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Antagonistic effect of disulfide-rich peptide aptamers selected by cDNA display on interleukin-6-dependent cell proliferation

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ABSTRACT

Several engineered protein scaffolds have been developed recently to circumvent particular disadvantages of antibodies such as their large size and complex composition, low stability, and high production costs. We previously identified peptide aptamers containing one or two disulfide-bonds as an alternative ligand to the interleukin-6 receptor (IL-6R). Peptide aptamers (32 amino acids in length) were screened from a random peptide library by *in vitro* peptide selection using the evolutionary molecular engineering method "cDNA display". In this report, the antagonistic activity of the peptide aptamers were examined by an *in vitro* competition enzyme-linked immunosorbent assay (ELISA) and an IL-6-dependent cell proliferation assay. The results revealed that a disulfide-rich peptide aptamer inhibited IL-6-dependent cell proliferation with similar efficacy to an anti-IL-6R monoclonal antibody.

1. Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine with a wide range of biological roles in immune regulation, hematopoiesis, inflammation and oncogenesis [1]. IL-6 mediates its effects on cells through the formation of a complex involving the membrane bound glycoproteins IL-6 receptor-alpha chain (IL-6R-alpha) and gp130. Assembly of this IL-6/IL-6R/gp130 complex triggers an intracellular signaling event. IL-6 also plays important roles in the pathogenesis of diseases such as rheumatoid arthritis, systemic juvenile idiopathic arthritis and Cattleman's disease [2]. Tocilizumab, a humanized anti-IL-6 receptor monoclonal antibody, has received significant attention as a promising drug for treating these diseases [3]. Pharmaceutical companies have initiated clinical development of hundreds of monoclonal antibodies for various disease indications [4]. However, antibodies have several noticeable disadvantages, including a large size and complex composition, low stability, and the high production costs of full-size antibodies under Good Manufacturing Practice conditions. Thus, novel alternative binding reagents (i.e., engineered protein scaffolds) have been developed by modifying the binding site of an existing rigid natural protein structure (e.g., lipocalins, fibronectin, protein A and ankyrin repeats) [5]. Small cystine knot miniproteins, which are stabilized by intramolecular disulfide bonds, as also represent promising therapeutic regents because of their high affinity and selectivity, small size and high biological stability [6].

We previously reported on peptide aptamers containing one or two disulfide-bonds as alternative ligands to IL-6R [7]. The 32-amino acids-long peptide aptamers were screened from a random peptide library by *in vitro* peptide selection using the evolutionary molecular engineering method "cDNA display" (Fig. 1). cDNA display is an *in vitro* display technology in which cDNA (i.e., the genotype) is covalently linked to peptide/protein (i.e., the phenotype) using a cell-free translation system (Fig. 1A). Since the selected peptide aptamers (namely Cys2 and Cys4) bound specifically to IL-6R with high affinity (Fig. 1C), we had considerable interest in defining the physiological properties of these aptamers. In this report, the physiological activities of the IL-6R peptide aptamers were examined by an *in vitro* competition enzyme-linked immunosorbent assay (ELISA) and an IL-6-dependent cell proliferation assay.

