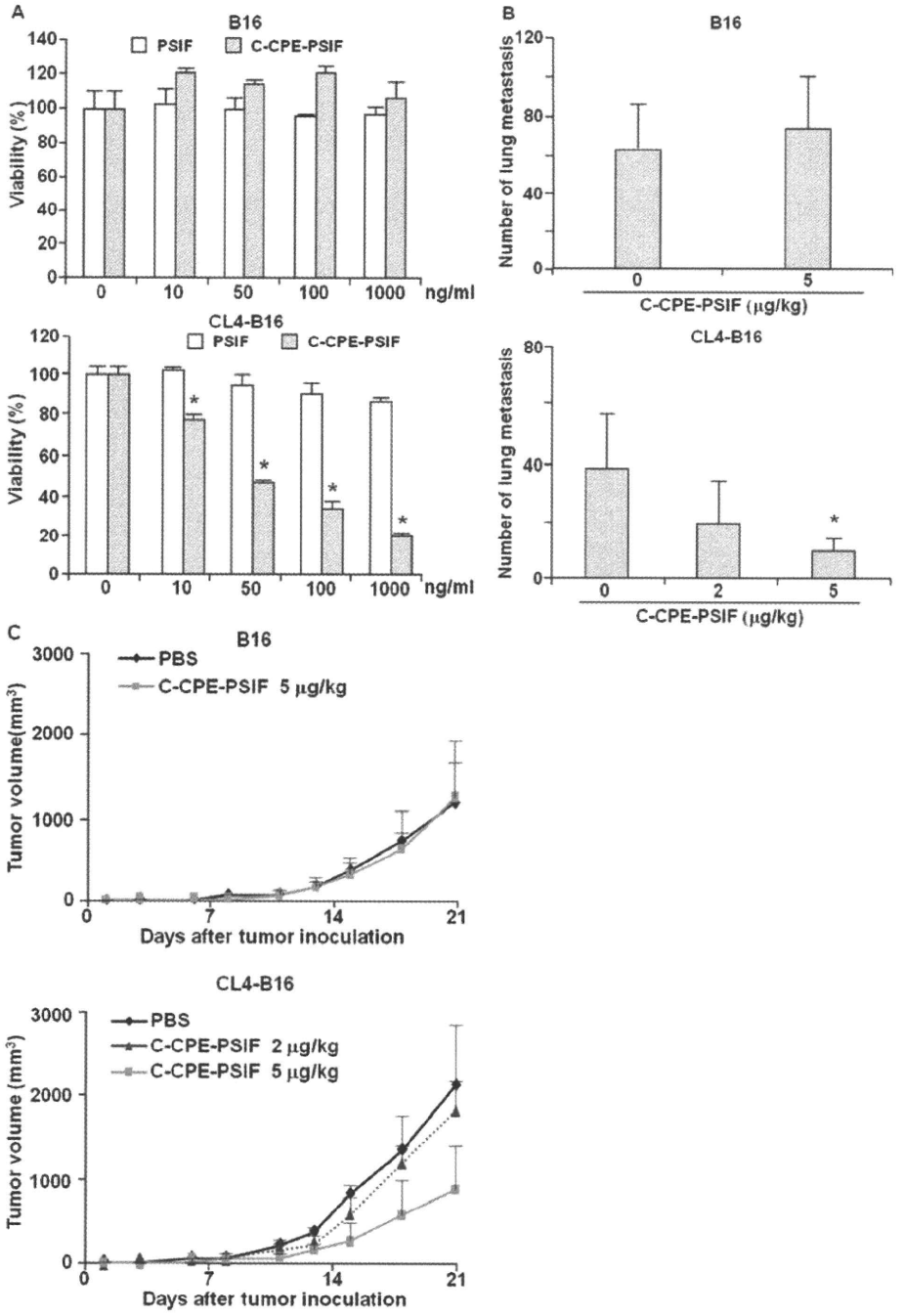

Fig. 2. Antitumor activity of C-CPE-PSIF for CL4-B16 cells. A, cytotoxicity of C-CPE-PSIF in CL4-B16 cells. B16 cells (top) and CL4-B16 cells (bottom) were treated with PSIF or C-CPE-PSIF at the indicated concentrations for 24 h. Cell viability (%) was measured by a WST-8 kit, according to the manufacturer's instructions (Nacalai Tesque). Data represent the mean ± S.D. ($n = 3$). *, significantly different from the vehicle-treated group ($p < 0.05$). B, antimetastatic activity of C-CPE-PSIF on lung metastasis of B16 (top) or CL4-B16 (bottom) cells. B16 or CL4-B16 cells (1×10^6 cells) were injected into the tail veins of mice on day 0, and vehicle or C-CPE-PSIF (2 or 5 μg/kg) was intravenously injected on days 0, 2, 4, 7, 9, 11, and 13. On day 14, the mice were sacrificed, their lungs were fixed, and the colonies on the lung surface were counted. Data are shown as means ± S.D. ($n = 5$). *, significantly different from the vehicle-treated group ($p < 0.05$). C, antitumor activity of C-CPE-PSIF on CL4-B16 subcutaneously inoculated allograft. B16 (top) or CL4-B16 (bottom) cells (1×10^5 cells) were intradermally inoculated into the right flank of mice on day 0, and PBS or C-CPE-PSIF (2 or 5 μg/kg) was intravenously injected three times a week. Tumor volume was monitored. Each point is the mean ± S.D. ($n = 5$). The data are representative of two independent experiments.

but did prevent lung metastasis. The circulating tumor cells might be more sensitive to C-CPE-PSIF than tumor cells in the solid tumor tissue. C-CPE-PSIF treatments did not cause a decrease in body weight (Fig. 3C), and there were no apparent biochemical side effects (Supplemental Fig. 1). ADR, which is frequently used in clinical chemotherapy, suppressed the tumor growth from 970.3 ± 278.4 to 458.6 ± 51.4 mm^3 at 4 mg/kg (Fig. 3D). As shown in Fig. 3E, 4 mg/kg ADR decreased the number of lung metastasis colonies (24 ± 13 colonies in the vehicle-treated group; 6 ± 4 colonies in the ADR-treated group). However, the ADR-treated mice experienced a 26% loss of body weight, which is a sign of side effects (Fig. 3F). Thus, the antitumor activity of C-CPE-PSIF might be more potent than that of ADR. These results indicate that claudin-4-targeting therapy might be a potent strategy for tumor therapy with a low level of side effects and a high level of antitumor activity.

Discussion

Most malignant tumors are derived from the epithelium, and metastasis is the major cause of death from cancers. In the present study, we found that systemic administration of a claudin-targeting molecule suppressed cancer metastasis, indicating that claudin targeting might be an effective therapy against cancer metastasis.

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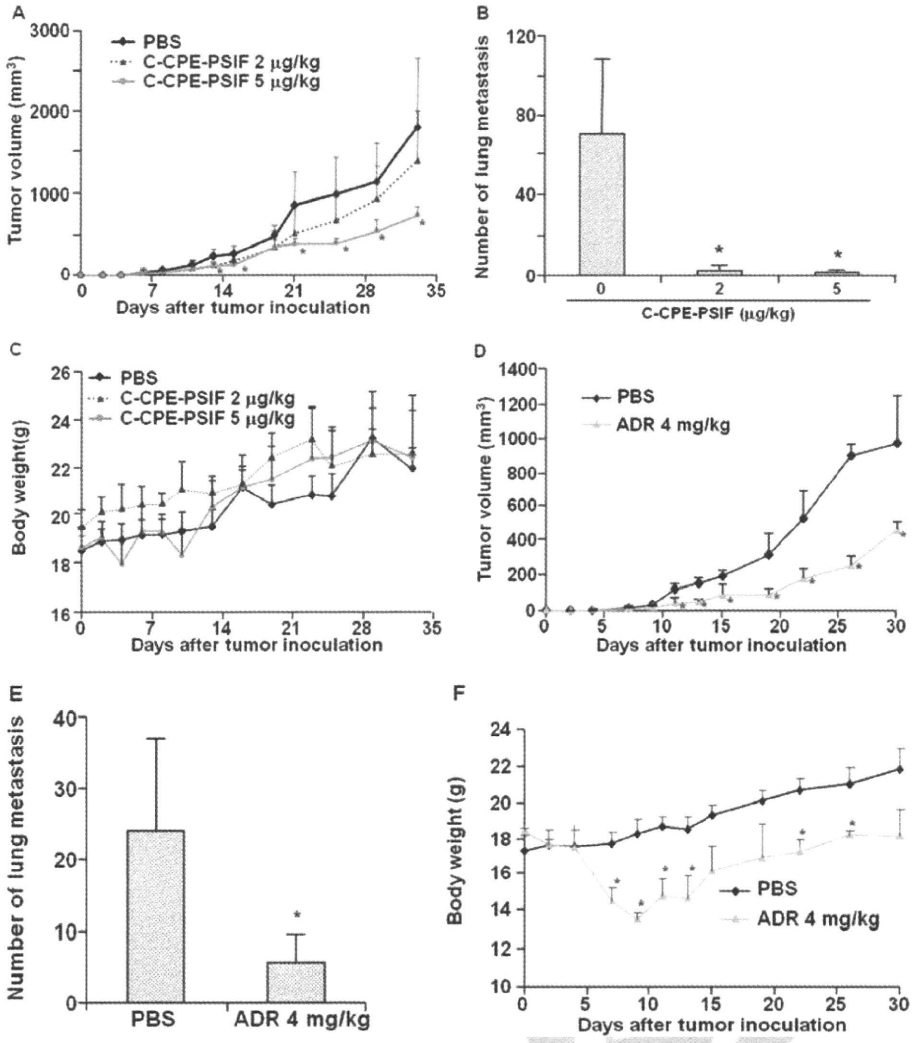

Fig. 3. Antitumor activity of C-CPE-PSIF for murine breast cancer 4T1 cells. 4T1 cells (1×10^5 cells) were intradermally inoculated into the right flanks of mice on day 0, and C-CPE-PSIF (A–C) or ADR (D–F) was intravenously injected three or two times a week at the indicated doses, respectively. Tumor volume (A and D) and body weight (B and E) were monitored. On day 35, the mice were sacrificed, their lungs were stained with India ink, and the number of spontaneous metastases was determined (C and F). Data are shown as means ± S.D. ($n = 5$). *, significantly different from the vehicle-treated group ($p < 0.01$). The data are representative of two independent experiments.

