Thus, EL was applied to rabbit skin *in vivo* with practical dose. Fig. 3 shows the amount of DPH in skin per unit area at 4 h after application of EL. Only 5% of the applied dose remained on or in the skin after application of TO1% EL. Rabbit skin resistance is less than that of human skin [13], DPH permeated the skin rapidly and almost all of the DPH was cleared by the bloodstream. Amounts of DPH in the SC, epidermis, and dermis after application of PMB4% EL were significantly greater than those after application of TO1% EL, which suggests that DPH permeation was controlled by DPH release from the vehicle when PMB4% EL was used.

3.4. Condition of EL after drying

Significant differences among formulations were observed after application of practical usage condition. Under practical usage conditions, only nonvolatile ingredients remained on the skin surface because of evaporation of water from the EL. Usually, an o/w emulsion converts into a w/o emulsion during drying because of water evaporation increasing the relative oil concentration. Thus, the weight of oil absorbed onto the paper was measured after drying EL on a glass plate (Fig. 4). An application of 20 μL EL contained 1 mg of DPH, 1 mg of SO, and 0.2 or 0.8 mg surfactant or polymer. For TO1% EL, the amount of oil absorbed was high as 70%. For PMB1% EL, the amount of absorbed oil was less than that of TO1% EL, and for PMB4% EL, only a very small amount of nonvolatile ingredients was absorbed onto the paper. These results indicate that PMB prevents absorption of the oil phase onto the test paper.

Since PMB is a polymer, it has the ability to form films that can prevent oil absorption onto the paper. Thus, two types of emulsions were prepared for comparison. One used TO as an emulsifier, adding PMB after preparation of the emulsion (TO1%+PMB4% EL). The other was pre-emulsified PMB4% only, without using a high-pressure emulsifying procedure with the Microfluidizer (PMB4%-pre EL). For TO1%+PMB4% EL, the amount of absorbed oil was similar to that of TO1% EL. Oil absorption was prevented for PMB4%-pre EL even though the effect was low, which indicates emulsification with PMB is necessary to prevent oil absorption onto the paper. A stable emulsion also is important. It appears as if PMB is adsorbed on the surface of the oil phase, and this condition is maintained after water evaporation.

3.5. Release of DPH

The features of dried PMB4% EL were different from those of other ELs. DPH release profiles from dried emulsions were compared (Fig. 5). The release of DPH after 2-h drying (time 0 h

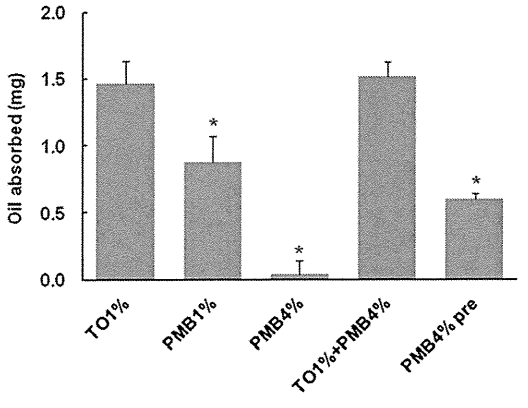

Fig. 4. The amount of oil phase absorbed onto the paper after 2 h drying the ELs. 20 μL EL was spread over 10 cm^2 on the glass plate. The column and bar show the mean ± SD of at least 3 experiments. *Significantly difference *versus* TO 1% EL.

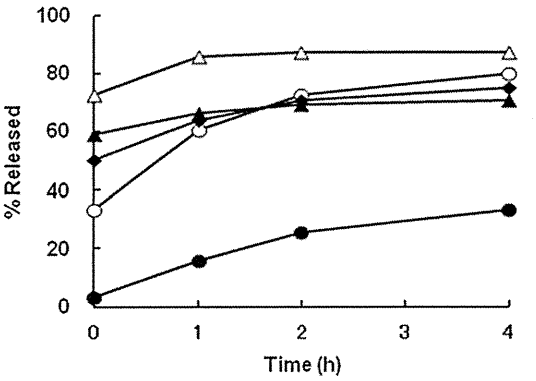

Fig. 5. Release profiles of DPH from various formulations. Twenty μL EL was spread over 10 cm^2 on the glass plate, followed by drying for 2 h, when the release test was initiated: △, TO1% EL; ○, PMB1% EL; ●, PMB4% EL; ▲, TO1%+ PMB4% EL; ♦, PMB4% -pre EL. Each point represents the mean of at least three experiments.

