptors (GPCRs) family, gastrin/cholecystokinin-2 receptor (CCK2R) plays a key role in the initiation and development of gastric cancer.

Objectives Targeting CCK2R by immunotherapeutics such as single-chain variable fragments (scFvs) may provide an effective treatment modality against gastric cancer. Thus, the main objective of this study was to isolate scFvs specific to CCK2R.

Methods To isolate scFvs specific to the CCK2R, we capitalized on a semi-synthetic diverse phage antibody library (PAL) and a solution-phase biopanning process. The library was panned against a biotinylated peptide of the second extracellular loop (ECL2) of CCK2R. After four rounds of biopanning, the selected soluble scFv clones were screened by enzyme-linked immunosorbent assay (ELISA) and examined for specific binding to the peptide. The selected scFvs were purified using immobilized metal affinity chromatography (IMAC). The binding affinity and specificity of the scFvs were examined by the surface plasmon resonance (SPR), immunoblotting and flow cytometry assays and molecular docking using ZDOCK v3.0.2.

Results Ten different scFvs were isolated, which displayed binding affinity ranging from 0.68 to 8.0 (nM). Immunoblotting and molecular docking analysis revealed that eight scFvs were able to detect the denatured form of CCK2R protein. Of the isolated scFvs, two scFvs showed high-binding affinity to the human gastric adenocarcinoma AGS cells.

Conclusions Based on our findings, a couple of the selected scFvs showed markedly high-binding affinity to immobilized CCK2R peptide and CCK2R-overexpressing AGS cells. Therefore, these scFvs are proposed to serve as targeting and/or treatment agents in the diagnosis and immunotherapy of CCK2R-positive tumors.

Keywords Phage display technology · Biopanning · Single-chain variable fragment · Gastric cancer · Gastrin receptor · Molecular docking

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Abbreviations

CCK2R	Gastrin/cholecystokinin-2 receptor
GPCRs	G protein coupled receptors
scFvs	Single-chain variable fragments
PAL	Phage antibody library
ECL2	Extracellular loop
ELISA	Enzyme-linked immunosorbent assay
IMAC	Immobilized metal affinity chromatography
SPR	Surface plasmon resonance
TM	Transmembrane
ICL	Intracellular loops
ECL	Extracellular loops
MALT	Mucosa-associated lymphoid tissue lymphomas
mAbs	Monoclonal antibodies
PAD	Phage antibody display
Ag	Antigen
g3p	Gene 3 minor coat protein
PDT	Phage display technology

Introduction

Gastric cancer, as one of the deadliest malignancies, appears to be the second leading cause of cancer-related mortality worldwide. Etiologically, the main risk factor for the gastric carcinogenesis is the infection with *Helicobacter pylori* [1, 2]. The chronic infection with *H. pylori* may cause atrophic corpus gastritis, resulting in a possible transformation of the benign mucosa into the gastric intestinal metaplasia, and ultimately to gastric cancer through virulence factors (e.g., urease, CagA, VacA, BabA) of bacteria [3]. In association with *H. pylori*-induced gastric carcinogenesis, the CCK2R/gastrin interaction has been reported to play central roles in the activation of key signaling pathways [4], which can be exploited for drug discovery. It seems that *H. pylori* infection can directly prompt trivial hypergastrinemia, which can be provoked by gastrin. Such phenomenon may induce some trophic impacts (e.g., increased volume/mass of the oxyntic mucosa) on the human gastric-intestinal-colorectal cells both in vitro and in vivo, in large part because of the activation of the CCK2R signaling pathway [5]. CCK2R is a member of G protein-coupled receptor (GPCR) superfamily. Structurally, it is characterized by several parts, including an extracellular N-terminus, seven transmembranes (TM) α -helices (i.e., TM-1 to TM-7) connected via three intracellular loops (ICL-1 to ICL-3) and three extracellular loops (ECL-1 to ECL-3), and finally an intracellular C-terminus [6]. Numerous studies have demonstrated that CCK2R signaling plays a key role in the development of several gastric tumors such as gastric adenocarcinoma, gastric mucosa-associated lymphoid tissue lymphomas (MALT), and gastric neuroendocrine tumors such as insulinomas and carcinoids [7, 8]. Targeting CCK2R by monoclonal antibodies (mAbs) was shown to inhibit the growth of the CCK2R-positive tumors both in vitro and in vivo xenograft animal model, indicating the usefulness of anti-CCK2R mAbs in gastric cancer [9]. Such an immunotherapy strategy might provide an effective treatment modality, though some issues must be resolved in terms of their translation into the clinical experiments. In fact, therapeutic mAbs have routinely been administered to cure and/or alleviate different diseases in the clinic [10, 11]. Accordingly, these treatment modalities continue to be expanded in different formats against various malignancies. By far, about 49 therapeutic mAbs have been approved by the United States FDA, while there exist much more under clinical trials. Of these, over 20 therapeutic Abs have been used for the treatment of different cancers, indicating the clinical potential of mAbs-based cancer immunotherapy [12].