Although the in vitro metastasis activity of CL4-B16 cells was higher than that of parental B16 cells, the in vivo lung metastasis of CL4-B16 cells was lower than that of B16 cells. As shown in Supplemental Fig. 2, claudin-4 increased the invasiveness and migration activity of B16 cells in vitro and decreased lung metastasis in vivo. A possible explanation for this discrepancy might be the experimental model, which evaluates a different stage of metastasis. The migration and invasion activity involved in the early stage of metastasis was investigated in the in vitro analysis, whereas extravasation and colonization to an organ involved in the late stage of metastasis was evaluated by the in vivo experiment. The altered expression of claudin-4 changed the metastasis of 4T1 cells to the heart and liver, suggesting that claudin affects organ-specific metastasis (Erin et al., 2009). Claudin-4 might suppress the lung metastasis of B16 cells.

Claudin is a structural and functional component of TJs (Furuse and Tsukita, 2006). What is the role of claudin in metastasis? Metastasis is composed of three steps: leaving the primary site, entering the blood flow, and invading the distant site. In the first step, the combination of claudin members in the TJ strands may be important. The claudin family contains at least 24 members. Claudin is believed to form homo- and hetero-type claudin strands, and the pattern of the strands differs among tissues and determines the properties of TJ seals (Furuse and Tsukita, 2006). For example, rigid TJ seals were formed when claudin-11 or claudin-15 was exogenously expressed in Madin-Darby canine kidney cells, whereas the expression of claudin-11 or claudin-15 reduced the TJ integrity in LLC-PK1 cells by its dominant negative effects on TJ sealing (Van Itallie et al., 2003). Dominant negative effects of claudin-4 on the TJ barrier might contribute to an acceleration in the detachment of cancer cells from the primary tumor tissue. In the second and third steps of metastasis, cancer cells must move through the extracellular matrix at the primary site and the distant site. Cancer cells must degrade the extracellular matrix by the expression of matrix metalloproteinase and increase their motility. Claudin expression enhanced invasion with increased matrix metalloproteinase activity (Agarwal et al., 2005). There is a relationship between the levels of claudin-1/claudin-4 and the metastasis of human cancers, including hepatic, colonic, ovarian, and gastric cancers (Miwa et al., 2001; Agarwal et al., 2005; Resnick et al., 2005; Halder et al., 2008; Lee et al., 2008). The overexpression of claudin suppressed cancer metastasis in human pancreatic and gastric cancers (Michl et al., 2003; Mima et al., 2005; Ohtani et al., 2009). Claudin-4 suppressed or accelerated in vitro and in

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vivo metastasis of human cancer cells (Agarwal et al., 2005; Ohtani et al., 2009). Cell–cell interaction through TJs regulates cell growth signaling (Matter et al., 2005). Taken together, these findings indicate that claudin family members might control several steps of cancer metastasis. The precise molecular mechanism and role of claudin in cancer metastasis remain to be determined.

Whether a claudin-4-targeting method causes severe side effects is critical for its clinical application in cancer therapy. Claudins play pivotal roles in TJ barrier and fence functions by maintaining cellular polarity in normal epithelium (Furuse and Tsukita, 2006). Claudins are believed to be more accessible in tumors than in normal epithelium. Claudins form TJ seals in lateral membranes between adjacent cells in normal epithelium, whereas claudins are exposed on the cell surface during tumorigenesis (Soler et al., 1999; Kominsky, 2006). Indeed, no local or systemic side effects have been observed after the intratumoral administration of CPE (Kominsky et al., 2007; Santin et al., 2007). Here, we also found that the systemic administration of C-CPE-PSIF causes no significant increase in biochemical markers (aspartate aminotransferase, alanine aminotransferase, and blood urea nitrogen) for toxicity at a therapeutic dose of 5 μg/kg (Supplemental Fig. 2). Thus, a claudin-targeting strategy might have weak side effects.

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It is difficult to prepare recombinant claudin protein because of its hydrophobic property, and claudin has low antigenicity. Until recently, an antibody against the extracellular loop domain of claudin had never been successfully prepared, and C-CPE was the only known claudin binder. Recently, Romani et al. (2009) prepared a single-chain antibody fragment against claudin-3 by using phage display technology. They found that the antibody fragment binds to ovarian and uterine carcinoma cells in vitro. More importantly, a therapeutic monoclonal antibody against claudin-4 was developed. Suzuki et al. (2009) successfully prepared anticlaudin-4 antibody by immunizing claudin-4-expressing tumor cells into a mouse with autoimmune disease. The antibody mediates antibody-dependent cellular cytotoxicity and both in vitro and in vivo antitumor activity. Although the preparation of anticlaudin antibody may lead to a breakthrough in cancer therapy, the immunogenicity associated with immunotoxin clinical therapies is a future problem (Kreitman and Pastan, 2006). The C-terminal 30 amino acids are the minimum functional domain of C-CPE to bind to claudin-4 (Hanna et al., 1991). The C-terminal 30-amino-acid fragment was used to deliver a cytokine to claudin-4-expressing cells by genetic fusion (Yuan et al., 2009). Humanized antibody and the claudin-4-targeting peptide may be useful for cancer therapy in the near future.

In summary, this is the first report to indicate that systemic injection of a claudin-targeting molecule suppresses tumor growth and metastasis. Hematologic cells do not develop TJs; therefore, a claudin-targeting therapy may have no hematologic toxicity. We anticipate that claudin targeting will be a potent strategy for cancer therapy.

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A Novel Screening System for Claudin Binder Using Baculoviral Display

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Abstract

Recent progress in cell biology has provided new insight into the claudin (CL) family of integral membrane proteins, which contains more than 20 members, as a target for pharmaceutical therapy. Few ligands for CL have been identified because it is difficult to prepare CL in an intact form. In the present study, we developed a method to screen for CL binders by using the budded baculovirus (BV) display system. CL4-displaying BV interacted with a CL4 binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), but it did not interact with C-CPE that was mutated in its CL4-binding region. C-CPE did not interact with BV and CL1-displaying BV. We used CL4-displaying BV to select CL4-binding phage in a mixture of a scFv-phage and C-CPE-phage. The percentage of C-CPE-phage in the phage mixture increased from 16.7% before selection to 92% after selection, indicating that CL-displaying BV may be useful for the selection of CL binders. We prepared a C-CPE phage library by mutating the functional amino acids. We screened the library for CL4 binders by affinity to CL4-displaying BV, and we found that the novel CL4 binders modulated the tight-junction barrier. These findings indicate that the CL-displaying BV system may be a promising method to produce a novel CL binder and modulator.

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Introduction

Tight junctions (TJ) are intercellular adhesion complexes in epithelial and endothelial cells; TJs are located in the most apical part of the complexes [1]. TJs have a barrier function and a fence function [2–4]. TJs contribute to epithelial and endothelial barrier functions by restricting the diffusion of solutes through the paracellular pathway. TJs maintain cellular polarity by preventing the free movement of membrane proteins between the apical and basal membranes [5]. Loss of cell-cell adhesion and cellular polarity commonly occurs in the early stages of cancer [6]. Modulation of the TJ barrier function can be a method to enhance drug absorption, and TJ components exposed on the surface of cancer cells can be a target for cancer therapy.

Biochemical analyses of TJs have identified TJ components, such as occludin, claudins (CLs) and junction adhesion molecule [7]. The CL family contains more than 20 integral tetra-transmembrane proteins that play pivotal roles in the TJ barrier and fence functions. CL1-deficient mice lack the epidermal barrier, while CL5-deficient mice lack the blood-brain barrier [8,9], indicating that the regulation of the TJ barrier by modulation of CLs may be a promising method for drug delivery. *Clostridium perfringens* enterotoxin (CPE) causes food poisoning in humans [10]. An interaction between the C-terminal domain of CPE (C-CPE) with CL4 deregulates the TJ barrier [11,12]. We previously found that C-CPE enhances jejunal absorption through its interaction with CL4, indicating that a CL binder is a potent drug-delivery system [13].

The majority of lethal cancers are derived from epithelial tissues [14]. Malignant tumor cells frequently exhibit abnormal TJ function, followed by the deregulation of cellular polarity and intercellular contact, which is commonly observed in both advanced tumors and the early stages of carcinogenesis [6]. Some CLs are overexpressed in various types of cancers. For example, CL3 and CL4 are overexpressed in breast, prostate, ovarian, pancreatic and gastric cancers. CL1, CL7, CL10 and CL16 are overexpressed in colon, gastric, thyroid and ovarian cancers, respectively [15,16]. These findings indicate that the CLs may be a target molecule for cancer therapy. A receptor for CPE is CL4 [11,12]. CPE has anti-tumor activity against human pancreatic and ovarian cancers without side effects [17,18]. The CLs binders will be useful for cancer-targeting therapy.

As above, recent investigations of CLs provide new insight into their use as pharmaceutical agents; for example, a CL binder may be used in drug delivery and anti-tumor therapy. Selection of a CL binder by using a recombinant CL protein is a putative method to

prepare a CL binder. However, CLs are four-transmembrane proteins with high hydrophobicity; there has been little success in the preparation of intact CL protein. Recently, a novel type of protein expression system that uses baculovirus has been developed. Membrane proteins are displayed on the budded baculovirus (BV) in their active form [19–21], indicating that the BV system may be useful for the preparation of a CL binder. In the present study, we investigated whether a CL binder was screened by using a CL-displaying BV.

Results

Preparation of CL4-displaying BV

C-CPE is the only known CL binder and modulator [12,13,22]. C-CPE has affinity to CL4 in a nanomolar range [23]. We chose C-CPE and CL4 as models of the CL binder and CL, respectively. Several reports indicate that membrane proteins expressed on the surface of BV are in an intact form [19–21]. To check the expression of CL4 on the BV, we performed immunoblot analysis of the lysate of CL4-BV against CL4. As shown in Fig. 1A, CL4 was detected in the virus lysates. To determine if the CL4 expressed on the virus has an intact form, we performed enzyme-linked immunosorbent assay (ELISA) with CL4-BV-coated immunoplates. C-CPE binds to the extracellular loop domain of CL4 [23]. After the addition of C-CPE to the CL4-BV-coated plate, the C-CPE bound to the CL4-BV-coated plate was detected by anti-his-tag antibody, followed by incubation with horseradish peroxidase-labeled antibody. C-CPE was dose-dependently bound to CL4-BV, whereas C-CPE did not interact with wild-BV (Fig. 1B). Deletion of the CL4-binding region (C-CPE303) attenuated the interaction of C-CPE with CL4-BV (Fig. 1C). Together, these results indicate that the CL4 displayed on BV may have an intact extracellular loop region.