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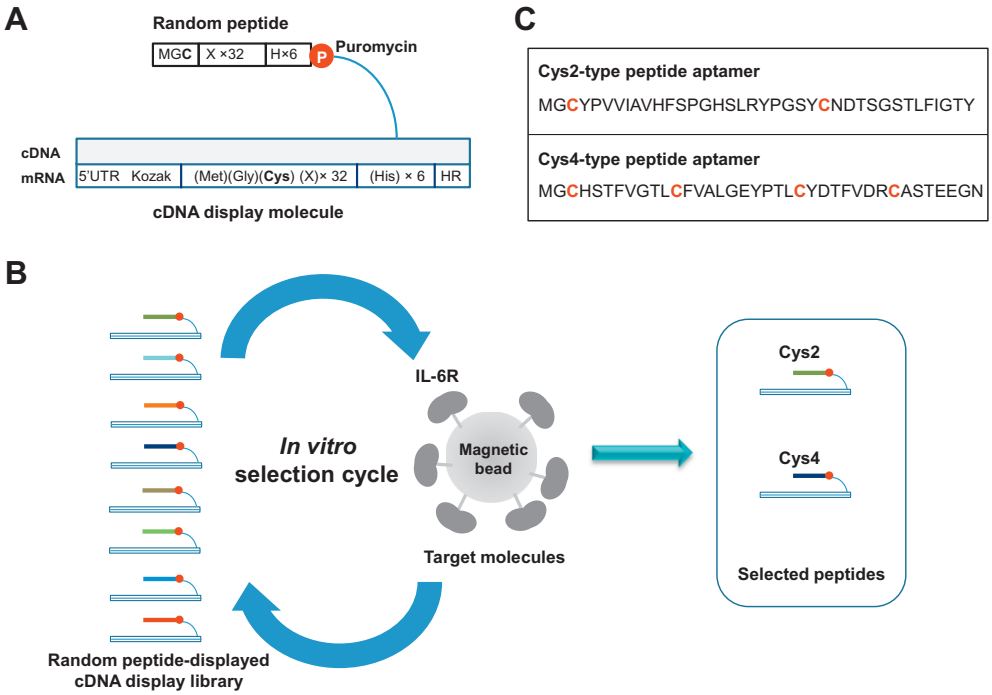

Fig. 1. Schematic diagram of *in vitro* selection from a random peptide library against the interleukin-6 receptor (IL-6R) by cDNA display. (A) cDNA display is based on the formation of a covalent fusion between cDNA (i.e. genotype) and its coding peptide (i.e., phenotype) via a puromycin attached to a DNA linker (puromycin–DNA linker) [7]. The random region of the peptide library is the 32 amino acids sequence. (B) After nine rounds of selection against IL-6R with the random peptide-displayed cDNA display library, Cys2-type and Cys4-type peptide aptamers were selected [7]. (C) The amino acids sequences of Cys2-type and Cys4-type peptide aptamers.

2. Materials and methods

2.1. Peptide chemical synthesis

Peptides containing two cysteine residues (Cys2-type) to form single disulfide bond, the N-terminus of which was attached to a biotin moiety, were synthesized by Toray (Tokyo, Japan). Peptides containing four cysteine residues (Cys4-type) bearing the patterns of two different disulfide bonds: (i) C1 and C4, and C2 and C3 (Cys4A); and (ii) C1 and C3, and C2 and C4 (Cys4B) were synthesized and characterized by HPLC to examine their topologies based on the retention time by the Peptide Institute (Osaka, Japan), as previously described [8].

2.2. Protein preparation

The DNA construct coding the maltose binding protein (MBP) fused to a Cys4 peptide was prepared using the pIVEX2.8d vector (Roche Applied Science, Switzerland). The protein was expressed by *in vitro* translation using RTS100 *E. coli* HY Kit (Roche Applied Science) and purified using His-tagged protein purification column (Qiagen, Germany).

2.3. Determination of the dissociation constant (Kd)

The binding affinities of the synthesized biotinylated peptides were assayed using a previously reported ELISA, with a few modifications [9]. Briefly, 10 nM of the biotinylated peptide was incubated with varying concentrations of free IL-6R (0.2–50 nM (Fig. 2C) and 1–500 nM (Fig. 2D)) in phosphate-buffered saline (PBS) at 25 °C for 1 h. The mixture was then incubated with IL-6R-coated beads for 30 min. After several washes with PBS containing 0.05% Tween 20 (PBS-T), streptavidin–horseradish peroxidase (SA–HRP; 1:2000 dilution) was added and incubated for 30 min. The supernatant was removed and the beads were washed 4–5 times with PBS-T. Next, 200 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added for color development and the reaction was terminated with 0.5 M H_2SO_4. The absorbance was measured at 450 nm. Data were plotted using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA).

2.4. Cys4 peptide aptamer inhibition assay

Inhibition of the interaction between IL-6 and IL-6R by the selected peptides was determined using competitive inhibition assays. A fixed amount of biotinylated IL-6 (45 nM, i.e., ~3 times the Kd) and varying concentrations of the peptide (50 nM–5 μM) were incubated with 350 nM IL-6R-coated beads in PBS containing bovine serum albumin at 25 °C for 1 h. After several washes with PBS-T, SA–HRP (1:2000 dilution) was added and incubated for 30 min. The supernatant was removed and the beads were washed 4–5 times with PBS-T. The TMB substrate (200 μL) was added for color development and the reaction was stopped with 0.5 M H_2SO_4. The absorbance was measured at 450 nm.