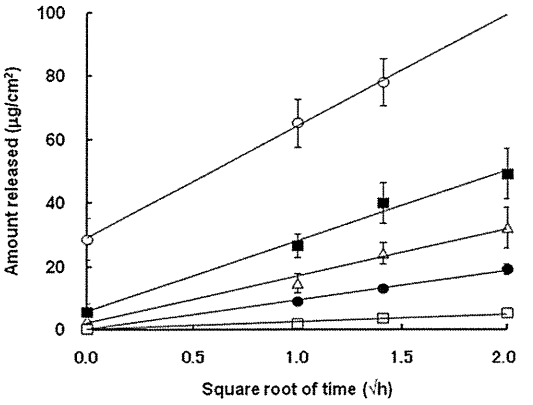

Fig. 6. Higuchi's plots of DPH release from the EL using various concentrations of PMB: ○,1%; ■, 2%; △, 3%; ●, 4%; □, 8%, Each point represents the mean ± SD of at least three experiments.

in the graph) was high for TO1% EL (70%), TO1%+PMB4% EL (60%), and PMB4%-pre EL (50%), and low for PMB4% EL (3%). These percentages were similar to the amount of oil absorbed to the paper, which indicates that DPH is released with SO.

The release profiles of DPH from PMB4% EL seem to obey Higuchi's equation (*i.e.*, a linear plot is obtained from a plot of released amount as a function of the square root of time). Fig. 6 shows the Higuchi plots of DPH release from ELs with various concentrations of PMB. In all cases, the plots show good linearity. For PMB1% EL, a burst of DPH release occurred at time 0. But when the concentration of PMB was greater than 2%, only a small amount of DPH was released at time 0. The slope of the approximation lines decreased with increasing PMB concentration in the EL, therefore, it was defined as the apparent release rate (k). The concentrations of SO and DPH varied from 1% to 15% and 3–8%, respectively. The release profiles were a Higuchi type in all cases, and k increased with increasing SO and DPH concentrations.

Table 3 summarizes the results of release tests. For experiments involving a high oil phase (DPH+SO) to PMB ratio (> 5), a burst was observed at time 0 h. The amount of DPH released (Q) at time t could then be described as:

$$Q = k\sqrt{t} + Q_0 \quad (1)$$

where Q_0 is released amount at time 0 h. Investigation of the effect of formulation on k revealed that the ratio of the amount of the oil phase (SO+DPH) at time 0 h (M_{oil}) to the amount of PMB

Table 3
The amount of ingredients after drying and apparent release rate obtained from Higuchi's plots.

	Formulation (%)			Applied amount (μg/cm²)			Released amount at 0 h (μg/cm²)		Residual amount at 0 h(μg/cm²)[a]			M_{oil}/M_{PMB}[b]	k (μg/cm²/√h)[c]	R^2[d]
	PMB	SO	DPH	PMB	SO	DPH	SO[e]	DPH[f]	PMB	SO	DPH			
Standard	4	5	5	80	100	100	3	0.3	80	97	100	2.46	9.2	0.996
PMB														
1%	1	5	5	20	100	100	54	29.0	20	75	71	7.28	35.3	0.999
2%	2	5	5	40	100	100	15	5.8	40	91	94	4.62	22.3	0.989
3%	3	5	5	60	100	100	5	2.3	60	97	98	3.25	14.8	0.984
8%	8	5	5	160	100	100	14	0.0	160	86	100	1.16	2.5	0.974
SO														
1%	4	1	5	80	20	100	11	2.4	80	11	98	1.36	7.7	0.999
3%	4	3	5	80	60	100	0	0.1	80	60	100	2.00	8.1	0.992
10%	4	10	5	80	200	100	5	0.5	80	196	100	3.70	16.2	0.982
15%	4	15	5	80	300	100	27	1.7	80	275	98	4.66	24.9	0.996
DPH														
3%	4	5	3	80	100	60	13	0.9	80	88	59	1.83	8.0	0.999
8%	4	5	8	80	100	160	19	1.6	80	83	158	3.02	17.5	0.999

[a] Calculated from applied amount and released amount.
[b] Calculated from residual amount of each ingredient; (DPH+SO)/PMB.
[c] Apparent release rate calculated from slope of Higuchi's plots of release study.
[d] Square of correlation coefficient of Higuchi's plot regression.
[e] Oil phase weight absorbed to paper.
[f] Y-intercept of Higuchi's plot of DPH release.