During the recent decades, phage antibody display (PAD) technology has been introduced as a robust combinatorial

approach for selection of fully-human mAbs against a wide range of antigens (Ags) using large and diverse functional phage antibody libraries (PALs) [13, 14]. The principle behind the PAD technology is the physical linkage between the genotype and the phenotype by cloning a designated gene encoding Ab fragment (i.e., Fv, scFv, and Fab) at the 5' end. Of the genes encoding a coat protein of a filamentous phage, gene 3 minor coat protein (g3p) has widely been used [15]. Technically, the g3p-Ab fragment fusion genes can be incorporated into the phage particles that also display g3p-Ab fragment fusion proteins on their surfaces [16]. The recombinant Ab fragment-displaying phages are applied to enrich the specific phage binders. To this end, the recombinant phage Abs are incubated with an immobilized Ag of interest, and then specific phage binders are eluted after washing off the unbound phages [17, 18]. The eluted specific phage binders are further amplified through the infection of *E. coli*, and literally prepared for the subsequent panning rounds [19]. In the current study, we capitalized on the PAD technology to isolate high-affinity scFvs against the CCK2R (a molecular marker in *H. pylori*-associated gastric tumors) to serve as targeting and/or immunotherapy agent. To this end, human single-fold scFv Tomlinson J library together was used in a solution-phase biopanning against a biotinylated form of the second extracellular loop of the CCK2R peptide. To further validate the isolated scFvs, three dimensional (3D) structures of the scFvs and CCK-B receptor were modeled. The binding activity of ten newly isolated scFvs with Cholecystokinin B receptor was computationally evaluated by using the in silico chemico-biological approaches.

Methods

Antibody reagents and bacterial strains

Human gastric adenocarcinoma AGS cells were obtained from the National Cell Bank of Iran (Pasture Institute, Teheran, Iran). All culture media and supplements, and ImmunoPure biotinylated-BSA were from Gibco, Thermo Scientific Pierce (Rockford, USA). Human single-fold scFv Tomlinson J library (with 1.37×10^8 transformants), KM13 helper phage, *Escherichia coli* strains TG1-Tr and HB2151 were obtained from Source BioScience (Nottingham, UK). The antibody specific to cholecystokinin B receptor was purchased from Abgent (San Diego, CA, USA). Bovine pancreas trypsin was from Sigma-Aldrich Co. (Taufkirchen, Germany). The primary anti-M13 mAb was from GE Healthcare (Little Chalfont, UK). The secondary goat anti-mouse IgG conjugated HRP and Dynabeads M-280 Streptavidin were obtained from Invitrogen Life Technology (Karlsruhe, Germany). ULTRA tablets and protease inhibitor cocktail were obtained from Roche (Basel, Switzerland).

Cell culture

AGS cells were cultured in Dulbecco's Modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO₂ incubator. The culture medium was changed every three days and sub-cultivation was performed routinely using trypsin-EDTA (0.25%) when cells reached 70–80% confluence [20].

PAL

The single-fold scFv Tomlinson J library is based on synthetic diversification of a human single framework for V_H (V3–23/DP-47 and J_H4b) and V_L (O12/O2/DPK9 and J_κ1) at CDR2 and CDR3 regions, in which the CDR1 is constant. ScFvs encoding sequences are cloned in an ampicillin resistant phagemid vector (pIT2) close to the minor coat protein 3 (pIII) as scFv-pIII fusion, which is followed by the c-myc and His 6 tags encoding sequences. ScFv Ab fragments, displayed on pIII of filamentous phage, consist of a single polypeptide with the V_H and V_L domains attached to each other by a flexible Gly-Ser linker [21].

Selection of gastrin receptor-specific phage Ab

To obtain high-affinity scFvs against CCK2R, the second extracellular loop of CCK2R (i.e., residues 190–219) was synthesized and biotinylated (i.e., biotin-mini-PEGPVYTVVQPVGPRVLQCVHRWPSARVRQTWS) with a mini-PEG at N-terminus (Biomatik, Cambridge, Ontario, Canada). It is used as a target Ag for biopanning. Phage scFv particles were prepared and rescued from the library J by super-infection using KM13 helper phage according to the library instruction. Biopanning was performed by means of a solution-phase procedure as described previously [22]. Briefly, about 6.8×10^{11} cfu phage displaying scFv from library J and “Dynabeads M-280 streptavidin” were separately blocked using blocking buffer (i.e., PBS containing 3% BSA and 0.05% Tween 20) at room temperature (RT) for 1 h. The blocked phage could bind the biotinylated peptide at RT for 2 h under rotation on an over-head rotator. Decreasing concentrations (from 200 nM to 50 nM) of biotinylated peptide were used for the selection rounds. The blocked Dynabeads were incubated with the phage-peptide mix at RT for 30 min to capture peptide-bound phage scFvs using a magnetic particle concentrator, DynaMag-2 (Thermo Fisher Scientific, Waltham, MA, USA). Then, unbound phages were removed by several washing steps with PBST (PBS- 0.1% Tween 20), as follows: 6, 12 and 24 times for rounds 1, 2/3 and 4. The specific phage-scFv binders were eluted by incubating Dynabeads-captured phages with 0.5 mL of bovine pancreas trypsin (1.0 mg/mL) at RT under

continuous rotation for 15 min. Finally, to proceed to the next round of selection, the eluted phages were amplified by infecting *E. coli* TG1 cells (at the exponential growth phase) according to the library protocol [23, 24]. To avoid any nonspecific phage binders to streptavidin, prior to all rounds of selection, the blocked phages were pre-absorbed with the blocked Dynabeads M-280 streptavidin at RT for 1 h.