Selection of C-CPE-phage by using CL4-BV

We next examined the interaction between C-CPE-phage and CL4-BV. As shown in Fig. 2A, C-CPE-phage bound to CL4-BV but not to wild-BV, and a scFv-phage did not bind to CL4-BV. To determine if CL-BV can be used to select CL binders, we prepared a mixture of C-CPE-phage and scFv-phage at a ratio of 2:10 and used CL4-BV to select CL4-binding phage in the mixtures. The amount of C-CPE-phage was increased to 11 of 12 clones in the mixture (Fig. 2B), indicating that CL-BV may be useful in the preparation of CL binders.

We previously found that each substitution of S304, S305, S307, N309, S313 and K318 with alanine increased the binding of C-CPE to CL4 [24]. Here, we prepared a phage library for C-CPE by randomly changing the functional 6 amino acids to any of the 20 amino acids. To confirm the diversity of the library, we checked the sequences of 17 randomly isolated clones. Each of the 17 clones had a different sequence, indicating that the library has a diverse population of C-CPE mutants (Table 1).

Then, we screened the CL4-binding phage by their affinity to CL4-BV. After addition of the C-CPE library to CL4-BV-adsorbed tubes, the CL4-BV-bound phages were recovered (1st screening). We repeated this screening process two more times (2nd screening and 3rd screening). If the number of CL4-bound phage is increased during the screening, the ratio of the incubated phage titers to the recovered phage titers will increase. As shown in Fig. 3A, the ratio was increased during screening from 4.5×10^{-7} to 5.5×10^{-5}, indicating that the screening system for CL4 binders may work. Indeed, the number of monoclonal phage clones with high affinity to CL4-BV was increased after the 3rd screening compared with that after the 2nd screening (Fig. 3B).

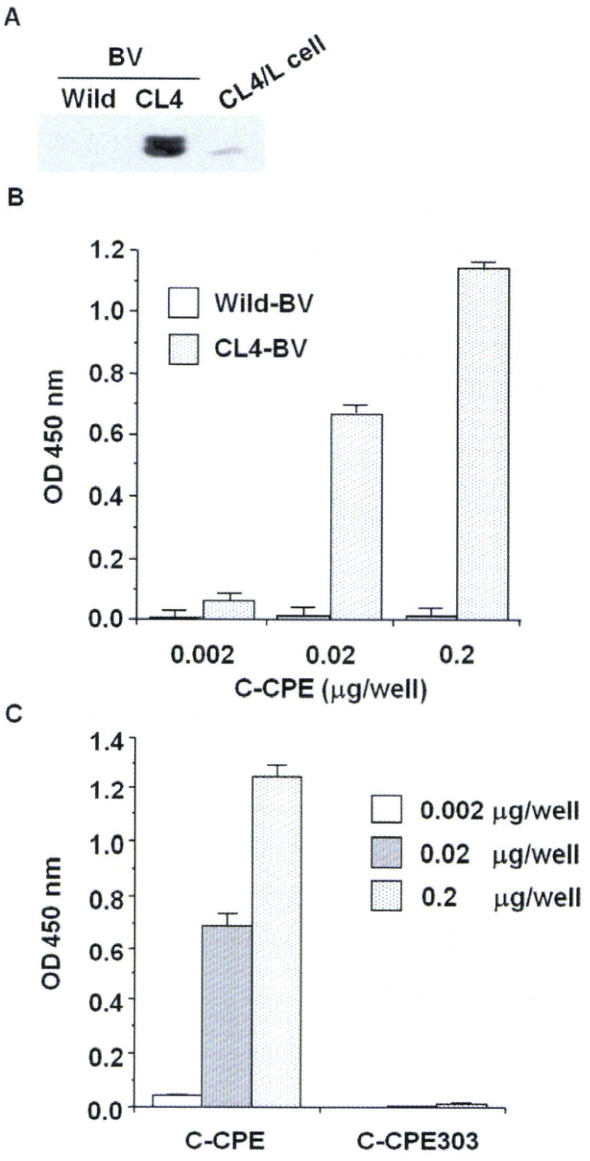

Figure 1. Preparation of CL4-displaying BV. A) Immunoblot analysis. Wild-BV and CL4-BV (0.1 μg/lane) were subjected to SDS-PAGE, followed by immunoblot analysis with anti-CL4 antibody. The lysate of CL4-expressing L (CL4/L) cells was used as a positive control. B, C) Interaction of a CL4 binder with CL4-BV. Immunotubes were coated with the wild-BV or CL4-BV, and C-CPE (B) or mutated C-CPE (C) was added to the BV-coated immunotubes at the indicated concentration. C-CPE bound to the BV-coated tubes was detected by ELISA with an anti-his-tag antibody.
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We analyzed the sequences of the CL4-BV-bound phages and got novel CL4-binder candidates with amino acid sequences that differed from the wild-type sequence (Table 2). To investigate their CL4-binding, we prepared the recombinant proteins of the binders and investigated their interaction with CL4 by ELISA with CL-BVs. As shown in Fig. 4A, the novel C-CPE derivatives had affinity to CL4 but not CL1. Next, we investigated whether the novel CL4 binders modulate TJ barrier in Caco-2 monolayer cell sheets, a popular model for the evaluation of TJ barriers [25].

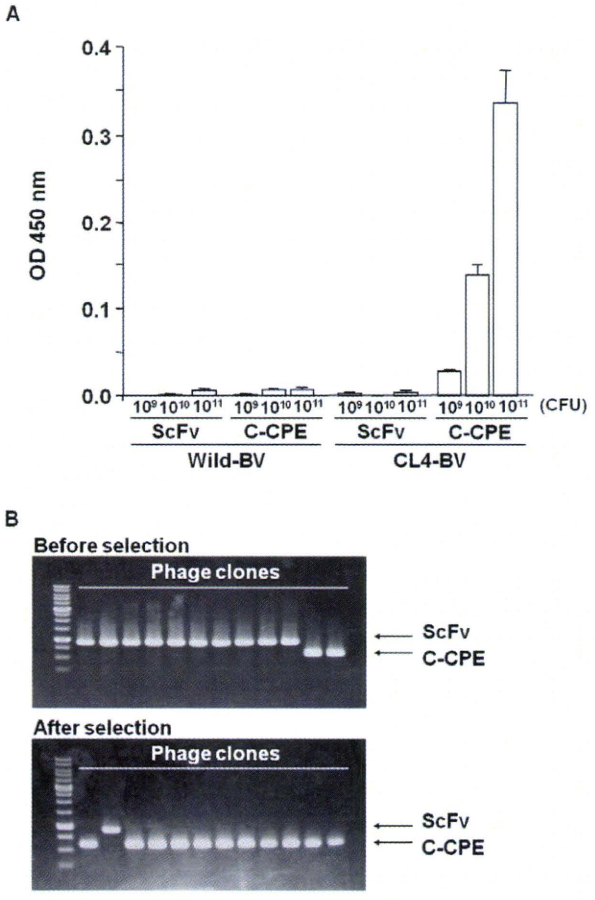

Figure 2. Selection of C-CPE-displaying phage by using the CL4-BV system. A) Interaction of C-CPE-displaying phage with CL4-BV. Wild-BV or CL4-BV was coated on an immunoplate, and then scFv-displaying phage or C-CPE-displaying phage was added to the BV-coated immunoplate at the indicated concentrations. The BV-bound phages were detected by ELISA with anti-M13 antibody as described in Materials and methods. Data are representative of two independent experiments. Data are means ± SD (n = 3). B) Enrichment of C-CPE-displaying phage by the BV system. A mixture of scFv-phage and C-CPE-phage (mixing ratio of scFv-phage to C-CPE-phage = 2:10) was incubated with a CL4-BV-coated immunotube, and the bound phages were recovered. Each phage clone was identified by PCR amplification, followed by agarose gel electrophoresis. Upper and lower pictures are before and after the selection, respectively. The putative sizes of the PCR products are 856 and 523 bp in scFv and C-CPE, respectively. The data are representative of two independent experiments.
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Treatment of the cells with C-CPE resulted in decreased transepithelial electrical resistance (TEER) values, a marker of TJ integrity, and the TEER values increased after removal of C-CPE. The C-CPE derivatives (clones 1–5) had TJ-modulating activity similar to that of C-CPE (Fig. 4B).

Discussion

CL is a promising target for pharmaceutical therapy. However, CL has low antigenicity, and there has been little success in the preparation of monoclonal antibody against the extracellular loop region of CL. The three-dimensional structure of CL has never been determined, so it is impossible to perform a theoretical design of a CL binder based on the structural information. In the present study, we developed a novel screening system for CL binders by using a BV system and a C-CPE phage display library, and we used this system to identify novel CL4 binders.

Table 1. C-CPE phage library.

	304	305	307	309	313	318
C-CPE	**S**	**S**	**S**	**N**	**S**	**K**
Clone 1	V	T	C	V	N	K
2	C	P	A	H	L	T
3	A	G	G	V	P	P
4	R	G	H	L	E	H
5	A	A	P	S	R	Q
6	P	A	P	D	P	A
7	C	T	T	T	N	K
8	H	P	S	P	G	H
9	R	G	G	R	N	R
10	A	P	S	T	Q	P
11	V	L	G	N	M	R
12	P	P	A	T	F	R
13	G	D	C	S	N	L
14	F	R	V	F	R	N
15	S	Q	Q	W	T	T
16	S	R	L	E	W	Q
17	K	R	E	R	Q	S

Phage clones were randomly picked up from the C-CPE phage library, and the amino acids sequences of C-CPE mutant were analyzed.
doi:10.1371/journal.pone.0016611.t001

In ligand screening, the preparation of a receptor for the ligand is very critical. Membrane proteins are especially difficult to prepare as recombinant protein with an intact structure. Functional membrane proteins such as cell-surface proteins are heterologously expressed on BV in their native forms [19–21]. Interactions between membrane proteins can be detected by using receptor-displaying and ligand-displaying BV [21]. In the present report, we found that CL4-BV interacts with a CL4 binder, C-CPE, but it does not interact with C-CPE303 that lacks the CL4-binding residues of C-CPE. The CL4-binding site of C-CPE corresponds to that of CPE; so, the second extracellular loop of CL appears to be the C-CPE-binding site [23,26]. These findings indicate that CL4 displayed on BV may have native form. We anticipate that CL-BV will be useful for the preparation of CL binders, such as peptides and antibodies.