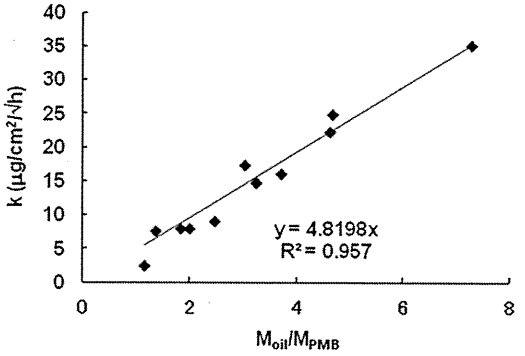

Fig. 7. The relation between the ratio of oil phase/PMB in the formulation and apparent release rate.

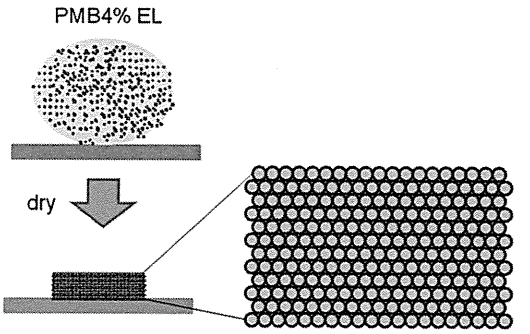

Fig. 8. Speculation of EL condition after application of the practical dose to the skin.

(M_{PMB}) showed good correlation (Fig. 7):

$$k = 4.8M_{oil}/M_{PMB} \quad (2)$$

For a homogeneous matrix, apparent release rate is expressed as

$$k = 2C_0(D/\pi)^{0.5} \quad (3)$$

where C_0 is DPH concentration in dried ELs and D is the diffusion constant in the matrix. In this case, C_0 is expressed as

$$C_0 = M_{DPH}/M_{total} \quad (4)$$

where M_{DPH} and M_{total} is residual amount of DPH and EL (SO+DPH+PMB) at time 0 h, respectively. From Eqs. (2), (3), and (4), D can be described as:

$$4.8M_{oil}/M_{PMB} = 2M_{DPH}/M_{total}(D/\pi)^{0.5}$$
$$(D/\pi)^{0.5} = 2.4(M_{oil}/M_{PMB})(M_{total}/M_{DPH})$$
$$= 2.4(M_{total}/M_{PMB})(M_{oil}/M_{DPH})$$

This shows that $D^{0.5}$ is correlated with the reciprocal of PMB concentration in residual EL and DPH concentrations in the oil phase.

A proposed mechanism is shown in Fig. 8. In the EL, the oil phase is covered with PMB, which acts like a nanocapsule. When the emulsion is applied on skin and dried, the emulsion is not converted into a w/o emulsion, but becomes a thin film of polymer containing the oil phase. The film is homogeneous from a macro view, so the release of DPH occurs in a controlled matrix-type diffusion. The dried EL consisted of two different phases. The DPH existed in the oil phase, and diffusion through the PMB layer was rate limiting; thus, the concentration of PMB in the matrix affected D.

4. Conclusions

An emulsion lotion with controlled release function was prepared. When a PMB EL was applied to skin with practical dose, a thin film formed after evaporation of water without phase conversion of the emulsion. The release pattern of DPH was of a matrix type and could be controlled by the ratio of the oil phase to PMB. The penetration of DPH into skin could be controlled even if the skin barrier function was compromised.

A PMB EL can function as a controlled release formulation for application to the scalp or large areas of skin.

Acknowledgments

We thank NOF and Nikko Chemicals for providing PMB and TO, respectively.

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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.