Polyclonal phage ELISA

The amplified and PEGylated polyclonal phages resultant from each round of the selection were analyzed by means of enzyme-linked immunosorbent assay (ELISA). The analysis was conducted through an indirect immobilization of the biotinylated peptide via streptavidin-biotinylated BSA system. Initially, both positive and negative polystyrene 96-well plates were coated with ImmunoPure® Biotinylated-BSA 2.0 µg/mL in PBS at 4 °C overnight. All incubations were followed by three washes each 5 min with PBS/0.1% Tween 20. After washing, 100 µL/well of streptavidin in PBS-0.5% gelatin was incubated at RT for 2 h. Then, positive plates were coated with the biotinylated peptide (100 µL/well of 200 nM), while the negative plates were incubated with blocking buffer (2% non-fat dried skimmed milk in PBS) at RT for 90 min. Following blocking of phage pools (10 µL) from each round of the selection, 2% M-PBS was added to each well and incubated at RT for 90 min. The detection of specific phage-scFv binders was accomplished by adding a primary anti-M13 mAb and a secondary goat anti-mouse IgG conjugated with HRP at a dilution of 1:5000 in 2% M-PBS. Staining was developed by tetramethylbenzidine (TMB) peroxidase substrate solution and stopped by adding 5% H₂SO₄. The optical density was read at OD₄₅₀-OD₆₅₀ using a microplate reader, BioTek ELx800™ (BioTek Instruments, Inc., Winooski, VT, USA) [23, 25].

Soluble scFv ELISA

To screen the positive soluble scFv clones, as a prerequisite step, *E. coli* host was changed from suppressor TG1 strain that produces scFv-displaying phage clones to a non-suppressor HB2151 strain that produce soluble scFvs without pIII. Therefore, the polyclonal phages eluted from the fourth round of biopanning were used to infect the *E. coli* HB2151 strain (OD_{600nm} of 0.4) at 37 °C for 30 min. After preparing ten-fold serial dilutions, to obtain a single colony, the infected bacteria were spread on TYE-AG plates (containing 100 µg/mL ampicillin and 1% glucose) and incubated at 37 °C overnight. The individual colonies were grown in 96-well culture plates with 100 µL of 2xTY-AG medium (containing 100 µg/mL ampicillin and 1% glucose) under shaking at 37 °C overnight. The next day, a small aliquot was transferred to another plate with 200 µL of 2xTY-AG (0.1% glucose) and incubated under

shaking at 37 °C until reaching the OD_{600nm} of ~0.9. The expressions of scFvs clones were induced by adding isopropyl b-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubating under shaking at 30 °C overnight. In order to extract the periplasmic scFvs, the culture plates were incubated with 3xPE buffer [periplasmic extraction buffer: 60% (w/v) sucrose, 150 mM Tris and 3 mM EDTA, pH 8] at 4 °C for 30 min. Then, the supernatant containing scFvs were collected by the centrifugation at 2000×g for 15 min [26]. In the soluble scFv ELISA, the Ag coating processes were performed similarly to that of the polyclonal phage ELISA with slight modification, in which 50 µL/well of the supernatant containing scFvs were incubated in the pre-coated plates at RT for 90 min. Then, the detection of specific scFvs was fulfilled using HRP-tagged protein A (with a dilution of 1:6000) at RT for 1 h.

PCR and sequencing

The PCR and sequencing were performed on pIT2 phagemid purified from the isolated positive scFv clones using QIAprep Spin Miniprep Kit. The PCR reaction was performed for 30 cycles (94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min) using LMB3 (5'-AGGAAACAGCTATGAC-3') and pHENseq (5'-CTATGCGGCCCATTC-3') primers. Then, the PCR products were subjected to the electrophoresis by running on 1% agarose gel to verify the presence of full-length V_H-V_L polypeptide. The sequencing was accomplished on the phagemid using LMB3 primer and analyzed by Chromas 2.33 software. To compare Ag binding regions, the amino acid sequences were aligned to the VBASE2 database upon the isolated scFv clones [27, 28].

Expression and purification of scFvs

For the expression of soluble scFvs, the positive scFv clones of HB2151 strain were cultured in 400 mL phosphate-buffered 2xYT-AG (i.e. 2xTY media containing 10% PBS, 100 µg/mL ampicillin and 2% glucose) under shaking (250 rpm) at 37 °C for 3 h, until reaching OD_{600nm} of ~0.9. Afterward, the culture media was changed to the induction media (i.e., 2xTY media containing 0.4 M sucrose and 1 mM IPTG). Then, the cells were further cultivated under shaking (250 rpm) at 30 °C for 5 h. For the preparation of periplasmic scFv, the induced bacterial cells were harvested by centrifugation (2800×g) at 4 °C for 10 min. Then, the pellets were incubated with 1/20 volume of the culture volume of TES buffer (100 mM Tris-HCl pH 8.0, 1 mM EDTA and 20% sucrose) containing complete protease inhibitors, ULTRA tablets (Roche, Basel, Switzerland) on ice for 1 h. The extracted periplasmic scFvs were collected by centrifugation (17,000×g) at 4 °C for 30 min, followed by dialysis against PBS at 4 °C overnight [29]. The IMAC method was

used for purification of the isolated scFvs. To this end, the extracted periplasmic scFvs were concentrated by Pierce Concentrator device with 9 kDa molecular weight cut-off (Thermo Scientific Pierce, Waltham, MA, USA) in 20 mL. They were then purified using Nab™ Protein L Spin Kit (Thermo Scientific Pierce, 0.2 mL) according to the manufacturer instruction. Briefly, 0.5 mL of the concentrated scFvs were incubated in a mini spin column for 15 min, followed by washing with binding buffer until reaching OD_{280nm} of zero. Subsequent to the elution of the bound scFvs by 0.2 M glycine-HCl buffer (pH 2.8), the scFvs containing fractions were collected and dialyzed against PBS and stored at -20 °C [30].