To the best of our knowledge, the preparation of CL binder has been performed by only four groups. Offner et al. prepared polyclonal antibodies against extracellular domains of CL3 and CL4 [27], Ling et al. screened peptide types of CL4 binder by using a 12-mer peptide phage display library and CL4-expressing cells [28], Suzuki et al. generated a monoclonal antibody against the second extracellular loop of CL4 from mice immunized with a human pancreatic cancer cell line [29] and Romani et al. screened scFv against CL3 by using a human antibody phage display library [30]. However, the CL modulators have never been developed; thus, C-CPE is the only known CL4 modulator [12]. In the present study, we prepared a C-CPE phage library containing C-CPE mutants in which each of the 6 functional amino acids was randomly replaced with an amino acid, and we isolated CL4 binders by using CL4-BV as a screening ligand. Interestingly, all of

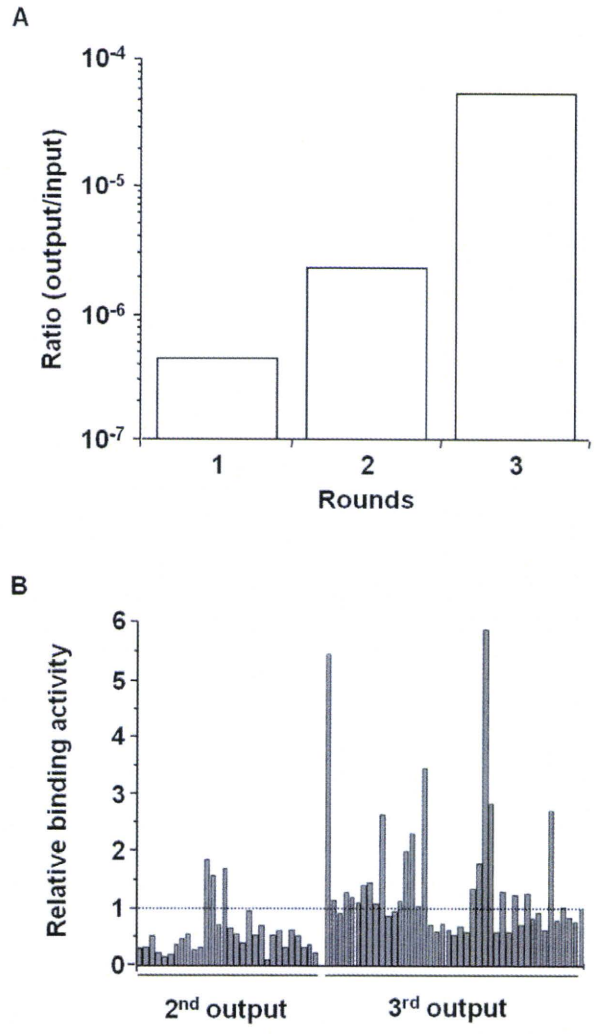

Figure 3. Screening of a novel CL4 binder. A) Enrichment of phages with affinity to CL4-BV. CL4-BVs coated on immunotubes were incubated with the C-CPE-derivative phage library at 1.6×10^{12} CFU titer (1^{st} input phage). The phages bound to CL4-BV were recovered (1^{st} output phage). The CL4-BV-binding phages were subjected to two additional cycles of the incubation and wash step, resulting in 2^{nd}, 3^{rd} output phage. The ratio of output phage to input phage titers was calculated. B) Monoclonal analysis of C-CPE-derivative phage. CL4-BV-bound phage clones were isolated from the 2^{nd} and 3^{rd} output phages, and the interaction of the monoclonal phage with CL4-BV was examined by ELISA with anti-M13 antibody as described in Materials and methods. Data are expressed as relative binding to that of C-CPE-phage indicated by the most right column.
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the CL4 binders modulated TJ barriers. We are investigating why the substitution of the amino acids with the other amino acids modulated CL4. These findings indicate that a BV screening system with a C-CPE library may be a powerful method to develop CL modulators.

The CL family forms various types of TJ barriers through combinations of its more than 20 members in homophilic/heterophilic CL strands [31,32]. Intercellular proteins ZO-1 and ZO-2 determine the localization of CL strands [33]. If a screening system to reconstitute heterogeneous CL strands with ZO-1 and/or ZO-2 is developed, then useful and effective CL modulators can be identified. In this point, the BV system has extremely superior features. G protein and G protein-coupled receptors have been

Table 2. CL4-binding phages.

	304	305	307	309	313	318
C-CPE	**S**	**S**	**S**	**N**	**S**	**K**
Clone 1	R	V	S	A	R	R
2	R	S	V	A	R	K
3	G	D	G	R	T	R
4	S	A	P	R	S	A
5	R	S	L	K	S	K

The sequences of C-CPE mutant in the CL4-binding phages were analyzed.
doi:10.1371/journal.pone.0016611.t002

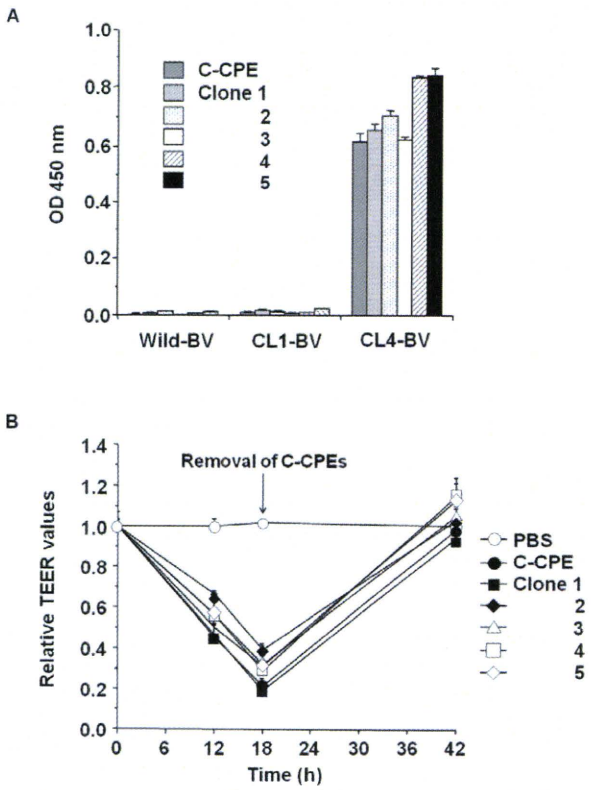

Figure 4. Isolation of a novel CL4 modulator. A) Interaction of the C-CPE derivatives with CL4. C-CPE derivatives were prepared as his-tagged recombinant proteins. The C-CPE derivatives (0.02 μg) were added to CL-BV-coated immunoplates, followed by detection of the C-CPE derivatives bound to CL-BV. Data are means ± SD (n = 4). B) Modulation of tight junction-barriers. Caco-2 cells were cultured on Transwell™ chambers. When TEER values reach a plateau, the cells were treated with C-CPE or C-CPE derivatives at the indicated concentrations. After 18 h of exposure to the C-CPEs, the cells were washed with medium to remove C-CPEs, and then the cells were cultured for an additional 24 h. Changes in TEER values were monitored during the C-CPEs treatment. Relative TEER values were calculated as the ratio of TEER values at 0 h. Data are representative of two independent experiments. The data are means ± SD (n = 4).
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functionally reconstituted in BV [20,34], and functional γ-secretase complexes have also been reconstituted on BV [35]. In the near future, the reconstituted CL system on BV will be developed and used for the screening of CL binders and modulators, hopefully leading to breakthroughs in pharmaceutical therapies that target CLs.

Materials and Methods

Recombinant BV construction and Sf9 cell culture

Recombinant BV was prepared by using the Bac-to-Bac expression system, according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD). Briefly, mouse CL1 and CL4 cDNA (kind gifts from Dr. M Furuse, Kobe University, Japan) were inserted into pFastBac1, and the resulting plasmids were transduced into DH10Bac *E. Coli* cells. Recombinant bacmid DNA was extracted from the cells. Sf9 cells were transduced with the bacmid coding CL, and the recombinant BV was recovered by centrifugation of the conditioned medium [36].

Preparation of the BV fractions

Sf9 cells (2×10^6 cells) were infected with recombinant BV at a multiplicity of infection of 5. Seventy-two hours after infection, the BV fraction was recovered from the culture supernatant of infected Sf9 cells by centrifugation. The pellets of the BV fraction were resuspended in Tris-buffered saline (TBS) containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and then stored at 4°C until use. The expression of CL1 and CL4 in the BV was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with anti-CL antibodies (Zymed Laboratory, South San Francisco, CA).

Preparation of mutant C-CPE library

C-CPE fragments in which the functional amino acids (S304, S305, S307, N309, S313 and K318) [24] were randomly mutated were prepared by polymerase chain reaction (PCR) with pET-H_{10}PER as a template, a forward primer (5′-catgccatggccgatatagaaaaagaaatccttgatt-tagctgctg-3′, Nco I site is underlined) and a reverse primer (5′-ttttccttttgcggccgcaaa*snn*ttgaaataatat*snn*ataagggta*snn*tcc*snn*ata*snn*snnat-tagcttt-3′, Not I site is underlined, and the randomly mutated amino acids are in italics). The PCR fragments were inserted into a pY03 phagemid at the NcoI/NotI sites [22]. The resultant phagemid containing the C-CPE mutant library was transduced into *E. coli* TG1 cells, and then the cells were stored at −80°C.

Preparation of phage

TG1 cells containing phagemid coding a scFv, C-CPE, C-CPE mutant or C-CPE mutant library were culture in 2YT medium containing 2% glucose and ampicillin. When the cells grew to a growing phage, M13K07 helper phages (Invitrogen) were added, and the medium was changed into 2YT medium containing ampicillin and kanamycin. After an additional 6 h of culture, the phages in the conditioned medium were precipitated with polyethylene glycol. The phages were suspended in phosphate-buffered saline (PBS) and stored at 4°C until use.

ELISA

Wild-BVs or CL-BVs (0.5 μg/well) were adsorbed onto an immunoplate (Greiner Bio-One, Frickenhausen, Germany). The wells were washed with PBS and blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). C-CPEs or phages were incubated in the immunoplate, and the BV-bound C-CPEs or phages were detected by using anti-his-tag antibody (Novagen, Darmstadt, Germany) or anti-M13 antibody (Amersham-Pharmacia Biotech, Uppsala, Sweden), respectively, horseradish peroxidase-labelled secondary antibody and TMB peroxidase substrate (Nacalai Tesque, Kyoto, Japan). The immunoreactive C-CPEs or phages were quantified by the measurement of absorbance at 450 nm. In the screening of phages, the data were normalized by the amounts of phages, which were quantified by ELISA for the FLAG-tag contained in the coat protein.

Selection of phage by using BV

A total of 0.5 μg of BV was adsorbed onto an immunotube (Nunc, Roskilde, Denmark). The tube was washed with PBS and blocked with TBS containing 4.0% BlockAce. The BV-coated tubes were incubated with mixture of phages, and then the tubes were washed 15 times with PBS and 15 times with PBS containing 0.05% Tween 20. The phages bound to the tube were eluted with 100 mM HCl. TG1 cells were infected with the eluted phages, and phages were prepared as described above. The resulting phages were subjected to repeated selection by using the BV-coated immunotubes.

