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Research paper

Claudin-4-targeting of *diphtheria* toxin fragment A using a C-terminal fragment of *Clostridium perfringens* enterotoxin

Hideki Kakutani [a], Masuo Kondoh [a,*], Rie Saeki [a], Makiko Fujii [b], Yoshiteru Watanabe [b], Hiroyuki Mizuguchi [c,d], Kiyohito Yagi [a,*]

[a] *Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan*
[b] *Department of Pharmaceutics and Biopharmaceutics, Showa Pharmaceutical University, Tokyo, Japan*
[c] *Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan*
[d] *Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan*

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ABSTRACT

Claudin (CL)-4, a tight junction protein, is overexpressed in some human neoplasias, including ovarian, breast, pancreatic and prostate cancers. The targeting of CL-4 is a novel strategy for tumor therapy. We previously found that the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) binds to CL-4. In the present study, we genetically prepared a novel CL-4-targeting molecule (DTA–C-CPE) by fusion of C-CPE and *diphtheria* toxin fragment A (DTA). Although DTA is not toxic to CL-4-expressing L cells, even at 20 μg/ml, DTA–C-CPE is toxic to CL-4-expressing L cells at 1 μg/ml. DTA–C-CPE-induced cytotoxicity was attenuated by pretreatment of the cells with C-CPE but not bovine serum albumin, indicating that DTA–C-CPE may bind to CL-4-expressing L cells through its C-CPE domain. To evaluate the specificity of DTA–C-CPE, we examined its cytotoxic effects in L cells that express CL-1, -2, -4 or -5. We found that DTA–C-CPE was toxic to only CL-4-expressing L cells. Thus, C-CPE may be a promising ligand for the development of cancer-targeting systems.

1. Introduction

Chemotherapeutic agents target the intracellular metabolic processes or growth rates that are different between malignant cells and normal cells, and rapidly growing cancer cells are sensitive to chemotherapies [1,2]. But, progressive cancer cells with a decreased growth rate respond poorly to chemotherapy [3]. Radiation therapy affects both the tumor and the surrounding normal tissue. These conventional therapies cause DNA damage, leading to genomic instability and susceptibility to neoplastic mutations [4]. Cancer cells often overexpress surface proteins, including growth factor receptors or antigens [5]; thus, targeting cancer cells by using the surface proteins is a promising strategy for cancer therapy. Ligands for growth factor receptors and cytokine receptors have been fused with fragments of bacterial toxins, such as *Pseudomonas* exotoxin and *diphtheria* toxin (DT) [3,6].

Tight junctions (TJs) form the apical junctional complex in epithelial cell sheets and play pivotal roles in the barrier of the epithelial cell sheets and the fence separating basal and apical components, such as receptors and transporters, on the membrane [7]. Epithelial TJs are dynamic structures that are modulated during neoplastic transformation [8]. The relationship between abnormal TJ function and epithelial tumor development has been suggested by earlier studies showing alterations in the TJ structures of epithelial cancers [9,10]. Loss of tight junction integrity may allow the diffusion of nutrients and other factors necessary for the survival and growth of the tumor cells [8]. Destruction of the fence function of TJs can lead to overproliferation of tumor cells [11,12]. If TJ components are exposed to the cell surface in cancer cells, they may be a promising target for cancer therapy.

Claudins (CLs) are key molecules in the formation of TJs; proteins in the 24-member claudin family contain four transmembrane domains [13]. CL-4 is frequently overexpressed in several neoplasias, including ovarian, breast, pancreatic and prostate cancers [12,14]. Thus, CL-4 may be useful as a target molecule in cancer therapy. CL-4 is a receptor for *Clostridium perfringens* enterotoxin (CPE), which is a single 35-kDa polypeptide that causes food poisoning in humans [15]. CPE exhibited anti-tumor activity

Abbreviations: C-CPE, the C-terminal fragment of *Clostridium perfringens* enterotoxin; DTA, *diphtheria* toxin fragment A; DTA–C-CPE, C-CPE-fused DTA; DT, *diphtheria* toxin; TJ, tight junction; CPE, *C. perfringens* enterotoxin; CL, claudin.