SDS-PAGE and Western blotting

An equal amount of expressed and purified scFv samples were run on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition using Miniprotean Tetra Cell system (Bio-Rad, Hercules, CA, USA). The scFv samples were treated with 5X SDS-sample buffer and boiled for 5 min, then the electrophoresis was performed at 200 V for about 1.5 h. Finally, Coomassie Brilliant Blue G-250 staining was done to visualize the resolved protein bands [31].

Expression and purification of scFvs were further verified by Western blotting. After SDS-gel electrophoresis, scFv samples were transferred onto a nitrocellulose membrane using a semi-dry Trans-Blot system (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skimmed milk at 4 °C overnight, washed (×3) by PBST (PBS containing 0.05% Tween 20) for 5 min, and incubated with the mouse anti-c-myc Ab-9E10 (Santa Cruz, Dallas, TX, USA) at a dilution of 1:1000 for 90 min. The membranes were washing (×3) with PBST and incubated with HRP-conjugated goat anti-mouse IgG (Abcam, Cambridge, UK) at a 1:4000 dilution for 1 h. The positive reactivity of the scFvs was visualized by an enhanced chemiluminescence system, ECL™ Prime Western blotting detection reagent (GE Healthcare, Little Chalfont, UK) [32].

Immunoblotting with purified scFv

Specific binding of the selected scFvs was characterized through immunoblotting of the lysate of the CCK2R-positive AGS cells. After washing (×3) with cold PBS buffer, AGS cells lysate was prepared using lysis buffer (50 mM Tris, 150 mM NaCl, 1 Mm EDTA, 1% Np40) supplemented with complete ULTRA tablets. After incubation at 4 °C overnight, the lysate was centrifuged (17,000×g) at 4 °C for 10 min. Total cell lysate (40 µg) was run on a 10% SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated

with the purified scFv at the concentration of 20 µg/mL in 2% MPBS for 2 h. After washing ($\times 3$) with PBST, the membranes were stained and developed as described previously to detect the specific binding of scFvs [33].

Flow cytometry analysis of purified scFvs

To determine the reactivity of purified scFvs with the native conformation of CCK2R, flow cytometry analysis was fulfilled in the CCK2R-positive AGS cells. The cells were cultivated to reach 50–70% confluency, then harvested by 0.25% trypsin-EDTA and centrifuged ($300\times g$) at 4 °C for 5 min. Then, approximately 2.5×10^5 cells per sample were blocked by FACS buffer (PBS containing 0.5% BSA and 0.1% Na₃N) for 30 min. The samples were washed with FACS buffer and incubated with 100 µL FACS buffer containing 10 µg scFv for 2 h. The cells were washed twice with FACS buffer, and then stained with the mouse anti-c-myc Ab-9E10 (10 µg/mL) for 1 h and the secondary goat anti-mouse IgG-PE conjugated Ab (0.5–1 µg) for 45 min. After the last washing step ($\times 2$), the cells were resuspended in 400 µL PBS. Detection of the fluorescently stained cells was performed using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). To exclude nonspecific binding of the secondary antibodies and hence the background staining, isotype controls which were the cells stained with both anti-c-myc and anti-mouse IgG-PE conjugated antibodies in the absence of scFv were included in each experiment. The cells stained with the commercial CCK2R Ab (Santa Cruz, Dallas, TX, USA) and the unrelated scFv were served as the positive and negative controls, respectively [34].

Surface plasmon resonance (SPR) analysis

The binding affinity of purified scFvs was determined by SPR using MP-SPR Navi 210A, instrument (BioNavis Ltd., Tampere, Finland). The biotinylated peptide was immobilized on the flow cell-1 (Fc1) of the streptavidin-coated sensor chip at a low concentration of 15 µg/mL, and a flow rate of 30 µL/min was applied for 7 min to obtain 100 response unit (RU). The Fc2 was applied as reference channel without peptide injection. For the affinity measurement, each scFv was injected onto the Fc1 and Fc2 at 5 different concentrations ranging from 50 to 1000 nM prepared in PBS [35]. Analysis of kinetic parameters was carried out using Data Viewer and TraceDrawer software (BioNavis Ltd., Tampere, Finland).

Statistical analysis

For the statistical analysis, one-way ANOVA followed by a multiple comparison post hoc test was used. A *p* value of less than 0.05 was considered for statistical significance.

Methods in in silico assays

Three-dimensional structure modeling

Three-dimensional (3D) structure of cholecystokinin type B receptor (CCK-BR), isoform 1 (UniProtKB ID: P32239–1), and ten scFvs (JC8, JC2, JE2, JC1, JD9, JB2, JA4, JB3, JA2, JC5) were modeled by using the MODELLER v9.18 [36]. Then, the NCBI blastp algorithm (Basic Local Alignment Search Tool, Protein-protein blast) was served against the Protein Data Bank (PDB) database to find the high-rank homologous structures as a template for homology modeling. The modeled structures were visualized via the Chimera v1.11 software [37].