Identification of a phage clone

To identify an isolated phage clone, we performed PCR or sequencing analysis. We amplified the inserted fragment into the phagemid by PCR using forward primer 5′-caggaaacagctatgac-3′ and reverse primer 5′-gtaaatgaattttctgtatgagg-3′. The resultant PCR products were subjected to agarose gel electrophoresis followed by staining with ethidium bromide. We performed a sequence analysis with primer 5′-gtaaatgaattttctgtatgagg-3′.

Measurement of phage titer

To quantify the concentration of phages, we measured the titer (colony formation unit (CFU)/ml) of the phage solution. Briefly, the phage solution was diluted to 10^{-5}–10^{-10} with PBS. The diluted solution was seeded onto Petrifilm™ (Tech-Jam, Osaka, Japan). After 24 h of incubation, the colonies were counted, and the titer was calculated.

Purification of C-CPE mutants

C-CPE and C-CPE303, in which the CL-4 binding region of C-CPE was deleted, were prepared as described previously [13]. To prepare plasmid containing C-CPE mutants, the C-CPE mutant fragment was PCR-amplified by using phagemids coding C-CPE mutants as a template. The resulting PCR fragment was inserted into pET16b, and the sequence was confirmed. The plasmids were transduced into *E. coli* strain BL21 (DE3), and production of mutant C-CPEs was induced by the addition of isopropyl-D-thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol) that was supplemented with 8 M urea when necessary. The lysates were applied to HiTrap™ Chelating HP (GE Healthcare, Buckinghamshire, UK), and mutant C-CPEs were eluted with buffer A containing 100–400 mM imidazole. The buffer was exchanged with PBS by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80°C until use. Purification of the mutant C-CPEs was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-his-tag antibody (Novagen). Protein was quantified by using a BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, Rockford, IL).

TEER assay

Caco-2 cells were seeded in Transwell™ chambers (Corning, NY) at a subconfluent density. The TEER of the Caco-2 monolayer cell sheets on the chamber was monitored by using a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). When TEER values reached a plateau, indicating that TJs were well-developed in the cell sheets, the Caco-2 monolayers were treated with C-CPE or C-CPE mutants on the basal side of the chamber. Changes in TEER values were monitored. The TEER values were normalized by the area of the Caco-2 monolayer, and the TEER value of a blank Transwell™ chamber (background) was subtracted.

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Author Contributions

Conceived and designed the experiments: MK TS TH KY. Performed the experiments: HK AT MK YS TY TS. Analyzed the data: HK AT MK KY. Contributed reagents/materials/analysis tools: HK AK TS TH. Wrote the manuscript: HK MK TY.

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Claudin as a Target for Drug Development

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Abstract: Tight junctions (TJs) play pivotal roles in the fence and barrier functions of epithelial and endothelial cell sheets. Since the 1980s, the modulation of the TJ barrier has been utilized as a method for drug absorption. Over the last decade, the structural and functional biochemical components of TJs, such as occludin and claudin, have been determined, providing new insights into TJ-based pharmaceutical therapy. For example, the modulation of the claudin barrier enhances the jejunal absorption of drugs, and claudin expression is deregulated in cancer cells. Claudin is a co-receptor for the hepatitis C virus. Moreover, claudin is modulated during inflammatory conditions. These findings indicate that claudins are promising drug targets. In this review, we discuss the seeds of claudin-based drug development, which may provide potential pharmaceutical breakthroughs in the future.

Keywords: Tight junction, claudin, cancer, inflammation, infection.

INTRODUCTION

Tight junctions (TJs) limit the movement of molecules through the intercellular space in epithelial and endothelial sheets, and they are located on the most apical part of cells [1, 2]. Electron microscopy has revealed that TJs appear as a series of continuous, anastomotic and intramembranous particle strands. Tsukita's group performed a series of biochemical analyses that clearly showed that the tetra-transmembrane proteins occludin and claudin are components of the TJ [3-5]. The claudin family contains more than 20 members. Interestingly, the expression profiles and the TJ-barrier function of the claudin family members are tissue-specific. For example, claudin-1 is involved in the epidermal barrier, and claudin-5 is involved in the blood-brain barrier [2, 6, 7]. It appears that claudin forms heteromeric and/or homomeric strands in TJs and that the combination and mixing ratios of different claudins determines the tissue-specific barrier properties of TJs [5, 8]. Epithelial cell sheets have bicellular TJs between adjacent cells and tricellular TJs at which three adjacent cells join together. Occludin and claudins are components of bicellular TJs. The occludin-related protein tricellulin has been recently identified to be a component of tricellular TJs [9]. Tricellulin is ubiquitously expressed in epithelial junctions of tissues and organs throughout the body. Down regulation of tricelluin mRNA by RNA interference resulted in disruption of epithelial barrier in an epithelial cell line [9]. However, human tricellulin mutations had no effect on epidermal, respiratory, renal or intestinal barrier [10]. Whether tricellulin can be a target for drug development is unclear.

Functions of TJs are classified as fence- and barrier- functions. Modulation of the TJ barrier has been a popular strategy used to promote drug absorption since the 1980s (See reviews [11, 12]). Sodium caprate is clinically used as an absorption enhancer of drug. Disturbance of either the TJ-fence function or the TJ-barrier function causes human diseases. Disturbance of the TJ-fence function followed by a loss of cellular polarity often occurs in tumorigenesis (See reviews [13-16]). TJs regulate the paracellular passage of ions, molecules, pathogens and inflammatory cells in epithelial and endothelial cell sheets [17-19]. The TJ-barrier becomes deregulated in various human diseases, including infections, inflammation and hereditary diseases (See reviews [20, 21]). Based on these findings, novel therapeutic strategies for TJ-related diseases have been proposed. In the present review, we discuss the seeds of claudin-based pharmaceutical therapies for human diseases relevant to TJs.

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CANCER AND CLAUDIN

Malignant tumors are a major cause of death. Approximately 7.6 million people worldwide died from cancer in 2007, and 90% of tumors are derived from epithelial tissue [22]. Normal epithelial tissues develop cellular polarity, whereas the epithelial polarity is often deregulated during tumorigenesis [23]. TJs are localized between adjacent epithelial cells and separate the apical and basolateral membrane domains, which vary in protein and lipid content, resulting in the maintenance of the cell polarity. Claudins are deregulated in various cancers [13-16]. Claudin may regulate cancer metastasis by modulating activation of matrix metalloproteinases [11]. In this section, we discuss recent breakthroughs in claudin-targeted cancer therapy.

Claudin as a Diagnostic Marker

Claudin proteins are frequently overexpressed in ovarian cancers. In ovarian cancer cells with a high level of claudin-4, the critical claudin-4 promoter region exhibits a low level of DNA methylation and a high level of histone H3 acetylation [24]. Claudin-4 was detected in the 32 of 63 plasma samples of patients with ovarian cancers. Among 50 patients without ovarian cancer, only one had claudin-4–positive plasma. Thus, claudin-4 has a high specificity for the detection of ovarian cancers *via* a blood test, indicating that claudin-4 may be a diagnostic marker for ovarian cancer [25]. Because of the high specificity of claudin expression patterns in cancers, claudin might be a novel non-invasive diagnostic marker for cancer therapy.

Anti-Claudin Antibody

One of the most popular strategies for claudin-targeted cancer therapy is the preparation of antibody against the extracellular region of claudin. However, attempts to prepare anti-claudin antibodies have had little success because claudin has low antigenicity and is highly conserved in various species. A strain of autoimmune mice, BXSB, was immunized with a human pancreatic cancer cell line, resulting in the successful preparation of anti-claudin-4 monoclonal antibody that recognizes the extracellular region of claudin-4 [26, 27]. Moreover, the antibody mediated antibody-dependent cell cytotoxicity (ADCC) and *in vivo* anti-tumor activity. ScFv against the extracellular region of claudin-3 was isolated by using the ETH-2 Gold phage display library, which is a synthetic human recombinant antibody library that contains $>10^9$ possible antibody combinations in an scFv format [28, 29]. Immunization with DNA encoding the first extracellular loop of claudin-18 made success on preparation of anti-claudin-18 monoclonal antibody [30]. These successes in the preparation of anti-claudin antibody are likely to lead to a breakthrough in the development of claudin-targeted cancer therapy.

Clostridium Perfringens Enterotoxin

Another approach to targeting claudin in cancer therapy is the use of *Clostridium perfringens* enterotoxin (CPE) . CPE is a single-chain polypeptide of 35 kDa that causes food poisoning in humans. The functional domains of CPE consist of the N-terminal cytotoxic region and the C-terminal receptor-binding region [31]. Claudin-3 and -4 serve as the receptors for CPE. CPE binds to the second extracellular loop of claudin-3 and -4 [32] (Fig. **1**). We previously prepared a claudin-targeting molecule (C-CPE-PSIF) by fusion of the C-terminal fragment of CPE (C-CPE) with the protein synthesis inhibitory factor (PSIF) derived from *Pseudomonas aeruginosa* exotoxin. C-CPE-PSIF, but not PSIF, is cytotoxic to claudin-4 expressing cells. TJ-undeveloped cells are more sensitive to C-CPE-PSIF than TJ-developed cells. Polarized epithelial cells are sensitive to the basolaterally applied C-CPE-PSIF, but they are less sensitive to the apically applied C-CPE-PSIF. A claudin-targeting molecule may recognize the cellular polarity. Intratumoral injection of C-CPE-PSIF reduced tumor growth. These findings indicate that C-CPE may be a novel molecule for drug delivery and cancer therapy [33]. The receptor-binding region of C-CPE fused to TNF was cytotoxic in human ovarian cancer cells [34]. Thus, CPE fragments might be a tool for claudin-targeting therapy. Treatment of mice with claudin-3 siRNA suppressed ovarian tumor growth and metastasis [35]. Claudin gene silencing with siRNA is also potent anti-tumor agents.

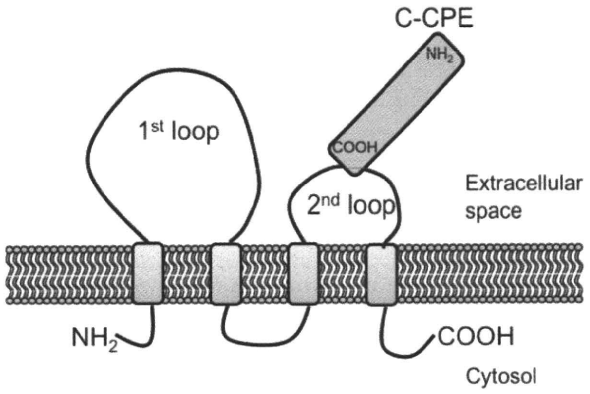

Fig. (1). Schematic illustration of interaction of C-CPE and claudin. Claudin is a tetra-transmembrane protein. C-CPE interacted with the 2nd loop region of claudin *via* its C-terminal domain [32, 76].