2.5. Cell culture and reagents

A lymphoblast cell line (DS-1 [10]) was obtained from the American Type Culture Collection (Manassas, VA, USA) and was cultured in RPMI 1640 complete culture medium (GIBCO, NY, USA) supplemented with 10% fetal bovine serum (GIBCO), 10 mM HEPES buffer (GIBCO), 10 U/ml human IL-6 (Sigma, St. Louis, MO, USA), penicillin (16 mg/L) and streptomycin (25 mg/L), at 37 °C in a humidified incubator with 5% CO_2. The cells, in 25 cm^2 flasks, were subcultured every 2–3 days. Cells were continuously cultured until harvested for analysis.

2.6. Cell proliferation assay

DS-1 cells in the logarithmic growth phase were transferred, with medium, to a centrifuge tube and centrifuged at 1400×*g* for 5 min, and washed twice with PBS. The cells were resuspended

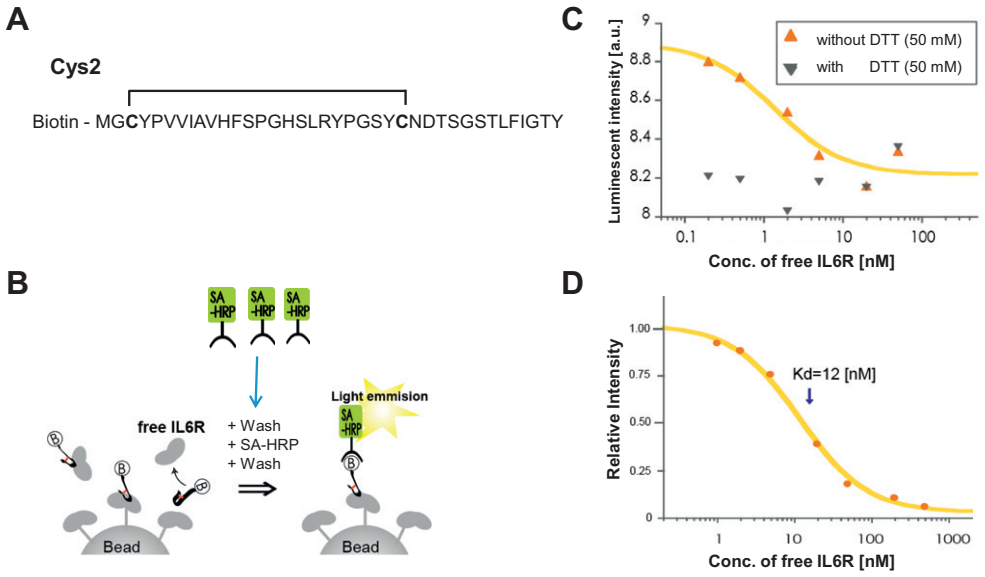

Fig. 2. Competitive enzyme-linked immunosorbent assay (ELISA) for the Cys2 peptide aptamer against IL-6R. (A) The amino acid sequence of the synthetic Cys2 peptide. Biotin is attached to the peptide's N-terminus. The two cysteine residues were chemically cross linked. (B) Schematic diagram of the ELISA used to determine the affinity of the Cys2 peptide IL-6R, as described in Section 2.3. (C) The affect of the presence/absence of the disulfide bond on the affinity to IL-6R. The affinity of the Cys2 peptide to IL-6R was measured in the absence and the presence of dithiothreitol (DTT). (D) The dissociation constant (Kd) of Cys2 peptide against IL-6R.

(2×10^4 cells/mL) in IL-6-free standard culture medium. The cells were then transferred to 24-well flat-bottomed microtiter plates (1 mL/well) and cultured for 24 h. The desired concentrations of anti-human IL-6R antibody (R&D Systems, Minneapolis, MN, USA) and human IL-6 were prepared in IL-6-free standard culture medium. The peptide aptamers were dissolved in dimethyl sulfoxide to a concentration of 10 mM and diluted to the specified concentrations using IL-6-free standard culture medium. After 24 h of culture, the cells were further cultured with human IL-6 (10 U/mL) and anti-human IL-6R antibody (0.2–13.7 nM) or the peptide aptamers (0.1 nM–10 μM) for 48 h, and cells were counted using a hemocytometer.