Energy minimization of 3D models and preparing for molecular docking

The possible internal interactions of the predicted structures were normalized by means of the Swiss-pdb viewer software [38]. This step was implemented through energy minimization tool of the Swiss-pdb viewer, within a vacuum and with the GROMOS96 43B1 parameters set without a reaction field. The 3D structures were initially prepared for docking by using the Dock Prep tool of Chimers program [39]. This tool accomplished some changes one-by-one including (i) deletion of solvent molecules, (ii) removal of alternate locations, (iii) keeping only the highest occupying positions, (iv) replacement of incomplete side chains by Dunbrack rotamer library, and (v) addition of hydrogen atoms and charges [40]. The AMBER ff14SB force field was measured for the standard residues and the AM1-BCC force fields for the other residues.

Homology modeling validation

The homology modeling quality was validated using the GA341 and Discrete Optimized Protein Energy (DOPE) scores of the MODELLER. The ProSA z-score, Verify3D and ERRAT online web-servers were also served for validation of the 3D structure modeling quality [41–44]. The model's geometry and stereochemical features were checked based on the psi/phi Torsion angles through the Ramachandran's plot that was mapped using the RAMPAGE online web-server [45].

CCKBR–scFv docking

The orientational docking of the scFvs and CCK-B receptor was performed by using ZDOCK server v3.0.2 [46], a cross-docking program that uses a Fast Fourier Transform method to perform an efficient 3D search between two proteins. This rigid-body docking tool utilizes a combination of scoring functions (e.g., shape complementarity, electrostatics, and

statistical potential) [47]. The molecular interactions (e.g., hydrophobic and hydrogen bindings) of the docked molecules were interpreted by using the DIMPLLOT command of the LIGPLOT software v1.4.5 [48].

Results

Analysis of biopanning progress by polyclonal ELISA

Polyclonal phage ELISA was used to evaluate enrichment of the biotinylated peptide-specific phages. For this purpose, amplified phage-scFvs after each round of panning were added to the plates coated with or without the biotinylated peptide (as the positive and control plates, respectively). As shown in Fig. S1 (Supplementary data), four rounds of the selection showed gradual increases in the signals from the unselected J library towards the fourth round, despite the decreased concentration of the biotinylated peptide during the successive rounds. The first round of panning with the signal value of 0.6 demonstrated the initiation of specific phage-scFvs enrichment as compared to the unselected J library with the weak signal value of 0.14. The third and fourth rounds of panning displayed a 20-fold increase in the signal, indicating a complete enrichment of specific phage-scFvs.

Screening for specific scFv clones

To characterize the specific scFv clones with binding activity to the biotinylated peptides, 96 individual HB2151 clones resultant from the fourth round of panning by soluble scFv ELISA were screened. Figure 1 shows that 38 scFv clones were able to produce signals at least 10-fold greater than that

of the negative control, which is about 40% of the scFv clones resultant from the fourth round of biopanning.

PCR and sequence of the positive clones

PCR amplification confirmed that the selected 35 Abs clones harbor full-length scFvs (i.e., 935 bp). To ensure the diversity of scFv clones, complete nucleotide sequences of V_H and V_L were determined for all the 35 ELISA positive clones. As shown in Table 1, DNA sequence analysis of all the 35 ELISA positive clones using CDRs comparison in VBASE2 database revealed that 10 scFv clones possessed amino acid similarity with three isolated clones JD9 (42%), JC8 (14%) and JC5 (14%), dominating the population.

Large-scale expression and purification of soluble scFv fragments

The selected scFv clones, which were expressed on a large scale and then soluble fractions extracted from the periplasmic, were subjected to the affinity chromatography purification. The SDS-PAGE electrophoresis and Western blotting analyses resulted in a clear band of protein with a molecular weight of approximately 28 kDa (Figs. 2a, b). Induction of periplasmic expressions was carried out at different time points (1–5 h). The expression of scFvs was found to be a time-dependent process as the intensity of a given protein band was enhanced by the expression time period. Most of the scFv clones were found to produce a higher amount of scFv after 4 to 5 h of the induction. The expression yield of scFv clones was in the range of 0.3–1.34 mg/L of culture (Table 1).

Fig. 1 Soluble scFv enzyme-linked immunosorbent assay (ELISA). Individual antibody clones (96 clones) from round 4 were screened for affinity binding to the biotinylated peptide through soluble scFv ELISA. Bacterial supernatants containing soluble scFv antibody fragments were

incubated with target plates. Target plates used in ELISA assay include the positive plate (sequentially coated with biotinylated-BSA, streptavidin and the biotinylated peptide, gray bars) and the negative control plate (coated with biotinylated-BSA and streptavidin, black bars)