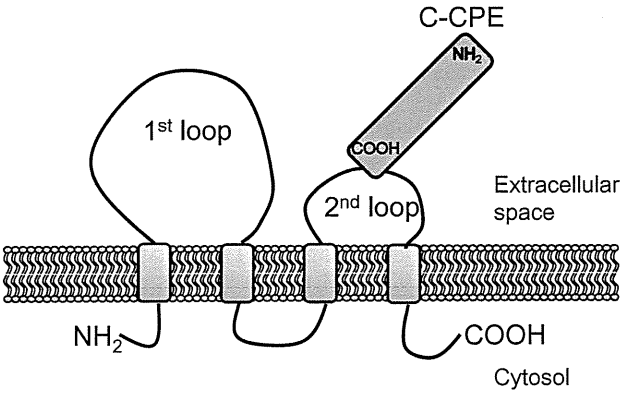

Fig. (1). Schematic illustration of interaction of C-CPE and claudin. Claudin is a tetra-transmembrane protein. C-CPE interacted with the 2nd loop region of claudin *via* its C-terminal domain [32, 76].

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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Peptides as Tight Junction Modulators

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Abstract: The first step in drug absorption is the passage of drug molecules across epithelial cell sheets. Epithelial cell sheets are pivotal for the maintenance of homeostasis in the body by acting as a biological barrier that separates the inside of the body from the outside environment. Intercellular space between the adjacent epithelial cells is tightly sealed by tight junctions (TJs), which prevent solutes from freely moving across the epithelial cell sheets. Modulation of the TJ barrier has been a potent strategy for drug absorption. Absorption enhancers have been investigated since the 1980s, and sodium caprate is clinically used as an absorption enhancer. However, the biochemical constituents and structures of TJs were not elucidated until 1993. Occludin, a tetra-transmembrane protein, was identified to be a structural component of TJs in 1993. Claudin, another tetra-transmembrane protein, was identified as a structural and functional component of TJs in 1998. Modulation of occludin- or claudin-barrier is novel methods to enhance drug absorption. Recently, synthetic TJ-binding peptides, a kinase of claudin and peptide fragments of toxins have been developed. In the present review, we summarize the recent progress in TJ-modulating peptides and discuss their potencies.

Keywords: Tight junction, occludin, claudin, drug absorption.

INTRODUCTION

Recent progress in proteome- and genome-based drug development has caused a paradigm shift in drug development. Biologics, such as peptides, nucleic acids and proteins, account for 30% of newly developed drugs. Most biologics are unable to efficiently across the cell membrane because of their hydrophilic properties and high molecular weights. Therefore, biologics must be injected for clinical use. The development of non-invasive administration systems for biologics would improve patient compliance and quality of life.

Epithelial cells prevent the free movement of solutes into the body and out of the body. Epithelial cell sheets function as a barrier that maintains homeostasis by separating the body from the external environment. Passing across the epithelial barrier is the first step in drug absorption, and overcoming the biological barrier has been a pivotal issue for non-invasive drug delivery. The two transport routes for drugs are the transcellular route and the paracellular route [1]. Small hydrophobic chemicals are often absorbed by passive transport via receptors and transporters or by simple diffusion in the epithelium. In contrast, it is difficult for hydrophilic drugs and large biopharmaceuticals to be absorbed through the transcellular route. Intercellular sealing components called tight junctions (TJs) prevent the leakage of solutes through the intercellular space. Therefore, modulation of the TJ seal in the epithelium would make it possible to non-invasively administer any type of drugs.

More than 50 years ago, freeze-fracture replica microscopy analysis identified TJs as tight seals across intercellular spaces [2]. Several methods, molecules, and excipients have been investigated for the safe and reversible opening of TJs to enhance drug absorption. Surfactants, chelators, bile salts, and fatty acids have been used to enhance the permeation of drugs. However, there are problems associated with their clinical use. The target molecules have low tissue-specificity, and the influx of toxic molecules and damage to the cell membrane may occur. Therefore, only sodium caprate is currently used as an absorption enhancer in pharmaceutical therapy [3].