3. Results

3.1. In vitro characterization of the peptide aptamers

The two peptide aptamers which previously selected, Cys2 and Cys4, are shown in Fig. 1. The Cys2-type peptide aptamers interact with IL-6R with Kd values of 4–100 nM [7]. Both peptide scaffolds have the potential to form disulfide bonds; however, it was not examined whether the disulfide bond of the Cys2 peptide (Fig. 2A) is essential for its affinity to IL-6R. Here, we examined the influence of the disulfide bond on the affinity of the Cys2 peptide to IL-6R by ELISA (Fig. 2B). In this assay, we found that 10 nM of the free Cys2 peptide barely bound to IL-6R immobilized on beads at concentrations of 0.2–50 nM of free IL-6R in the presence of 50 mM dithiothreitol (DTT). In contrast, in the absence of DTT the aptamer was observed to bind to the IL-6R on beads (Fig. 2C). The resulting Kd value was 12 nM (Fig. 2D). However, the Cys2 peptide did not inhibit the interaction between IL-6 and IL-6R (data not shown). Thus, the Cys4 peptide was synthesized as an MBP fusion construct using a cell-free translation system (Fig. 3A). To examine the inhibitory effect of the Cys4 peptide on the interaction between IL-6 and IL-6R, we performed a competitive inhibition assay (Fig. 3B). In this assay, native IL-6 inhibited the binding of biotinylated IL-6 to IL-6R with an IC_{50} value of 80 ± 3 nM [11], whereas the Kd value for the Cys4 peptide was between 50 and 500 nM (Fig. 3C). These results indicate that the Cys4 peptide should inhibit the interaction between IL-6 and IL-6R.

3.2. Inhibition of IL-6-dependent cell proliferation by the peptide aptamers

We synthesized two kinds of Cys4 peptides with different disulfide bond (S–S) linkages, Cys4A and Cys4B, by solid-phase peptide synthesis (Fig. 4A). As previously reported, Cys4A strongly binds to IL-6R whereas Cys4B weakly interacts with IL-6R [7]. In this report, to confirm the physiological activities of these peptide aptamers, influence to the proliferation of DS-1 cells (i.e. a process that is dependent on IL-6) was examined. As a positive control of antagonistic activity, cells were treated with an anti-IL-6R antibody. As shown in Fig. 4B, the anti-IL-6R antibody inhibited IL-6-dependent proliferation of DS-1 cells in a concentration-dependent manner, with an IC_{50} of 1.7×10^{-9} M. The antagonistic properties of the peptide aptamers Cys4A, Cys4B and Cys2 were tested using the same procedure. In this assay, Cys4A was observed to effectively inhibited IL-6-dependent proliferation of DS-1 cells ($IC_{50} = 2.36 \times 10^{-7}$ M), whereas Cys4B only weakly inhibited DS-1 cells and Cys2 did not inhibit cell proliferation (Fig. 4C).

4. Discussion

Miniproteins, rigid scaffolds containing α-helices, β-sheets and disulfide-constrained secondary structural elements are currently under development as alternative, non-immunoglobulin proteins for the generation of novel binding motifs [12]. Peptide aptamers, which differ from other classes of miniproteins, are promising candidate ligands because of their small size, simple design and disulfide-independent folding [13]. In general, peptide aptamers selected by the yeast two-hybrid method are artificial recognition molecules that consist of a variable peptide region of 8–20 amino acids and are displayed with a scaffold protein [14]. As an alternative to peptide aptamer selection by the yeast two-hybrid method, ligand-like linear short peptide aptamers can be selected by phage display against FLASH (FLICE-associated huge protein) [15] and by cDNA display against cathepsin E [16] from random peptide