INFECTION AND CLAUDINS

Twenty million people die from infectious diseases each year. Most pathogens enter the body through nasal, pulmonary, intestinal and genital mucosa, and the mucosal epithelial cell sheets play a pivotal role as the first line of defense against the pathogens. Invading pathogens are distributed throughout the organ *via* endothelial cells of the blood vessels. TJs seal intercellular spaces between adjacent cells, preventing entry of the pathogens into the body and into the organ across the paracellular spaces. Disruption of mucosal TJ seals allows pathogens to enter into the body and the organ. In this section, we review the recent findings on the relationship between infections and claudins.

West Nile Virus (WNV) and Claudin

WNV, a neurotropic flavivirus, is a human pathogen that targets neurons and causes potentially lethal encephalitis in 1% to 2% of WNV-infected febrile patients [36]. No therapeutic agents or vaccines have been approved for use against WNV infection. Langerhans cells in the skin become infected with WNV by the bite of a carrier mosquito. WNV replicates in the regional tissues and lymph nodes, which results in the dissemination of the virus into the bloodstream. The following second replication proceeds at several sites in the host, including epithelial cells in the skin, kidney, intestine and testis, and then WNV may ultimately invade the brain [37]. The infection of the nervous system is characteristic of the most severe cases of WNV disease, and it often results in death or long-term neurologic sequelae [38]. Understanding the mechanism of the second infection and the viral entry into the brain is critical for the development of therapies against WNV. In WNV-infected epithelial cells, claudin-1, -2, -3 and -4 are degraded, followed by a disruption in the TJ barrier without cell death. The capsid of the WNV was responsible for the modulation of the TJ barrier [39]. These findings suggest that an inducer of claudin may be a promising candidate for pharmaceutical agents to inhibit the dissemination of WNV. Whether or not the WNV modulates the blood-brain barrier *via* the modulation of claudin-5 is an unsettled question.

Human Immunodeficiency Virus (HIV) and Claudin

HIV encephalitis (HIVE), including behavioral, motor, and cognitive impairments, is a common condition in the late stage of HIV-associated dementia [40]. Invasion of HIV into the brain and the transmigration of HIV-infected lymphocytes into the brain are the major causes of HIVE [41]. The blood-brain barrier (BBB), which is responsible for the regulation of solutes and cells between the peripheral circulation and the central nervous system, is comprised of the brain microvascular endothelial cells. Adjacent brain microvascular endothelial cells are connected by TJs that limit paracellular flux and restrict permeability [42]. The BBB frequently breaks down in patients with HIVE [41]. Claudin-5 plays a pivotal role in the BBB [7]. Treatment of human brain microvascular endothelial cells with HIV Gp120 envelope glycoprotein decreased the claudin-5 levels, followed by a disruption of the TJ barrier [43]. Claudin-5 levels were lower in brain microvessels from HIV patients with HIVE compared with brain microvessels from HIV patients without HIVE [44]. The deregulation of the claudin-5 barrier by HIV may be responsible for the breakdown of the BBB in HIV patients. Cannabinoids, the active ingredients in marijuana, reduce pain and improve the quality of life in HIV patients [45]. HIV activates signal transducers and activators of transcription-1 (STAT-1) [46]. Cannabinoids and an inhibitor of STAT-1 prevented the down-regulation of claudin-5 in the HIV Gp120- and HIV-treated human brain microvascular endothelial cells, respectively [43, 44]. These findings indicate that an inducer of claudin-5 may be a pharmaceutical agent for HIVE.

Hepatitis C Virus (HCV) and Claudin

Approximately 170 million people worldwide are infected with HCV. More than 80% of acute infections become persistent, resulting in liver fibrosis, cirrhosis, and hepatocellular carcinoma [47]. HCV infects human hepatocytes but not murine hepatocytes, and the detailed mechanism responsible for this difference has remained obscure. There is no pharmaceutical agent that prevents HCV infection. HCV attaches to tetraspanin CD81 and scavenger receptor class B type I (SR-BI) on host cells through its envelop glycoprotein [48, 49]. However, when CD81 and SR-BI were expressed in non-primate cell lines, the cells were still resistant to HCV entry [50, 51]. Recent studies to identify the additional factors that are needed to render non-human cells susceptible to HCV entry revealed that claudin-1 and occludin are co-receptors for HCV entry [51, 52]. HCV envelop proteins interact with the first extracellular loop region of claudin-1 and the second extracellular loop region of occludin [51, 52]. Binders to CD81, SR-BI, claudin-1 or/and occludin are expected to inhibit HCV entry. The HCV genome is frequently mutated; thus, pharmaceutical agents that recognize host molecules, such as the receptors, may be promising candidates for the prevention of HCV infection.

INFLAMMATORY BOWEL DISEASE (IBD) AND CLAUDIN

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is characterized by an activated mucosal immune system that leads to impaired epithelial barrier function and tissue destruction with relapsing diarrhea [53, 54]. Ulcerative colitis is characterized by chronic inflammation and ulcers in the colon, while Crohn's disease causes ulcers and swelling of the mucosa on all areas of the digestive tract from the mouth to the anus. A common feature of IBD is enhanced permeability of the intestinal epithelium and disruption of the epithelial barrier. In this section, we summarize the recent findings on the relationship between IBD and claudins.

Changes of Claudins in IBD

The epithelial barrier function is impaired in ulcerative colitis, and ulcerative colitis is associated with decreased numbers of TJ strands in the epithelial barrier [55]. Biochemical analysis of TJ components in rectal biopsy specimens from patients with active ulcerative colitis revealed that the protein and mRNA levels of claudin-4 and -7 were decreased, whereas the protein and mRNA levels of claudin-2 were increased, as compared with control patients [56]. Overexpression of claudin-2 led to a decrease in the TJ barrier in an epithelial cell line, whereas claudin-4 or -7 transfection elevated the epithelial barrier function [57, 58]. Thus, the down-regulation of claudin-4/7 and the up-regulation of claudin-2 can lead to altered TJ structure, resulting in impaired epithelial function in active ulcerative colitis. However, claudin-deficient mice or claudin-overexpressing mice did not reproduce the pathology of IBD. Whether change in claudins is cause of IBD or result from IBD remains to be proved.

Although the precise etiology of IBD remains unknown, it is well accepted that IBD results from a deregulated mucosal immune response to environmental factors in genetically susceptible hosts. In IBD patients, the primary defect may be due to an abnormal intestinal epithelial barrier function [59]. The SAMP1/YitFc (SAMP) mouse strain is a spontaneous model of IBD that closely resembles Crohn's disease due to its histological features and localization to the terminal ileum [60]. The deregulated epithelial barrier function in SAMP mice is accompanied by an increase in claudin-2 and a decrease in occludin [61, 62].

FoxO4 is a member of the forkhead box transcription factor O (FoxO) subfamily, which has unique cell type–specific functions that regulate target genes and are involved in the regulation of immune responses [63, 64]. FoxO4-null mice were more susceptible to trinitrobenzene sulfonic acid–induced colitis [65]. FoxO4 deficiency increased the intestinal epithelial permeability and down-regulated the TJ proteins ZO-1 and claudin-1. Immunohistochemical analysis revealed that epithelial expression of FoxO4 was significantly down-regulated in patients with active ulcerative colitis as compared to patients with inactive ulcerative colitis [66]. Thus, FoxO4 might be a target for ulcerative colitis therapy.

A Potent Pharmaceutical Agent for IBD

Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ, are key mediators for the disruption of the epithelial barrier associated with Crohn's disease [55, 66, 67]. Expression of claudin-2 was increased by TNF-α in epithelial cells [68]. Experimental colitis model mice showed the down-regulation of occludin and up-regulation of claudin-2. Deletion of TNF-α receptor attenuated these changes of occludin and claudin-2 in the experimental colitis model. Importantly, anti-TNF treatment infliximab, which is currently used in Crohn's disease and ulcerative colitis, suppressed the reduction of occludin and elevation of claudin-2 in the experimental colitis model [69].

n-3 polyunsaturated fatty acids (PUFAs), which are abundant in fish oil and include eicosapentaenoic acid and docosahexaenoic acid, have beneficial effects on IBD [70-72]. In an experimental IBD model induced by treatment with trinitrobenzene sulfonic acid, the distribution of TJ proteins, including occludin and claudin-1, was affected; however, the administration of n-3 PUFAs prevented this redistribution of TJ proteins [73].

Probiotics are living bacteria that, when ingested in sufficient quantity, improve the health of the host beyond their inherent basic nutrition [74]. Probiotics have anti-inflammatory effects in IBD. VSL#3, a mixture of 8 probiotic bacterial strains, provided protection against intestinal inflammation in an experimental colitis model. Probiotics also attenuated the enhancement of epithelial permeability and the reduction of TJ components, including occludin, claudin-1 and -4 in the experimental model [75]. Therefore, compounds that enhance the TJ barrier function are candidates for IBD therapy.

CONCLUSIONS

Epithelium and endothelium are located between the outer and inner components of the body or tissues. Most malignant tumors are derived from epithelium. Moreover, epithelium and endothelium are also barriers that prevent invading pathogens and inflammatory cells from entering into the body and tissues. Therefore, the epithelium and endothelium are excellent targets for drug delivery systems, anti-tumor agents, anti-infection agents and anti-inflammatory agents.

Recent studies have revealed the involvement of claudin in some human diseases relevant to TJs (Table 1). Claudin is often overexpressed in human cancers [13-16]. Therefore, a cancer therapy approach that uses claudin ligands is sought. Suzuki *et al.* used autoimmune mice to successfully prepare an anti-claudin-3 monoclonal antibody that mediated ADCC [26]. We anticipate that a novel claudin-targeted cancer therapy will be forthcoming. TJ components are also associated with infections. Claudin-1 and occludin are co-receptors for HCV [51, 52]. The claudin-5 level was reduced in brain microvessels of patients with HIVE [44], and cannabinoids, a clinically used agent for HIV patients, prevented the down-regulation of claudin-5 [43]. These findings indicate that a

Table 1. Perspective on Claudin-Targeted Therapies

Applications	Claudins	References
A diagnostic marker for ovarian cancers	Claudin-4	[25]
Inhibitor of WNV dissemination	Claudin-1~4	[39]
Inhibitor of HIV encephalitis	Claudin-5	[43-45]
Inhibitor of HCV infection	Claudin-1	[51]
Inhibitor of intestinal inflammation in IBD	Claudin-1~4	[69, 73, 75]

WNV, west nile virus; HIV, human immunodeficiency virus; HCV, hepatitis C virus; IBD, inflammatory bowel disease.

claudin/occludin binder and an inducer of claudin-5 may be an inhibitor of HCV infection and a therapeutic agent for HIVE patients. Disruption of the intestinal epithelial barrier is a common feature in patients with IBD. A chemical compound that strengthens the claudin barrier function will be a promising drug for IBD.