Table 1 CDRs diversity of the scFv clones selected from Tomlinson J library

Selected Clones	Frequency % (number of occurrence)	V _H chain			V _L chain			Yield of ScFvs (mg/L)
		CDR1 (27–34)	CDR2 (56–63)	CDR3 (105–113)	CDR1 (27–32)	CDR2 (56–58)	CDR3 (105–113)	
JD9	42% (15/35)	GFTFSSYA	IHERGTKT	AKPGDDFDY	QSISSY	AAS	QQSYSTPNT	1.34
JC8	14% (5/35)	GFTFSSYA	IHPGGAQT	AKPGSEFDY	QSISSY	AAS	QQSYSTPNT	1.25
JC5	14% (5/35)	GFTFSSYA	IAPGGAQT	AKPGAEDFY	QSISSY	EAS	QQSSLAPQT	1.26
JB2	8% (3/35)	GFTFSSYA	IETQGTKT	AKHGTDFDY	QSISSY	GAS	QQELRLPTT	1.12
JE2	5% (2/35)	GFTFSSYA	IPMNGGLT	AKIGDDFDY	QSISSY	AAS	QQFTRLPLT	1.22
JA4	2% (1/35)	GFTFSSYA	IANGGAQT	AKPGADFDY	QSISSY	DAS	QQSSLAPQT	0.52
JB3	2% (1/35)	GFTFSSYA	IAPGGAQT	AKPGAEDFY	QSISSY	GAS	QQYHAFPGT	0.68
JC1	2% (1/35)	GFTFSSYA	IAASGSLT	AKLTHTFDY	QSISSY	SAS	QQVVNMPET	0.8
JC2	2% (1/35)	GFTFSSYA	IAASGSLT	AKLTHTFDY	QSISSY	KAS	QQQSPSPMT	0.31
JA2	2% (1/35)	GFTFSSYA	IAASGSLT	AKLTHTFDY	QSISSY	HAS	QQMGHRPLT	1.29

DNA sequences of 35 positive scFv clones were aligned in the VBASE2 database and results displayed as CDRs comparison. Sequences are given in single-letter amino acid code. Numbering and assignment of CDRs were according to IMGT. Frequency shows the occurrence of scFv clones in ELISA-positive scFvs screened

After purification of scFvs by affinity chromatography using protein L, SDS-PAGE, and Western blotting analyses were conducted to validate the purity of periplasmic scFvs. As

shown in Fig. 2 (panels C and D), the purification procedure resulted in a high purity of a single protein band corresponding to full-length scFv (approximately 28 kDa). The

Fig. 2 SDS-PAGE and Western blot analyses of the expression and purification processes. The expression of periplasmic scFvs was induced with 1 mM IPTG, and then total cell lysates after 5 h cultivation were analyzed. Panels **a** and **b** respectively represent the SDS-PAGE and Western blotting analyses for uninduced (UN) and induced (IN), periplasmic extraction samples 1–5 h after induction and uninduced periplasmic sample 5 h (UNP). Panels **c** and **d** respectively

show the SDS-PAGE and Western blotting analyses after the purification of periplasmic scFvs by protein L affinity chromatography. UN and UNP served as negative controls. Con.P: concentrated periplasmic fraction; F: Flow-through; W1-W2: Washed fractions; E1-E4: Elution fractions; MM: Molecular mass marker in kDa; IPTG: Isopropyl β -D-1-thiogalactopyranoside

purification process of major scFv clones confirmed that the highest amount of scFvs were eluted in fraction 2. The expression and purification of all selected scFv clones exhibited similar results (data not shown).

Experimental binding affinity determination

The k_{on} , k_{off} and K_D values of 7 purified scFvs, which were able to recognize the CCK2R peptide either through immunoblotting or flow cytometry analyses, are summarized in Table 2. The K_D values of the scFvs varied from 0.68 to 8 nM. Selection of the scFvs with a sub-nanomolar range of affinities appeared to be very important findings because the selection of scFvs was performed using phage library with the moderate diversity (1.37×10^8 transformants). Kinetics data obtained by SPR analyses indicated that the scFv clones JB3 and JD9 displayed strong binding kinetic with the slowest off-rates of 12.5 and $0.25 \times 10^{-4} \text{ s}^{-1}$, respectively.

Specificity analysis of scFvs

To assess the binding potential of the selected scFvs towards CCK2R, the purified scFvs were examined by the immunoblotting and flow cytometry. For the immunoblotting, the AGS cells lysates were used. As shown in Fig. 3, the immunoblotting analysis revealed that 8 out of 10 selected scFvs (i.e. JA2, JA4, JB3, JC1, JC2, JC8, JD9, and JE2) were able to specifically detect the CCK2R protein in the AGS cells lysate. Consistent with the commercial positive Ab specific to the cholecystokinin B receptor, a single protein band of 48 kDa corresponding to molecular weight of CCK2R was detected, indicating specific reactivity of the scFvs.

Besides, binding properties of scFv Ab fragments to the native CCK2R expressed on the cell surface were further evaluated by the flow cytometry analysis using AGS cells. Based on staining intensity profiles obtained by the flow cytometry analysis (Fig. 4), the scFvs were categorized into three groups,

Table 2 Affinity and kinetic parameters of the 7 scFvs measured for binding to the biotinylated peptide by SPR

ScFv clones	k_{on} ($10^5 \text{ M}^{-1} \text{ S}^{-1}$)	k_{off} (10^{-4} S^{-1})	K_D (10^{-9} M)
JE2	3.94	10	2.54
JC8	32.9	266	8.09
JC1	7.71	15.9	2.06
JA4	4.79	13.2	2.76
JA2	8.25	18.6	2.26
JB3	14.6	12.5	0.86
JD9	3.72	0.25	0.68

Association (k_{on}) and dissociation (k_{off}) constants were determined by MP-SPR Navi 210A for each purified scFv antibody and equilibrium binding constant (K_D) calculated as k_{off}/k_{on}

including strong (JC8 and JE2), weak (JA4, JD9, JC1, JB3, JA2, JC2) and negative (JB2 and JC5) binders. Overall, these results indicated that the scFv clones JC8 and JE2 were able to recognize the conformational structure of the CCK2R protein on the cell surface as compared to the commercial Ab specific to the cholecystokinin B receptor as the positive control.