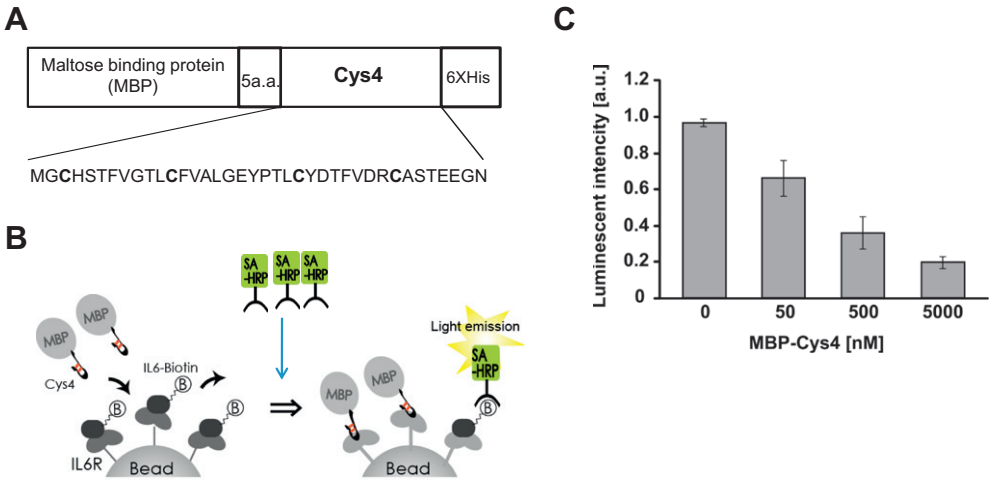

Fig. 3. *In vitro* inhibitory effect of the Cys4 peptide on the interaction between IL-6 and IL-6R. (A) Structure and sequence of the Cys4 peptide, a maltose binding protein (MBP) fusion construct. (B) Schematic diagram of the competitive inhibition assay used to examine the effects of the Cys4 peptide on the interaction between IL-6 and IL-6R, as described in Section 2.4. (C) MBP-Cys4 dose-dependently inhibits the interaction between IL-6 and IL-6R. Values are means ± standard error ($n = 4$).