* Corresponding author. Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan. Tel.: +81 6 6879 8196; fax: +81 6879 8199 (M. Kondoh), tel.: +81 6 6879 8195; fax: +81 6879 8195 (K. Yagi).

E-mail addresses: masuo@phs.osaka-u.ac.jp (M. Kondoh), yagi@phs.osaka-u.ac.jp (K. Yagi).

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in CL-expressing cancers, such as breast [16], ovarian [17] and pancreatic cancers [18]. They did not observe side effects from CPE treatment, indicating that a ligand for CL-4 may be a promising candidate for cancer-targeting therapy.

CL has very low antigenicity, and there are few antibodies to the extracellular region of CL. CPE is composed of N-terminal cytotoxic domain and C-terminal receptor-binding domain [15]. C-CPE is the C-terminal receptor-binding domain, and C-CPE is the first CL-4-binder [19]. In the present study, we prepared a CL-targeting agent (DTA–C-CPE) consisting of C-CPE coupled to a protein synthesis inhibitory factor, fragment A of DT [20]. DTA–C-CPE had CL-4-specific cytotoxicity; thus, C-CPE may be a promising ligand for the development of cancer-targeting systems.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium (WST-8) and phosphatase inhibitor cocktail were purchased from Nacalai (Kyoto, Japan). Protease inhibitor cocktail and anti-β-actin mAb were obtained from Sigma–Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-labeled antibodies were obtained from Chemicon (Temecula, CA). Anti-His-tag antibody was purchased from Novagen (Madison, WI). All other reagents were of research grade.

2.2. Cell culture

L cells, a mouse fibroblast cell line, and mouse CL-expressing L cells were kindly provided by Dr. S. Tsukita (Kyoto University, Japan). Cells were cultured in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FBS) at 37 °C.

2.3. Preparation of DTA–C-CPE

DTA (CRM45) cDNA was kindly provided by Dr. K. Kohno (Nara Institute of Science and Technology, Japan) [21]. The plasmids containing DTA fused with C-CPE were prepared as follows. DTA was amplified by polymerase chain reaction (PCR) with pTA–DTA as a template, a forward primer (5′-GCGGTACCATGGGCGCTGATGATGTTGTTG-3′, *Kpn*I site is underlined) and a reverse primer (5′-CCTTAATTAATCGCCGTACGCGATTTCCTG-3′, *Pac*I site is underlined). The resulting PCR fragments were subcloned into *Kpn*I/*Pac*I-digested pETH$_{10}$PER (kindly provided by Dr. Y. Horiguchi, Osaka University, Japan), and the sequence was confirmed (pET–DTA–C-CPE). Double-stranded oligonucleotide of G/S linker was prepared by annealing (heating at 95 °C for 5 min and chilling at room temperature for 60 min) of single-strand oligonucleotides, a forward oligonucleotide (5′-TGGAGGAGGAGGATCTGGAGGAGGAGGATCTGGAGGATACCCATACGACGTCCCAGACTACGCTAT-3′, *Pac*I site is underlined) and a reverse oligonucleotide (5′-AGCGTAGTCTGGGACGTCGTATGGGTATCCTCCAGATCCTCCTCCTCCAGATCCTCCTCCTCCAAT-3′, *Pac*I site is underlined). The resulting oligonucleotides were subcloned into *Pac*I-digested pET–DTA–C-CPE, and the sequence was confirmed (pET–DTA-linker-C-CPE).