Construction of 3D models and validation analysis

The CCK-B receptor was subjected to homology modeling based on the blastp against the crystallized protein structures in PDB for finding its structural templates. According to the homology finding method, we found a maximum 28% identity with our sequence that belongs to the chain A of 4S0V, the human Ox2 orexin receptor (PDB ID: 4S0V). The tertiary structure of CCK-BR, its second extracellular loop (residues 190–219), and one of the scFvs, as a sample are shown in Fig. 5. The MODELLER validation scores (GA341 and DOPE), ProSA z-score, Verify3D/1D score, ERRAT, and the Ramachandran plot results are summarized in Table S1.

The ProSA web-server provide an overall z-score for 3D structures, and the stabilized models have more negative z score values. As shown in Table S1, the ProSA z-score of the 3D structures is within the range of values that commonly belong to the experimentally identified proteins. However, the z-score of the second extracellular loop of CCK-2R (−4.55) was less than the scFv 3D models. An acceptable 3D model should have an ERRAT score more than 50%. Moreover, The Verify3D/1D score indicates the percentage of residues that have scored greater than or equal to 0.2. The model with more negative DOPE scores can count as a reliable structure. However, this score is not normalized with respect to the protein size and cannot be as a comparative index between the different sequences. The range of GA341 score is from zero to 1.0, where the model with GA341 scores near 1.0 can be distinguished as a good model. According to these validation scores and based on the Ramachandran plot analysis, the quality of the modeled scFvs is satisfying. Nonetheless, due to the low similarity percentage (28%) between the query sequence (CCK-2R) and the template PDB structures, the quality of homology modeling in the case of CCK-2R is not favored.

In silico simulation of the CCK2R-scFv docking

According to the experimental assays, we expected to get a range of binding affinities between scFvs and the extracellular domain of the CCK-B receptor (aa190–219). In this case, we carried out the orientational docking for all the ten scFv clones including JC8, JE2, JA4, JD9, JC1, JB3, JA2, JC2, JB2, and JC5. Then, the quality and quantity of the amino acids that involved in the interacting sites between scFvs and CCK-BR were evaluated using DIMPLLOT software (Table S2). The Ligplot analysis showed the JC5 and JB2 scFv clones have

Fig. 3 Specificity analysis of the selected scFvs using Immunoblotting. AGS cell lysate containing CCK2R protein was separated on 12% SDS-PAGE and then transferred onto the membrane. The membranes were incubated with purified scFv fragments: JA2 (a), JE2 (b), JC2 (c), JC1 (d), JC8 (e), JB3 (f), JA4 (g), JD9 (h), as well as, a commercial anti-

CCK2R antibody as a control (i). Then, the membranes were stained with anti-c-myc tag and HRP-conjugated goat anti-mouse IgG antibodies and subsequently staining was developed by ECL. Arrowhead shows the location of CCK2R protein band (48 kDa) detected by scFv and control antibodies

the lowest hydrophobic and hydrogen binding affinity with CCK-BR and JC2, JE2, JC1, and JC8 showed a more binding activity with the CCK2R, which agrees with our FACS and immunoblotting result.

Discussion

To date, over 50 FDA-approved therapeutic mAbs have been commercialized for treatment of various diseases (e.g., cancer, inflammation, infection and metabolic diseases), while many others are in different stages of clinical trials [49]. Majority of the FDA-approved mAbs are recombinant mAbs, chimeric and humanized. Targeted therapy of cancer using mAbs provide promising outcomes [50]. However, most of the mAbs are routinely produced by hybridoma technology and recombinant engineering to reduce immunogenicity such as human anti-mouse Ab response (HAMA) [51, 52]. Moreover, in comparison with the conventional hybridoma method, the PAD technology has several advantages, including: (i) high affinity antibody isolation against any Ag and even toxic substances as well as self-Ags, (ii) fully human antibody selection with no potential immunogenicity in a time- and cost-effective manner, and (iii) by-passing laborious works such as animal immunization necessary for the hybridoma technology [19, 53].

To design non-immunogenic fully human mAbs, various combinatorial human Ab displayed libraries have been used, including phage, ribosome, mRNA, *E. coli*, yeast, and mammalian cell display systems. Among them, phage display technology (PDT) has extensively been used for the isolation of recombinant Ab fragments such

as scFv and Fab [54–61]. PDT in combination with relevant different selection platforms (e.g., whole cell panning, solution or solid-phase selection, and paramagnetic proteoliposomes) have been considered as a robust biopharmacological technology for developing mAbs against a few GPCRs family (e.g., C3aR, C5aR, CCR4, CCR5, and CXCR4); well-reviewed by Hutchings et al. [62]. In the current study, we aimed to develop scFvs against the CCK2R, which plays an important role in the initiation and progression of human gastrointestinal tumors. A biotinylated peptide encompassing the ECL2 of CCK2R was used for generation of scFvs. The ECL2 of CCK2R was used as a potential target for the selection of scFvs because it is the most diverse and accessible loop of the class A GPCRs presumably contributing to the activation of receptors via ligand recognition, binding, and signaling [6, 63]. To this end, we applied “human single fold scFv library J” and a solution-phase biopanning against the ECL2 for the selection of scFvs, whose specificities were further validated by immunoblotting and flow cytometry. About 20-fold enrichment of specific phage-scFv binders was achieved after four rounds of the solution-phase biopanning (Fig. S1). Soluble scFv-based ELISA screening after the fourth round of selection (Fig. 1) showed high hit rate of 40% when is compared with the positive scFv clones screened from the solution-phase biopanning against a large diversity of proteinous antigens [64]. The PCR and sequencing results (Table 1) confirmed that all the selected clones harbor full-length inserts that contained 10 different scFvs sequences. Such diverse panel of scFvs isolated against the biotinylated peptide indicate that they might recognize different epitopes and/or bind to the same epitopes probably with