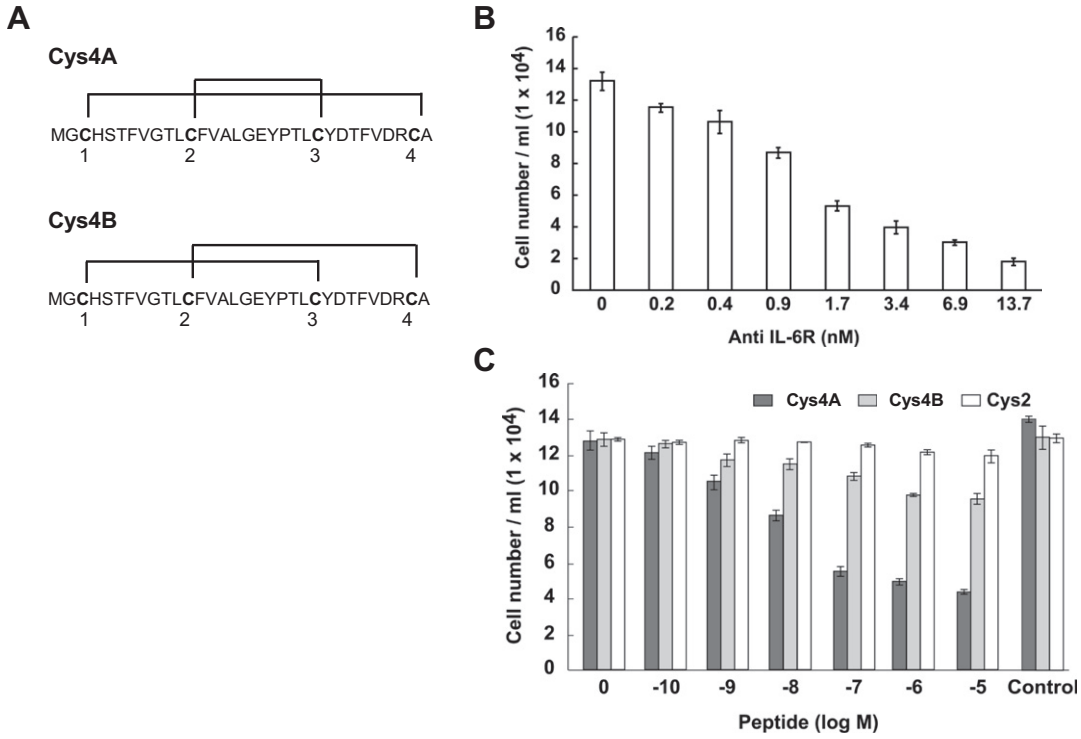

Fig. 4. Anti-IL-6R antibody and peptide aptamers dose-dependently inhibit IL-6-dependent cell proliferation. (A) Amino acids sequences and the disulfide bonds in Cys4A and Cys4B. (B, C) DS-1 Cell numbers determined at 48 h after treatment with IL-6 and the anti-IL-6R antibody or peptide aptamers. The anti-IL-6R antibody and Cys4A inhibited the IL-6-dependent proliferation of DS-1 cells with IC_{50} values of 1.7×10^{-9} M and 2.36×10^{-7} M, respectively. Cys4B weakly inhibited and Cys2 did not inhibit cellular proliferation. Control = cells treated with 0.1% dimethyl sulfoxide (the concentration used in the 10^{-5} M peptide condition). IC_{50} values were calculated based on 50% inhibition of cell proliferation. Values are means ± standard error ($n = 3$).

libraries of 12 amino acids and 8 amino acids in length, respectively. In this report, the 30 or more residue peptide aptamers were selected from a random peptide library without a constant protein using a cDNA display as in the case of DNA and RNA aptamers selected from random nucleotide libraries. Although the peptide aptamers are apparently linear flexible peptides, they have constrained and looped structures caused by disulfide cross-links (Fig. 2C), which is promoted by "disulfide shuffling" using cDNA display [7]. In our previous study, a three-finger scaffold (MicTx3) containing four disulfide bonds was successfully used to obtain ligands to a target protein (IL-6R) [11]. However, until now, it was unclear whether the artificial disulfide-rich peptide aptamers selected from a random peptide library are biologically active. This is the first report to show that an artificial looped peptide aptamer, without a scaffold protein, has antagonist activity (Figs. 3 and 4). Thus, artificial disulfide-rich peptides of $\geqslant$20 residues selected from random peptide libraries may represent novel peptide aptamer categories. In addition, although the Cys4 peptide aptamers inhibit the IL-6-dependent cell proliferation, the Cys2 peptide aptamers have negligible effect. This observation indicates that the number of disulfide

bonds within the peptides may be important in facilitating the biological activity of the peptide. Thus, the disulfide shuffling method by cDNA display represents a potential approach for the screening of functional cysteine-rich peptides with bioactivity.

Comparison of peptides with bioactivity against IL-6R may reveal valuable information. The Cys4 peptide aptamer has a loop structure similar to the IL-6R inhibitor peptide 13-L1 (LV**C**YQLLASRPGTLETGPDDFT**C**V; 24 residues), which is part of a three-finger scaffold [11]; however, their amino acid sequences are completely different. This shows that there are multiple sub-optimal peptide sequences against an identical function, such as receptor inhibition. Therefore, the fitness of the selected sequences may depend on the local optima of a rugged "fitness landscape", as previously reported [17,18].

In general, the handling of disulfide-rich peptides appears to be formidable because the determination of the cross-links is a challenging process. However, because the peptide aptamers are selected by cDNA display using cell-free translation systems, the folding of the peptide, such as the MBP-Cys4 (Fig. 3A), is performed during the translational and post-translational reactions. Thus, the peptide aptamers may be selected based on their propensity to form a unique loop structure and act as an active binder during the *in vitro* selection cycle. This results in a simple conformation that can be easily evaluated, unlike the natural cystine knot miniproteins, such as the conotoxins [19].

The formation of disulfide bonds in the cysteine-rich aptamers should provide stability to these ligand moieties in a similar manner to the natural cystine knot miniproteins. However, further experiments examining the thermal stability and proteolytic resistance of the disulfide-rich peptide aptamers are required.

We have improved high-throughput screening using a "one-pot" cDNA display method [20]. With such technical improvements, and the increasing adoption of *in vitro* display technologies [21], we anticipate that many peptide aptamers will be easily and cost-effectively selected from peptide libraries. Since an engineered cystine knot miniprotein has been successfully used for tumor imaging and several others are marketed as analgesics or have entered preclinical/clinical testing [22], we believe that disulfide-rich peptide aptamers represent a promising new class of drug molecules.

Acknowledgments

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