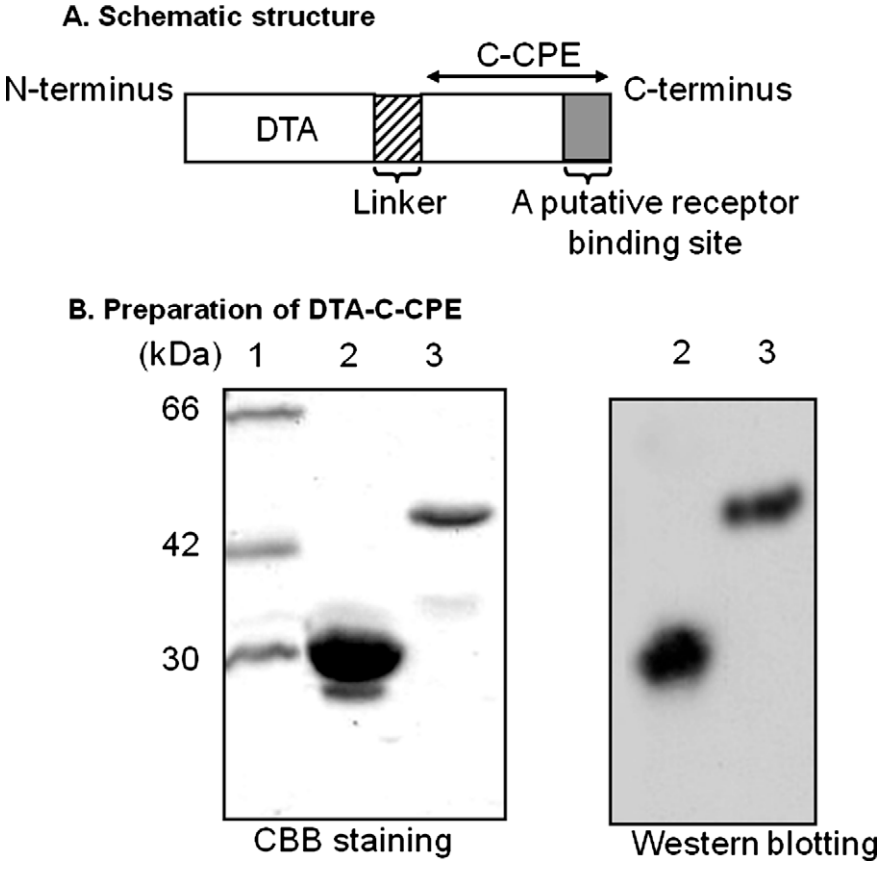

Fig. 1. Preparation of DTA–C-CPE. (A) Schematic structure of DTA–C-CPE. DTA–C-CPE is a fusion protein of DTA and C-CPE with a linker indicated by a slashed column. A dark column indicates a putative receptor-binding region of C-CPE. (B) Preparation of DTA–C-CPE. DTA or DTA–C-CPE was produced by a conventional expression system of *E. coli*, and the proteins were purified by His-tag affinity chromatography with Ni-resins. The purification of DTA–C-CPE was confirmed by SDS–PAGE followed by staining with Coomassie Brilliant Blue (CBB) (left panel in B) and by Western blotting using an anti-His-tag mAb (right panel in B). Lane 1, a marker of molecular size; lane 2, DTA; lane 3, DTA–C-CPE. The putative molecular sizes of DTA and DTA–C-CPE were 30 and 43.2 kDa, respectively.

The plasmid, pET–DTA-linker-C-CPE, was transduced into *Escherichia coli* strain BL21 (DE3), after which the cells were cultured in LB medium supplemented with 100 μg/ml ampicillin at 37 °C until the logarithmic phase. Isopropyl-D-thiogalactopyranoside (0.25 mM) was added to the medium, and the cells were cultured for an additional 3 h. The cells were harvested and then lysed in buffer A (10 mM Tris–HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol). The lysates were centrifuged, and the resultant supernatant was applied to HiTrap Chelating HP (GE Healthcare, Little Chalfont, UK). DTA–C-CPE was eluted by buffer A containing imidazole. The solvent was exchanged with phosphate-buffered saline by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80 °C until use. Purification of DTA–C-CPE was confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue and immunoblotting with anti-His-tag antibody. Protein was quantified by using a protein assay kit (Pierce Chemical, Rockford, IL) with BSA as a standard.