The mechanism by which TJs sealed the intercellular space remained unclear until the identification of TJ components in the 1990s. In 1993, Furuse and colleagues identified a component of TJs, occludin, a ~65-kDa protein with four transmembrane domains [4]. Although occludin was expected to be a pivotal constituent of the TJ barrier, the knockdown of occludin did not cause the loss of TJ barrier function. In 1998, another TJ protein, claudin, was identified [5]. Claudin is a ~23-kDa protein with four transmembrane domains, and the claudin family contains at least 24 members [6]. The exogenous expression of claudin led to the formation of TJ strands in fibroblast cells that lacked claudins [7]. Interestingly, claudin expression profiles and barrier functions differ among tissues [6, 8]. For example, claudin-1 and claudin-5 are expressed in a variety of tissues, and claudin-1- and claudin-5-deficient mice lose the barrier function of the epidermis and the blood-brain barrier, respectively [9, 10]. Thus, claudins play an essential role in the formation of the barrier. TJs are composed of transmembrane proteins, such as occludin and claudins, and intracellular proteins, such as the ZO family and actin [11]. The ZO family determines the localization of TJs [12]. In 2005, Ikenouchi *et al.* discovered a novel TJ protein, tricellulin, which shares partial homology with occludin and is expressed in the tricellular contacts of epithelial cells [13]. Tricellulin also participates in the barrier function and organization of the bicellular junction.

Absorption enhancers have been developed since the 1960s, when TJs components had not yet been identified. Thus, the first-generation absorption enhancers were developed without knowledge of the molecular structure of TJs. Second-generation absorption enhances, which were developed by using TJ components [3, 14], provide novel strategies for the mucosal absorption of drugs. Most of these TJ modulators are peptides (Fig. **1**).

In this review, we discuss recent progress in the development of second-generation TJ-modulating peptides, and we examine the potencies of these peptides.

TJ MODULATOR PEPTIDES BINDING OCCLUDIN

Electron microscopy analysis revealed that TJs form a continuous strand in the lateral membrane and that the adjacent strands associate with each other [2, 15, 16]. These findings indicate that TJ binders or modulators may modulate TJ sealing. Occludin is the

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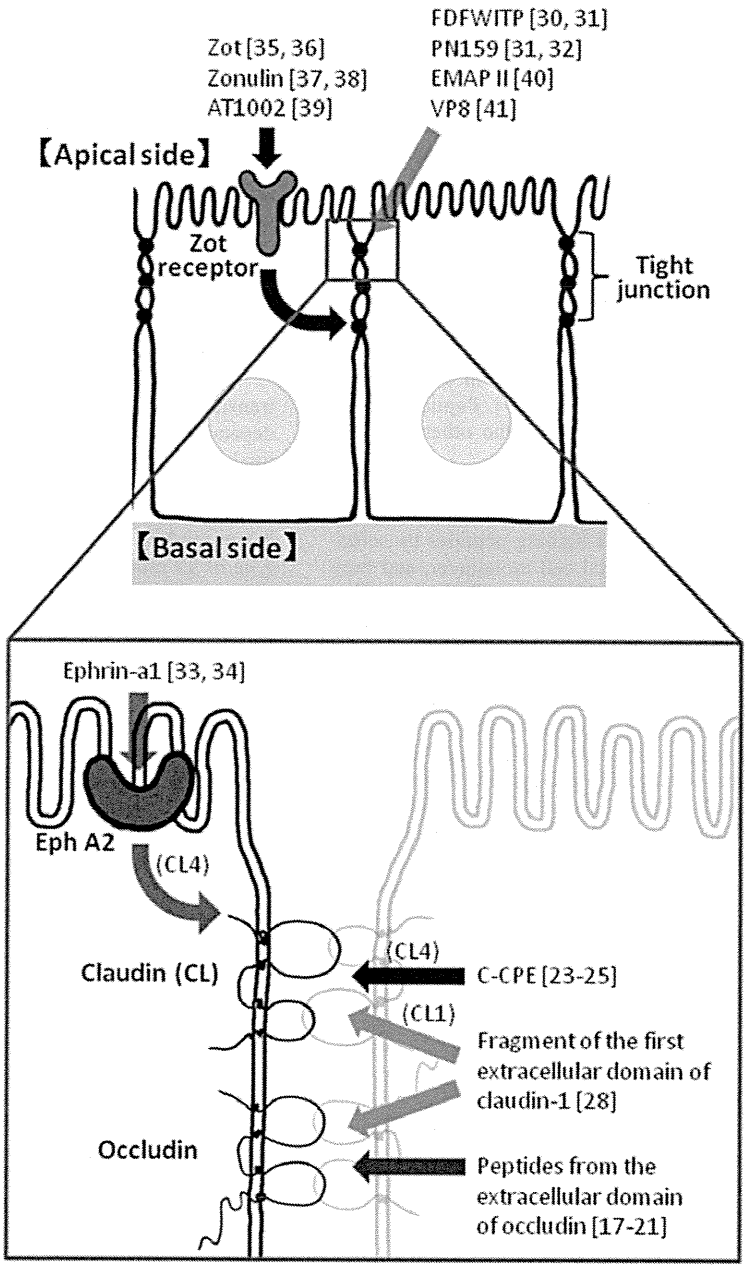