Biochemical and functional information regarding TJs has accumulated since the identification of occludin in 1993, and the deregulation of claudins has been observed in several human diseases [16, 20, 21]. The potential of TJ-based therapies is promising. We believe that TJ-targeted therapies might provide a breakthrough in pharmaceutical therapy in the future.

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ABBREVIATIONS

TJ	=	Tight junction
ADCC	=	antibody-dependent cell cytotoxicity
CPE	=	*Clostridium perfringens* enterotoxin
C-CPE	=	the C-terminal fragment of CPE
PSIF	=	protein synthesis inhibitory factor
WNV	=	West Nile virus
HIV	=	human immunodeficiency virus
HIVE	=	HIV encephalitis
BBB	=	blood-brain barrier
STAT-1	=	signal transducers and activators of transcription-1
HCV	=	hepatitis C virus
SR-BI	=	scavenger receptor class B type I
IBD	=	inflammatory bowel disease
FoxO	=	forkhead box transcription factor O
TNF	=	tumor necrosis factor
PUFAs	=	n-3 polyunsaturated fatty acids

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Promising Targets for Anti-Hepatitis C Virus Agents

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Abstract: Hepatitis C virus (HCV) infection is a serious global health problem, with 3-4 million new cases reported each year. Chronic HCV infection places 170 million people at risk of developing liver cirrhosis and hepatocellular carcinoma. However, difficulties in preparing HCV particles *in vitro* have delayed development of effective anti-HCV therapies. In 2005, Wakita *et al.* developed an *in vitro* method to prepare HCV particles, thereby enabling researchers to better understand the mechanism of HCV infection. Other recent advances include development of a virus-free system for evaluating HCV replication and the identification of HCV receptors, such as claudin-1 and occludin, that may serve as targets for anti-HCV drugs. In this review, we discuss recent findings in HCV infection research, including discovery of new potential targets for anti-HCV therapy.

Keywords: Hepatitis c virus, CD81, claudin-1, NS3 helicase, cyclophilin, miRNA122.

INTRODUCTION

It is estimated that approximately 170 million people worldwide are infected with hepatitis C virus (HCV). Chronic HCV infection induces cirrhosis of the liver or hepatocellular carcinoma. Currently, no vaccines or inhibitors that block HCV entry into cells are approved for clinical use. Standard therapy for chronic HCV infection is the combination of pegylated interferon (IFN) and ribavirin (RBV); however, only 50% to 60% of infected patients get a sustained anti-viral response by this therapy. In addition, the severe side effects typical of IFN and RBV treatment often lead patients to stop treatment, and development of novel treatments with fewer serious side effects are therefore necessary.

Hepatitis C virus is a single-stranded RNA virus belonging in the family *Flaviviridae*. The viral genome is approximately 9,600 nucleotides, containing a 5'untranslated region (5' UTR), a region encoding a polyprotein of about 3,000 amino acids, and a 3' UTR. An internal ribosome-entry site (IRES) in the 5' UTR induces cap-independent translation. Once translated, the viral polyprotein is proteolytically processed by cellular signal peptidases and viral proteases into at least 10 mature viral proteins. Three of these proteins (Core, E1, and E2) are the structural proteins included in virions. It is unclear whether protein p7 is included in virions. Non-structural proteins (NS) include NS2, NS3, NS4A, NS4B, NS5A, and NS5B, and all except NS2 are necessary for formation of the complex associated with viral replication. In this review, we summarize recent developments in anti-HCV agents and discuss potent targets for anti-HCV agents.

INHIBITORS OF HCV ENTRY INTO CELLS

HCV Receptors

Hepatitis C virus contains two glycosylated envelope proteins, E1 and E2. While the role of E1 in infection is poorly understood, E2 is known to play a critical role through binding to the cell surface receptor and facilitating virus entry. Several receptors and co-receptors are involved in HCV infection, including CD81, scavenger receptor class B type I (SR-BI), low-density lipoprotein receptor (LDLR), claudin-1, and occludin [1-5]. Although it has been demonstrated that both CD81 and SR-BI directly bind to E2, there is no evidence that claudin-1 and occludin bind the HCV envelope, suggesting that claudin-1 and occludin may interact with other co-receptors to induce HCV entry.

Development of inhibitors that block envelope protein E2 from interacting with cellular receptors is an important area of anti-HCV research. One such class of inhibitors, (ssDNA) aptamers that recognize the HCV E2 protein, was isolated using a living cell surface technique (Systematic Evolution of Ligands by Expotential Enrichment). The ssDNA aptamer ZE2 binds to E2 with high affinity and inhibits its interaction with CD81, and was shown to block HCV infection *in vitro* [6].

Other inhibitors of HCV infection include proteins that bind to or modulate the activity of CD81 and prevent its interaction with CD81 with E2. Salicylate derivatives identified through virtual screening inhibit HCV infection by binding to the open conformation of the large extracellular loop (LEL) of CD81 and preventing its binding to E2. Benzyl salicylate inhibits the interaction of CD81-LEL with E2 by 25% at 50 μM [7]. Another modulator of CD81 activity is PSCK9, a regulator protein of membrane-bound receptors such as LDLR, ApoER2, and very low-density lipoprotein receptor. A recent study showed that PSCK9 deregulates the cell surface localization of CD81. Soluble PCSK9 inhibits HCV infection *in vitro* in a dose-dependent manner [8].

Claudin-1 has been identified as a co-receptor involved in HCV entry into cells, and its interaction with CD81 may help facilitate the early and late stages of HCV entry [4]. Claudin-1 is estimated to be a co-receptor that interacts with CD81. Recently, a claudin-1 antibody was developed that helped elucidate the role of claudin-1 in HCV infection. Anti-claudin-1 inhibited HCV infection at the same stage of HCV entry at which an anti-CD81 antibody did [9]. Since there is no evidence that claudin-1 binds directly to any HCV envelope proteins, it is believed that claudin-1 interacts with CD81 to form a complex that enables HCV cell entry, and may thus serve as a target for development of new HCV entry inhibitors.

Several HCV entry inhibitors that target neither the HCV envelope proteins nor cellular receptors have also been developed. One such inhibitor is C5A, an amphipathic α-helical peptide derived from the membrane anchor domain of HCV NS5A. C5A prevents initiation and spread of HCV infection by destabilizing virions, and has been shown to destroy the integrity of other viral particles, including other *Flaviviridae* (West Nile virus and dengue virus), some paramyxoviruses, and human immunodeficiency virus [10, 11]. C5A might recognize lipid composition of virus membranes, leading to the antiviral activity of C5A to the other viruses [11].

Arbidol is a broad-spectrum antiviral agent that inhibits virus-induced membrane fusion [12-14]. Arbidol is an effective inhibitor of both hepatitis B and C, as well as a wide range of other viruses, including influenza A and B, parainfluenza virus 3, respiratory syncytial virus, and rhinovirus 14. Other agents that block viral entry into host cells include Peptide 75, a peptide derived from the HCV E2 protein transmembrane domain [15], and the Lamiridosins, compounds extracted from *Lamium album* [16]. While the mechanisms through which these agents act to inhibit viral infectivity are poorly understood, continued research may lead to development of additional novel series of inhibitors.

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Protein p7 Inhibitors

Viroporin protein p7 has two membrane-spanning helices and oligomerizes to form a cation-selective ion channel or pore [17-19]. However, the function of p7 and its importance in the HCV infection cycle is unclear. Recent studies revealed that p7 is critical for HCV entry as well as the release of infectious virions both *in vitro* and *in vivo* [20-23]. While p7 is not required for HCV RNA replication [24, 25], the protein is necessary for assembly of the viral particle [22], suggesting that p7 may be a virion component.

Amantadine is an inhibitor of the influenza A virus M2 protein, which is also a viriporin protein. Amantadine also blocks HCV p7 ion channel activity [26, 27]. Several clinical trials showed that amantadine treatment may be effective in patients with chronic hepatitis C infection [28-33]. Other inhibitors of protein p7 have been also identified, including iminosugar derivatives, hexamethylene amiloride, rimantadine, and GSK1-3 [20, 34-37]. More importantly, three-dimensional structure and functional amino acids of protein p7 have been determined [38, 39]. These findings will facilitate the development of new inhibitors against this important HCV protein.

INHIBITORS OF REPLICATION-ASSOCIATED VIRAL PROTEINS AND THE VIRAL GENOME

IRES Inhibitors

The internal ribosome-entry site (IRES) is a well-defined structure of about 340 nucleotides in the 5' UTR of the HCV genome [40]. The host 40S ribosomal subunit and eukaryotic initiation factor 3 (eIF3) recognize the HCV IRES and synthesize viral polyproteins in a cap-independent manner.

The IRES consisting of three domains (domain II-IV) is an attractive target for antiviral drugs because the sequences of subdomains IIIe and IIId are well conserved in all HCV sub-types. Oligodeoxynucleotides targeting IRES domain IIId had antiviral effects [41]. The structures of sub-domain IIa and IIIb are also highly conserved among many HCV sub-types [42, 43]. The L-shaped conformation of sub-domain IIa directs the apical hairpin loop of domain IIb towards the ribosomal E site in the proximity of the active site [44, 45]. The L-shaped architecture of domain II is essential for binding of the 40S ribosome to the IRES. Benzimidazole targets domain IIa and inhibits HCV replication by inducing a widening of the RNA interhelical angle in sub-domain IIa, thereby leading to inhibition of IRES-driven translation [46]. Sub-domain IIIb consists of a hairpin loop, an internal loop and two mismatched cytosine bases. The sequence is not well conserved, but the three-dimensional structure is well conserved. Recognition of the structure of sub-domain IIIb by eIF3 is essential for IRES-dependent translation, indicating that modulation of sub-domain IIIb conformation may inhibit the HCV replication [47]. IRES domain IV contains the HCV translation start codon. The GCAC sequence near the initiator AUG codon is also essential for ribosome assembly [48, 49]. Several inhibitors, including shRNA, siRNA, and hammerhead ribozyme, target the GCAC sequence and efficiently inhibit HCV replication by blocking the initiation of translation [50-52].