Fig. 4 Specificity analysis of the selected scFvs using flow cytometry: AGS cell expressing CCK2R protein was incubated with purified scFvs including JE2 (**a**), JC8 (**b**), JC2 (**c**), JD9 (**d**), JC1 (**e**), JB2 (**f**), JB3 (**g**), JA2 (**h**), JC5 (**i**), JA4 (**j**), and then binding of scFvs (orange) were identified with c-myc tag and anti-mouse IgG-PE conjugated antibodies. An

unrelated scFv (**l**) and a commercially available anti-CCK-BR antibody (**k**) were served as negative and positive controls, respectively. White histograms as an isotype control (staining of AGS cells in the absence of scFv or anti-CCK2R antibody) were included in the experiment to subtract background fluorescence

different affinities. After the large-scale expression, the selected scFvs were verified for binding to whole CCK2R antigen through immunoblotting, flow cytometry, and protein-protein docking methods. The yield of periplasmic expression for the selected scFv clones using a phagemid vector, pIT2, was found to be in a range of 0.3–1.34 mg/L of culture. These results are in consensus with the expression yield of 0.1–5 mg/L for scFv clones isolated from Tomlinson libraries [21, 65, 66].

To obtain high-affinity scFvs, the panning process was performed under highly stringent conditions in terms of

washing and purification steps (Fig. 2). The peptide concentration was also decreased during successive rounds of selection from 200 nM (round 1) to 50 nM (round 4). Having this biopanning procedure, we successfully isolated scFvs with the high binding affinity (< 10 nM) directly from Tomlinson J library (Table 2). The immunoblotting analysis revealed that 8 purified scFvs were able to specifically recognize the CCK2R in AGS cells lysate (Fig. 3). Flow cytometry analysis was accomplished to investigate the potential binding affinity of the selected scFvs to the intact CCK2R antigens expressed on the surface of

Fig. 5 Homology modeling of CCK2R and scFv and their molecular docking. **a** Three-dimensional structure of CCK2R. **(b)** Three-dimensional structure of scFv. The docking result is represented as 3D

(b) and 2D **(c)**. The 3D structures are visualized using the Chimera program and the intermolecular interactions between docked CCK2R-scFv is displayed as 2D and via Ligplot software

AGS cells (Fig. 4), indicating the highest binding activity of two scFvs to AGS cells. Two scFvs (i.e., JC5 and JB2) failed to recognize the CCK2R peptide in both immunoblotting and flow cytometry assays, which may be explained by either the enrichment of phage binders specific to biotin or mini-PEG linker molecules during panning. To overcome such issues, Abs reactive with biotin/ mini-PEG should be removed through pre-depletion of PAL [67]. Less accessibility of conformational epitope(s) recognized by the six scFvs may attribute to the slight reactivity of some of the isolated scFvs in the flow cytometry, perhaps due to masking of the epitope by N-glycosylation or other membrane proteins [68]. The binding affinity is a function of the stability of the ligand-receptor formational pairs and optimizes the new bonds (e.g., hydrogen and hydrophobic) that affect the biological activity of a

complex molecule. Protein-protein docking programs such as ZDOCK can predict the conformation of protein pairs and intermolecular interactions without a crystalized structure (Fig. 5). Our findings highlight that the solution-phase biopanning is a well-suited approach for the isolation of high affinity and conformational-specific scFvs, which has successfully used for the selection of scFvs against CCK2R. Here, we modeled the 3D structure of the produced scFvs antibodies to be used in protein-protein docking process to investigate the CCK2R-scFv interaction activity.

The *in vitro* and *in silico* binding analysis showed that some of the newly isolated scFvs can efficiently bond to the CCK-B receptors. This finding can be used as an advanced pharmaceutical platform for the future specialized immunotherapy programs in the CCK2R-positive tumors.

Conclusions

Various types of Abs and Ab/non-Ab scaffolds have been developed to combat various perennial malignancies [69–71]. Of different technologies used to produce Abs, the phage display technology offers a great possibility for generation of fully human mAbs against various antigenic targets [54, 60, 72]. Even though CCK2R is involved in the initiation and progression of gastrointestinal tumors, surprisingly, no GPCR-specific immunotherapy has so far been used in the clinic against this superfamily. As a result, we capitalized on PDT and a solution-phase biopanning to develop therapeutic mAbs against CCK2R. According to our wet- and dry-lab findings, the selected scFvs showed high binding affinity and specificity against native conformation of CCK2R. Altogether, the selected scFvs are proposed to serve as targeting and therapeutic agents that might provide potential applications in both research and clinic.

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Compliance with ethical standards

Conflict of interest The authors of this study declare that they have no conflict of interest.

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