Fig. (1). Schematic drawing of tight junction.

first TJ-sealing structural component to be identified [4]. Peptides from the extracellular domain of occludin have been used to modulate intercellular sealing. Junctional sealing is impaired by 9-mer or 10-mer oligopeptides that are homologous to segments of the first extracellular loop of occludin [17]. A synthetic peptide corresponding to 14 amino acids at the N terminus of the first extracellular loop of occludin increased the permeability of TJs in intestinal and airway epithelia [18, 19]. A 44–amino acid synthetic peptide corresponding to the second extracellular loop of occludin perturbs the TJ permeability barrier and increases the turnover of cellular occludin rather than decreasing occludin synthesis [20]. A testis-specific, ligand-fused occludin peptide, a 22–amino acid peptide corresponding to the second extracellular loop of occludin fused to follicle-stimulating hormone mutant protein, specifically perturbed the blood-testis barrier in rats [21]. Thus, the fusion of tissue-specific ligand to an occludin modulator may provide a novel method to deliver drugs to target tissues.

TJ MODULATOR PEPTIDES BINDING CLAUDINS

Claudin was the first TJ functional component to be identified [5]. *Clostridium perfringens* enterotoxin (CPE) is responsible for the symptoms of a common food poisoning in humans [22]. Claudin-4 is a CPE receptor, and there are other claudin receptors as well [23-25]. The functional domains of CPE can be separated into a receptor-binding region (C-terminal of CPE, C-CPE) and cytotoxic region (N-terminal of CPE). C-CPE binds to claudin-4 and disrupts the barrier function of TJs formed by claudin-4 with no cytotoxicity [24]. We previously found that C-CPE was 400-fold more potent than sodium caprate, the only clinically used absorption enhancer, at enhancing the jejunal absorption of dextran [26].

C-CPE enhanced nasal but not jejunal and pulmonary absorption of a biologic, human parathyroid hormone derivative, hPTH(1-34) [27]. C-CPE is hydrophobic with low solubility, but deletion of 10 amino acids at the N-terminal of C-CPE increased its solubility by 30-fold. The truncated C-CPE enhanced jejunal and pulmonary absorption of hPTH(1-34) [27]. Thus, claudin modulator may be a promising absorption enhancer of biologics. However, C-CPE is a nontoxic fragment, and its antigenicity must be overcome prior to its clinical application.

A 27-amino acid peptide corresponding to half of the first extracellular domain of claudin-1 reduced the TJ barrier in epithelial cells [28]. The claudin-1 peptide interacted with both claudin-1 and occludin, and the claudin-1 peptide modulated gastric mucosal permeability but not intestinal mucosal permeability. There are 24 members of the claudin family, and the combination of the members determines the properties of TJ barriers [29]. Peptides corresponding to the extracellular loop domains of the other claudins may enhance the intestinal absorption of drugs.

OTHER TJ-BINDING PEPTIDES

Johnson and coworkers screened TJ-binding peptides by selection with purified TJ protein or epithelial cell monolayer, and they isolated a TJ-binding peptide, FDFWITP, from a 7-mer peptide–displaying phage library [30, 31]. They prepared the cyclic peptide and found that it dose-dependently modulated the TJ barrier with a peak of 80% reduction at 100 μM for 15 min. The disrupted TJ barrier was recovered 2 h after the removal of the peptide. Another peptide, PN159, was identified from a custom library of peptides designed based on a wide range of structural and physical-chemical properties. PN159 showed reversible TJ-barrier modulating activity in an *in vitro* model of nasal epithelial tissue [31, 32]. PN159 improved the permeation of human peptide YY derivative (PYY_{3-36}) across the nasal epithelial barrier without apparent toxicity *in vitro* and *in vivo*. The underlying mechanisms for TJ modulation by FDFWITP and PN159 have never been determined. The determination of these mechanisms may provide a novel strategy for enhancing the absorption of biologics.