2.4. Cytotoxic activity

Cell viability was determined by using a tetrazolium-based colorimetric assay or lactate dehydrogenase (LDH) assay. Briefly, cells were seeded into a 96-well plate at 1×10^4 cells per well. On the following day, the cells were treated with DTA or DTA–C-CPE (0–20 μg/ml) for 48 h. In the colorimetric assay, WST-8 was added to the wells, mixed thoroughly and incubated for 1 h. Then, the absorbance was measured at 450 nm. In the LDH assay, the release of LDH from the cells was analyzed by using a CytoTox96 NonRadioactive Cytotoxicity Assay kit (Promega, Madison, WI), according to the manufacturer's protocol. The LDH release was calculated by using the following equation: percentage of maximal LDH release = LDH in the culture medium/total LDH in the culture dish.

2.5. Competition assay

Cells (1×10^4 cells) were pretreated with 0–40 μg/ml C-CPE or BSA for 2 h, and then 1 μg/ml of DTA–C-CPE was added. After an additional 48 h of culture, a colorimetric assay was performed as described previously.

3. Results

3.1. Preparation of DTA–C-CPE

When DTA enters the cytosol, it inhibits elongation factor 2 through ADP-ribosylation and induces the inhibition of protein synthesis, leading to cell death [20,22]. C-CPE is a receptor-binding domain of CPE, and the CL-4-binding region is located on the C-terminal of C-CPE [23]. To prepare a CL-4-targeting molecule, we genetically fused DTA with C-CPE at the N-terminal of C-CPE and C-terminal of DTA. A schematic illustration of DTA–C-CPE is shown in Fig. 1A. DTA–C-CPE was produced in *E. coli* and was purified by affinity chromatography with Ni-resins. The molecular size of DTA–C-CPE, as determined by SDS–PAGE and immunoblotting, was identical to its putative size (43.2 kDa, Fig. 1B).

3.2. Cytotoxic properties of DTA–C-CPE

To examine the cytotoxicity of DTA–C-CPE, we investigated the effects of DTA–C-CPE on CL-4-expressing L (CL4/L) cells. DTA had no effect on CL4/L cells at 20 μg/ml, whereas DTA–C-CPE dose-dependently decreased the viability, reaching 39.7% relative viability at 1 μg/ml (Fig. 2A). Similar results were observed in the LDH-release assay. As shown in Fig. 2B, 5 μg/ml of DTA did not cause a release of cellular LDH; but, DTA–C-CPE at 5 μg/ml significantly increased the release of cellular LDH from 4.7% to 25.0%.

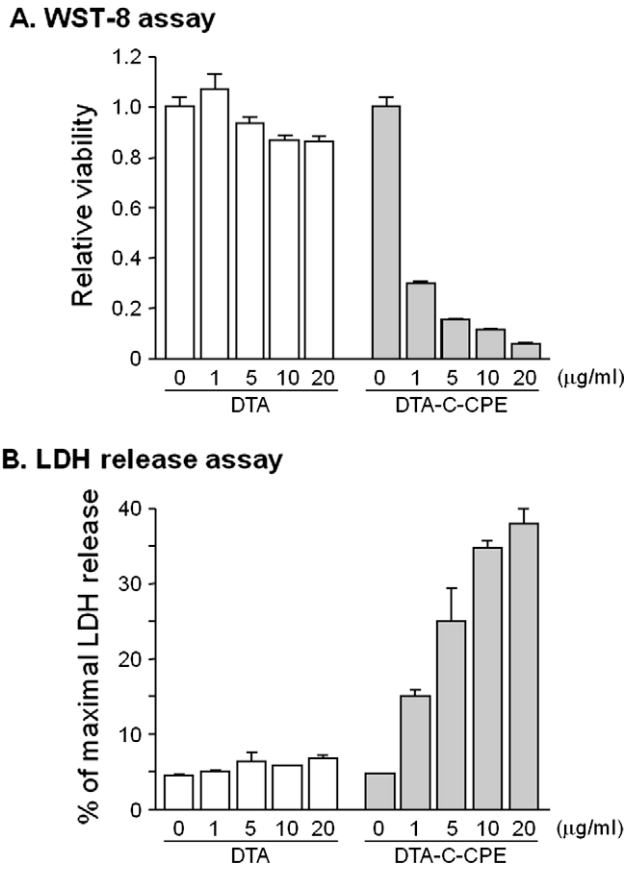

Fig. 2. Cytotoxicity of DTA–C-CPE. CL4/L cells were treated with DTA or DTA–C-CPE at the indicated concentration for 48 h. The cellular viability was measured by WST-8 assay (A) or LDH-release assay (B). Data are the mean ± SD ($n = 3$). The data are representative of three independent experiments.