NS3 Helicase Inhibitors

Helicase NS3 possesses multifunctional enzymatic activities and plays an essential role in HCV replication [53]. The N-terminus of NS3 is a serine protease involved in viral polyprotein processing, while the C-terminus is an RNA helicase/nucleotide triphosphatase [54]. A number of inhibitors of NS3 protease activity, such as boceprevir, telaprevir, SCH-900518 and VX-813, have been clinically used as inhibitors of HCV replication [55, 56]. NS3 helicase unwinds RNA in a 3' to 5' direction on a 3' overhang region, using any NTPs or dNTPs as an energy source [57-60]. There are a variety of known NS3 helicase inhibitors with diverse modes of action. Benzimidazole and benzotriazole derivatives, acridone-4-carboxylic acid derivatives, triphenylmethane derivatives, QU663, and NS3 peptide (p14) have all been identified as inhibitors of the NS3 helicase [55, 61-65]. Acridone-4-carboxylic acid derivatives intercalate into RNA and inhibit both NS3 helicase and NS5B polymerase activities, while triphenylmethane derivatives inhibit NS3 helicase by preventing NTPase hydrolysis and RNA substrate binding. QU663 is a nucleotide-mimicking compound that inhibits NS3 helicase activity by competing with the enzyme for nucleic acid substrates. Finally, peptide p14, a highly conserved arginine-rich sequence of NS3 helicase, inhibits the enzyme by binding to domain I.

NS4A

NS4A forms a stable heterodimeric complex with NS3 and has serine protease activity. The NS3/4A heterodimer cleaves viral polyprotein into mature viral proteins. An important inhibitor of NS4A is ACH-806 [1-(4-pentyloxy-3-trifluoromethylphenyl)- 3-(pyridine-3-carbonyl)thiourea] (ACH-806). EC50 values for ACH-806 were 30 and 14 nM in genotype 1a and 1b replicon systems, respectively [66]. ACH-806 has synergistic activity with the NS3 protease inhibitor as well as the NS5B polymerase inhibitor, and has no cross-resistance to either inhibitor [67, 68]. A clinical study has revealed that ACH-806 is an effective antiviral agent against HCV genotype 1 [69].

NS4B

NS4B is believed to induce the formation of intracellular membrane structures termed the membranous web [70]. HCV replication complex consisting of NS4A, 5B and other NS proteins is co-localized with HCV RNA in the membranous web. An amphipathic N-terminal helix in NS4B mediates membrane association and forms the replication complex [71]. An arginine-rich motif in the C-terminus of NS4B specifically binds the 3' terminus of the negative HCV RNA strand, which is essential for HCV replication. Clemizole hydrochloride inhibits binding of NS4B to the negative RNA strand and thereby disrupts HCV replication [72]. However, the underlying mechanism has not been currently understood.

NS5A

NS5A is a 56- to 58-kDa membrane-associated phosphoprotein consisting of three domains (domains I, II, and III). In its basally phosphorylated form (p56), NS5A is active in viral replication, whereas the hyperphosphorylated form (p58) is active in viral packaging [73, 74]. Domain I, located in the N-terminus of NS5A, contains a membrane anchoring helix and zinc- and RNA-binding motifs. Domain I has multiple functions, including promoting membrane association of the replication complex, zinc-binding, RNA-binding, and dimerization of NS5A. NS5A is localized in the replication complex on endoplasmic reticulum *via* the domain I, and NS5A interacts with 3'-ends of HCV plus and minus RNA strands. A class of compounds with a thiazolidinone core structure (BMS-824, -858, and -665) inhibits HCV replication *in vitro* by interfering with one or more of the functions of NS5A domain I. These compounds target 76 N-terminal amino acids of NS5A, and they may interfere with RNA-binding or NS5A dimerization. The resultant inhibition of hyperphosphorylation of NS5A might inhibit HCV replication [75]. Another class of NS5A inhibitors, the piperazinyl-N-phenylbenzamides, prevents HCV replication by blocking dimerization of NS5A [76].

Cyclophilin

The immunosuppressant compound cyclosporin A (CsA) is one of the most well known HCV inhibitors. Cyclosporin A acts by

targeting cellular proteins involved in HCV replication [77, 78]. Several sub-types of cyclophilin (CyP), CyPA and CyPB, have been reported to be CsA targets [79-81]. A recent study involving the knockdown of individual CyP sub-types revealed that CyP40 is a novel target of CsA [82]. CyPB facilitates HCV replication *via* the regulation of the RNA binding ability of NS5B [81]. CyPA and CyPB are likely to play different roles in HCV replication than does CyPA. Non-immunosuppressive CsA analogs, such as NIM811, Debio-025, and SCY635, inhibit both CyPA and CyPB [83-85], while CyPB and CyP40 facilitate HCV replication in CsA-resistant cells. There is thus considerable interest in development of CyPB or CyP40 inhibitors [82].

Lupus Autoantigen (La)

Many cellular proteins that interact with IRES elements and stimulate IRES-driven translation have been reported, including the lupus autoantigen (La), polypyrimidine tract binding protein (PTB), poly rC binding protein 2 (PCBP2), C23 nucleolin, and NS1-associated protein 1 [86-94]. These transacting proteins are termed IRES-transacting factors (ITAFs). Granzyme H interferes with La-mediated HCV-IRES translational activity by cleaving the La protein [95]. A synthetic peptide (named LAP) that corresponds to the 18 N-terminal amino acids of La efficiently blocks HCV replication [96]. It is believed that LAP competitively blocks La from interacting with the ITAFs PTB and PCBP2, suggesting the possibility that ITAFs would be suitable targets for inhibition of HCV replication [97].

microRNAs

A few microRNAs (miRNAs) associated with HCV replication have been reported. A liver-specific miRNA, miR-122, facilitates HCV RNA replication by binding the 5' UTR of the viral genome [98]. It has been suggested that down-regulation of miR122 is involved in the anti-HCV activity of IFN [99]. Recently, development of a novel therapeutic agent targeting miR122 was reported. Silencing miR-122 with a locked nucleic acid (LAN)-modified phosphorothioate oligonucleotide (SPC3649) efficiently blocked HCV RNA replication in chronically infected chimpanzees [100].

Another miRNA, miR-199a, has anti-HCV activity that is independent of the IFN pathway. The target sequence of miR-199a is a

Table 1. Targets for HCV Entry

Targets	Anti-HCV agents	Mechanisms
E2	ssDNA aptamers (ZE2)	Inhibit the interaction of E2 with CD81 by binding to E2
CD81	Salicylate derivatives	Inhibit the interaction of E2 with CD81 by binding to LEL of CD81
	Soluble PSCK9	Deregulate the cell surface localization of CD81
Claudin-1	Claudin-1 antibody	Inhibit cell entry of HCV by blocking cladin-1
Viral membrane	C5A	Destabilize virions
Cellular membrane	Arbidol	Inhibit virus-induced membrane fusion
Unknown	Peptide 75	Not understood
	Lamiridosins	Not understood
p7	Amantadine	Inhibit p7 ion channel activity
	Amino sugar derivatives	Inhibit p7 ion channel activity

Table 2. Targets for HCV Replication

Targets	Anti-HCV agents	Mechanisms
IRES	Benzimidazole	Widen the RNA interhelical angle in sub-domain IIa
	shRNA targeting 322-340 of the 5'UTR	Inhibit ribosome assembly
	siRNA targeting 331-350 of the 5'UTR	Inhibit ribosome assembly
NS3	Boceprevir, Telaprevir, SCH-900518, VX-813	Inhibit protease activity
	Benzimidazole derivatives	Inhibit NS3 helicase activity
	Benzotriazole derivatives, Acridone-4-carboxylic acid derivatives	Intercalate into RNA and inhibit NS3 helicase activity
	Triphenylmethane derivatives (QU663)	Inhibit NTPase hydrolysis
	NS3 peptide (p14)	Bind to NS3 domain I
NS4A	ACH-806	Inhibit NS3/NS4A protease activity
NS4B	Clemizole hydrochloride	Inhibit binding of NS4B to HCV negative RNA strand
NS5A	Thiazolidinone	Inhibit a function of NS5A domain I
	Piperazinyl-N-phenylbenzamides	Inhibit dimerization of NS5A
CyP	CsA	Inhibit PPIase activity of CyPs
	NIM811, Debio-025, SCY635	Inhibit PPIase activity of CyPs
La	Granzyme H	Cleave La protein
	LAP	Competitively inhibit binding of La protein to ITAFs
miR-122	SPC3649	Silence miR-122

highly conserved region among HCV sub-types located in domain II of the HCV IRES. Thus, miRNAs are also attractive targets for development of new HCV inhibitors [101].

CONCLUSIONS

The development of an *in vitro* amplification system for HCV by the Wakita group in 2005 has had a profound impact on studies of this important virus [102]. This amplification system has enabled researchers to produce viral particles in sufficient quantities to obtain a better understanding of the molecular mechanism underlying HCV infection, and has aided in the development of inhibitors of a variety of viral target molecules. The targets for anti-HCV therapeutic agents that have been discussed here can be classified into molecules involved in HCV entry (HCV receptors and p7) and in HCV replication (HCV and host cellular components) (Tables **1** and **2**). Inhibitors of NS3/4A protease or NS5B polymerase are promising anti-HCV agents among them. However, frequent mutation of HCV during proliferation has led to the emergence of drug-resistant viruses. To address this issue, numerous efforts have been paid on identification of cellular factors involved in viral replication and infection. One such promising anti-HCV agent is the LAN-modified oligonucleotide that targets the liver specific miRNA associated with HCV replication. This agent showed anti-HCV activity in chronically infected chimpanzees without apparent side effects for an extended period [100]. Very recently, monoclonal antibodies against claudin-1 prevented infection of highly variable HCV quasispecies [103]. We believe that recent progress in understanding the biology of HCV combined with advances in medicinal chemistry will lead to additional breakthroughs in anti-HCV therapy.

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ABBREVIATIONS

HCV	=	hepatitis C virus
IFN	=	interferon
RBV	=	ribavirin
UTR	=	untranslated region
IRES	=	internal ribosome-entry site
NS	=	nonstructural protein
SR-BI	=	scavenger receptor class B type I
LDLR	=	low density lipoprotein receptor
LEL	=	large extracellular loop
eIF3	=	eukaryotic initiation factor 3
CsA	=	Cyclosporin A
CyP	=	cyclophilin
La	=	lupus autoantigen
PTB	=	polypyrimidine tract binding protein
PCBP2	=	poly rC binding protein 2
ITAFs	=	IRES-transacting factors
miRNA	=	microRNA
LNA	=	locked nucleic acid

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