EPH AND EPHRIN

Eph receptor tyrosine kinases and ephrin ligands are widely expressed in epithelial cells and mediate cell-cell interactions. EphA2 associates with claudin-4 via their extracellular domains and leads to the phosphorylation of the cytoplasmic carboxyl terminus of claudin-4. This phosphorylation decreases the integration of claudin-4 into sites of cell-cell contact and enhances paracellular permeability in Madin-Darby canine kidney cells [33]. Ephrin-a1 and EphA2 are also expressed in distal normal lung vasculature, and their expression is increased in injured lung. Intravenous injection of ephrin-a1 caused a large increase in the extravasation of labeled albumin into the lungs of rats [34]. Ephrin-a1 stimulation disrupts TJs in the endothelium, resulting in reduced endothelial barrier integrity. Thus, a modulator of EphA2 would be a novel type of mucosal absorption enhancer.

ZONULA OCCLUDENS TOXIN

Zonula occludens toxin (Zot) produced by *Vibrio cholerae* reversibly increases intestinal permeability by interacting with a specific surface receptor with subsequent protein kinase Cα-dependent polymerization of actin microfilaments strategically located to regulate the paracellular pathway [35, 36]. Zonulin is an intestinal Zot analogue that also induces TJ disassembly and an increase in the passage of inulin [37, 38]. Recently, AT1002, a 6-mer synthetic peptide (H-FCIGRL-OH), was isolated from the active fragment of Zot and enhanced the absorption of polyethylene glycol (molecular weight, 4000) and inulin after intranasal administration [39]. This promising result suggests that this peptide may be a useful enhancer for peptides.

ENDOTHELIAL-MONOCYTE-ACTIVATING POLYPEPTIDE

Endothelial-monocyte-activating polypeptide II (EMAP II) is a pro-inflammatory cytokine that increases the permeability of the blood-tumor barrier. EMAP II is located between brain tumor cells and microvessels formed by highly specialized endothelial cells in the rat model of C6 glioma [40]. After EMAP II injection, the mRNA and protein expression levels of ZO-1, occludin and claudin-5 were significantly decreased. Thus, EMAP II may be a clinically applicable tool to locally open the blood-tumor barrier and deliver antitumor drugs to tumors. Moreover, EMAP II might also be used in tumor dormancy therapy to target tumor vessels.

VP8

VP8 is a rotavirus protein of the outer capsid that is capable of transiently opening TJs [41]. VP8 reduced the TJ barrier in a dose-dependent and reversible manner, and VP8 also enhanced the intestinal absorption of orally administered insulin through the paracellular pathway. Intriguingly, VP8 contains amino acids corresponding to part of the external loops of claudins and occludin. Peptides corresponding to the functional domain of VP8 might be a novel type of TJ modulator with broad-ranging specificity for TJ components.

CONCLUSIONS

TJ modulators have been developed since the 1980s. Recent breakthroughs in epithelial cell biology have clarified the biochemical constituents and structures of TJ seals, in which occludin and claudins play pivotal roles in TJ sealing and intracellular proteins, such as the ZO family, determine the localization of TJs [6, 12]. These findings indicate that TJ modulators targeting the transmembrane proteins and the intracellular proteins may be novel drug delivery systems [4, 5]. Several peptides that modulate the TJ barrier have been developed, and some of these peptides enhance the mucosal absorption of peptide drugs (Fig. **1**). Claudin is often a target of TJ-modulating peptides. Claudins form homo- and heterotypic strands in the lateral membrane, and the adjacent claudin strands associate with each other [29]. The combination of claudins determines tissue-specific TJ-barrier functions [6]. Importantly, various studies of claudin-overexpression and -knockdown showed that the claudin strands may control charge- and size-selective paracellular transport of solutes [6]. Properties of the selective paracellular transports are estimated to be determined by the combination of the claudin members. Modulation of appropriate claudin strands may enhance paracellular transport of selective molecules and reduce non-specific paracellular influx of solutes except for drug. Claudin modulators may be a promising mucosal absorption enhancer. The blood-brain-barrier is the one of the most challenging biological barriers from therapeutic point of view. A claudin-5 modulator may be a new method to deliver drugs across the blood-brain-barrier into the central nervous system [10].