3.3. Targeting properties of DTA–C-CPE

To confirm the CL specificity of DTA–C-CPE, we evaluated the cytotoxicity of DTA–C-CPE in L cells that expressed CL-1, -2, -4 or -5. DAT-C-CPE did not show severe cytotoxicity in L, CL1/L, CL2/L and CL5/L cells, even at 5 μg/ml, whereas DTA–C-CPE reduced the viability of CL4/L cells to 35.0% and 23.3% of the vehicle-treated cells at 1 and 5 μg/ml, respectively (Fig. 3A). To determine whether DTA–C-CPE bond to CL4/L cells via its C-CPE domain, we performed a competition assay. As shown in Fig. 3B, pretreatment of the cells with C-CPE dose-dependently attenuated the cytotoxic activity of DTA–C-CPE from 41.3% to 90.9% of viability at 0–40 μg/ml of C-CPE. In contrast, pretreatment of the cells with BSA at 40 μg/ml did not affect the cytotoxicity of DTA–C-CPE, indicating that DTA–C-CPE bound to the cells via its C-CPE domain. Thus, fusion of C-CPE gives a CL-4-targeting property to DTA, producing a CL-4-specific cytotoxic agent.

4. Discussion

CL-4 is often overexpressed in some malignant tumors, such as breast, prostate, ovarian, pancreatic and gastric cancers [12,14,17]. CL-4 targeting is a promising method for tumor-targeting therapy. In the present study, we prepared a fusion protein of DTA, a protein