Two challenges to the development of claudin modulators are the lack of structural information for claudin and the limited purification of claudin. Claudin is a tetra-transmembrane protein, and the extracellular domains are approximately 50 and 15 amino acids of the first and second loop, respectively. The three-dimensional structure of claudin has not been determined. Moreover, claudin-4 is the only claudin family member that has been purified. Thus, the underlying mechanism for the interaction between the peptides and claudin has never been understood. The future analysis of claudin structure will contribute to the optimization of absorption-enhancing claudin-binding peptides.

Claudin has little antigenicity; therefore, the preparation of antibodies against the extracellular loop domain of claudin has been delayed. Recently, Suzuki *et al.* successfully prepared monoclonal antibody against the extracellular domains of claudin-4 [42]. The future identification of the epitope of the anti-claudin-4 antibody

and the determination of the complex structure may lead to the design of a claudin-binding peptide based on structural information. The structure of C-CPE, a popular claudin-4-binder, has been determined [43]. Moreover, the mode of interaction between CPE and claudins has been proposed [8, 25, 44]. The underlying mechanism for the interaction between C-CPE and claudins will be clear in the near future and contribute to the theoretical design of claudin-binding peptide by using C-CPE as a prototype.

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ABBREVIATIONS

TJ	=	Tight junction
CPE	=	*Clostridium perfringens* enterotoxin
C-CPE	=	C-terminal fragment of *Clostridium perfringens* enterotoxin
Zot	=	Zonula occludens toxin
EMAPII	=	Endothelial-monocyte-activating polypeptide II

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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, and 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 subdomain IIa and IIIb are also highly conserved among many HCV sub-types [42, 43]. The L-shaped conformation of subdomain 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 subdomain IIa, thereby leading to inhibition of IRES-driven translation [46]. Subdomain 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 subdomain IIIb by eIF3 is essential for IRES-dependent translation, indicating that modulation of subdomain 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. CsA 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]. CyPB and CyP40 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 miR-122 is involved in the anti-HCV activity of IFN [99]. Recently, development of a novel therapeutic agent targeting miR-122 was reported. Silencing miR-122 with a locked nucleic acid (LNA)-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 highly conserved region among HCV sub-types located in domain

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

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 LNA-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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Spiral progression in the development of absorption enhancers based on the biology of tight junctions ☆

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ABSTRACT

Epithelium covers the body and, therefore, separates the inner body from the outside environment. Passage across the epithelium is the first step in drug absorption. Tight junctions (TJs) seal the space between adjacent epithelial cells and prevent the free movement of solutes through the paracellular space. Modulation of the epithelial barrier is the most important strategy for enhancing drug absorption. Development of the strategy has accelerated with progress in understanding of the biology of the TJ seal. The first-generation absorption enhancers were screened on the basis of their absorption-enhancing activity in vivo. However, TJs were not well understood initially. The identification of TJ components, including those based on occludin and claudins, has led to the development of new strategies for drug absorption. Accumulation of knowledge of claudins has provided new insights into the paracellular transport of drugs. This review examines the relationship between advances in understanding of TJ biology and paracellular transport of drugs and discusses progress in the development of mucosal absorption enhancers.

Contents

1. Introduction

The philosopher Hegel proposed that "change moves in spirals, not circles." In other words, things of the past will reemerge with progress.

Drugs are administered by routes that include oral, nasal, pulmonary, and epidermal routes or injection. Noninvasive routes are better than invasive ones, but drug administration via the nasal, pulmonary, and epidermal routes requires specialized equipment.

Abbreviations: TJ, tight junction; EDTA, ethylenediaminetetraacetic acid; AJ, adherens junction; DS, desmosome; GP, gap junction; CPE, *Clostridium perfringens* enterotoxin.

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