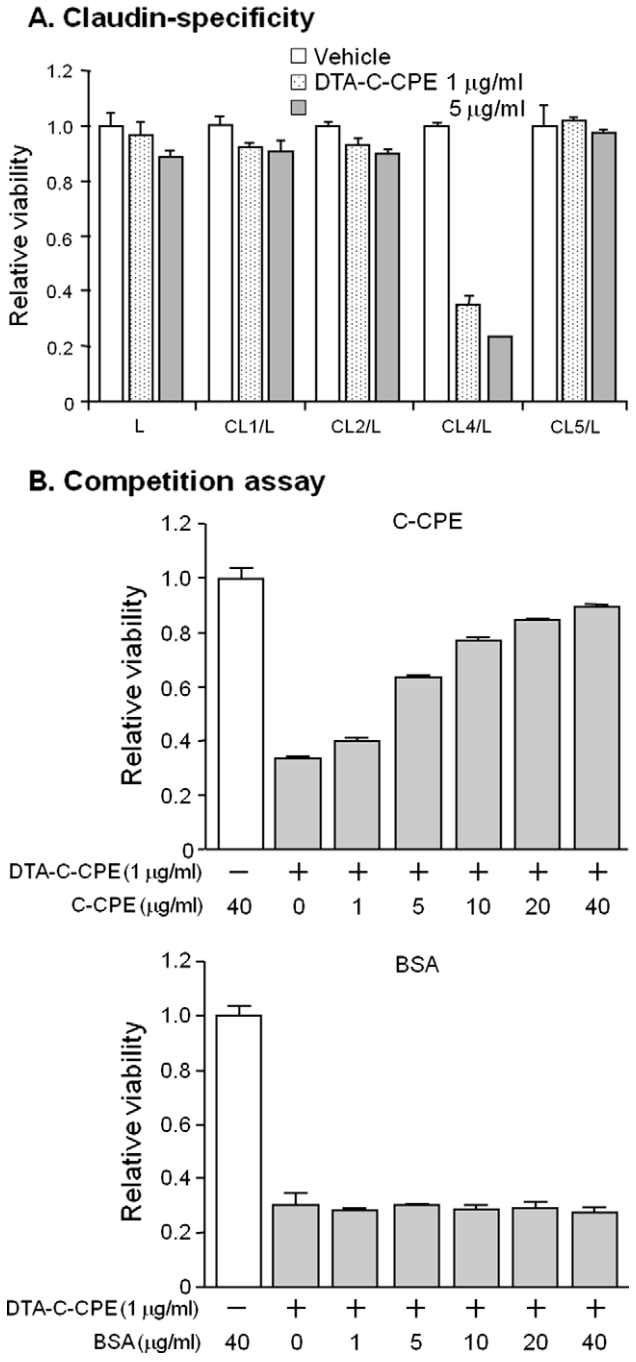

Fig. 3. Cytotoxic properties of DTA–C-CPE. (A) Claudin-specificity. L, CL1/L, CL2/L, CL4/L or CL5/L cells were treated with DTA–C-CPE at the indicated concentration for 48 h. After incubation, the cellular viability was measured by WST-8 assay. Data are the mean ± SD (n = 3). The data are representative of three independent experiments. (B) Competition assay. CL-4/L cells were pretreated with C-CPE (upper panel) or BSA (lower panel) at the indicated concentration for 2 h, and then the cells were treated with DAT-C-CPE (1 μg/ml) for 48 h. The cellular viability was measured by WST-8 assay. Data are the mean ± SD (n = 3). The data are representative of three independent experiments.

synthesis inhibitory factor, and C-CPE, which binds to CL-4, and we found that the fused protein (DTA–C-CPE) is toxic to CL-4-expressing cells.

DTA kills cells by inactivating elongation factor 2 when one molecule of this protein is introduced into the cytosol [24]. DTA permits the successful targeting of cells displaying only a limited number of tumor-specific growth factor receptors or antigens overexpressed on their surface, and immunotoxins containing DTA, ONTAK and DT 388GMCSF are used clinically for cancer-targeted therapy [25–27]. Therefore, we selected DTA as a cytotoxic molecule for the present study.

A CL-4-targeting molecule containing DTA needs to bind to CL-4 and enter the cytosol. C-CPE is the receptor-binding domain of CPE, and the CL-4-binding region is located on the C-terminal of C-CPE [15,23,28]. CL-4 has a sorting signal to clathrin-coated vehicles, and CL-4 is expected to be taken up by clathrin-mediated endocytosis [29–31]. CL-4 bound to DTA–C-CPE may be taken up by the endocytotic pathway, followed by release of DTA from endosomes into the cytosol. Further studies are needed to elucidate the detailed mechanism of DTA–C-CPE-induced cell death.

Reduced side effects and increased anti-tumor effects are pivotal characteristics needed for anti-tumor agents. Targeting cancer cells by using ligands for growth factor receptors or antigens that are overexpressed on the cell membrane is a potent strategy, and the success of the targeted therapy depends on the target molecule selection. The CL family has attractive characteristics for their use as targets in tumor therapy. First, CL has two extracellular loop domains that can be target sites [12]. Second, CLs are overexpressed in nine of 12 cancer types, creating a differential expression profile between tumor cells and normal cells [12,14]. Third, CLs are often exposed on the apical membrane in cancer cells, whereas CLs are located in the intercellular junction between adjacent cells in normal cells [14]. Even if the CL level in tumors is not more than the level in normal tissues, CL may be more accessible in the tumor. Thus, CLs have great promise as targets for tumor therapy. C-CPE is a CL ligand. We prepared C-CPE-PSIF, a lead compound for tumor therapy, by using the CL-4-targeting ligand C-CPE [32]. We already determined the functional domains of C-CPE as a CL-4-targeting molecule, and we are using C-CPE as a prototype to develop a novel CL ligand. This is the first study to produce CL-4-targeted DTA. Future development of the CL-4-targeting immunotoxin using DTA and a CL ligand will provide a novel tumor-targeted therapy.

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