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Effect of 70-nm silica particles on the toxicity of acetaminophen, tetracycline, trazodone, and 5-aminosalicylic acid in mice

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Exposure to nano-sized particles is increasing because they are used in a wide variety of industrial products, cosmetics, and pharmaceuticals. Some animal studies indicate that such nanomaterials may have some toxicity, but their synergistic actions on the adverse effects of drugs are not well understood. In this study, we investigated whether 70-nm silica particles (nSP70), which are widely used in cosmetics and drug delivery, affect the toxicity of a drug for inflammatory bowel disease (5-aminosalicylic acid), an antibiotic drug (tetracycline), an antidepressant drug (trazodone), and an antipyretic drug (acetaminophen) in mice. Co-administration of nSP70 with trazodone did not increase a biochemical marker of liver injury. In contrast, co-administration increased the hepatotoxicity of the other drugs. Co-administration of nSP70 and tetracycline was lethal. These findings indicate that evaluation of synergistic adverse effects is important for the application of nano-sized materials.

1. Introduction

Nano-sized particles, which have a diameter of less than 100 nm, are widely used in medicine, food, and machinery. With their smaller size, the physical and chemical properties of their constituents change, so that they may be toxic, for example to the lungs or liver, even though macro-particles of the same materials are not (Byrne and Baugh 2008; Nishimori et al. 2009b). Some nano-sized particles show long-term accumulation or a wide distribution in the body (Byrne and Baugh 2008; Nishimori et al. 2009b; Xie et al. 2009; Yang et al. 2008).

Recent reports indicate that some nano-sized particles can generate reactive oxygen species (ROS) on their surfaces, leading to cellular injury (Jin et al., 2008; Sharma et al. 2007; Ye et al. 2010). There are also many drugs that cause adverse effects through the generation of ROS (Ali et al. 2002; Kovacic 2005; Xu et al. 2008). Thus, nano-sized particles might enhance the side-effects of some pharmaceutical drugs. Indeed, we have shown that 70-nm silica particles (nSP70) cause liver injury but that macro-sized silica particles with a diameter of 300 and 1000 nm do not (Nishimori et al. 2009b). Also, when co-administered to mice, nSP70 but not the macro-sized silica particles enhance the toxicity of cisplatin and paraquat (Nishimori et al. 2009a). Surprisingly, co-administration of cisplatin and nSP70 was lethal, suggesting that each chemical may have different synergistic effects in the presence of nano-sized materials.

In the current study, to clarify the influence of nano-sized materials on the adverse effects of chemicals, we assessed the toxicity in mice of 5-aminosalicylic acid (an agent for treating inflammatory bowel disease), tetracycline (a broad-spectrum antibiotic), trazodone (an antidepressant), and acetaminophen (a common antipyretic analogue) in the presence or absence of nSP70.

2. Investigations and results

Several reports indicate that 5-aminosalicylic acid, which is used to treat inflammatory bowel disease, causes liver injury and interstitial nephritis (Deltenre et al. 1999; Margetts et al. 2001). Administration of 5-aminosalicylic acid caused an increase in ALT, AST and BUN levels (Fig. 1). Also, nSP70 dose-dependently elevated ALT and AST levels. Co-treatment with 5-aminosalicylic acid and nSP70 resulted in higher levels of ALT and AST than nSP70 alone. In contrast, changes in BUN levels in response to 5-aminosalicylic acid were not affected by nSP70.

Next, we investigated effect of nSP70 on tetracycline, a broad-spectrum antibiotic. As shown in Fig. 2A and 2B, administration of tetracycline did not elevate biochemical markers for liver injury. In contrast, co-administration with nSP70 resulted in the synergistic induction of liver injury. However, nSP70 alone did not cause kidney injury. Importantly, co-administration of 30 and 50 mg/kg nSP70 with tetracycline resulted in the death of 1 of 4 and 2 of 4 mice, respectively.

Finally, we investigated effect of nSP70 on toxicity of the antidepressant trazodone and the antipyretic analgesic acetaminophen. We found that nSP70 did not have a synergistic effect on the toxicity of trazodone (Fig. 3). In contrast, co-administration of acetaminophen with nSP70 caused synergistic liver injury (Fig. 4).

3. Discussion

In this study, we showed that nSP70 synergistically enhance the toxicity of 5-aminosalicylic acid, tetracycline, and acetaminophen but not trazodone. To avoid direct interac-

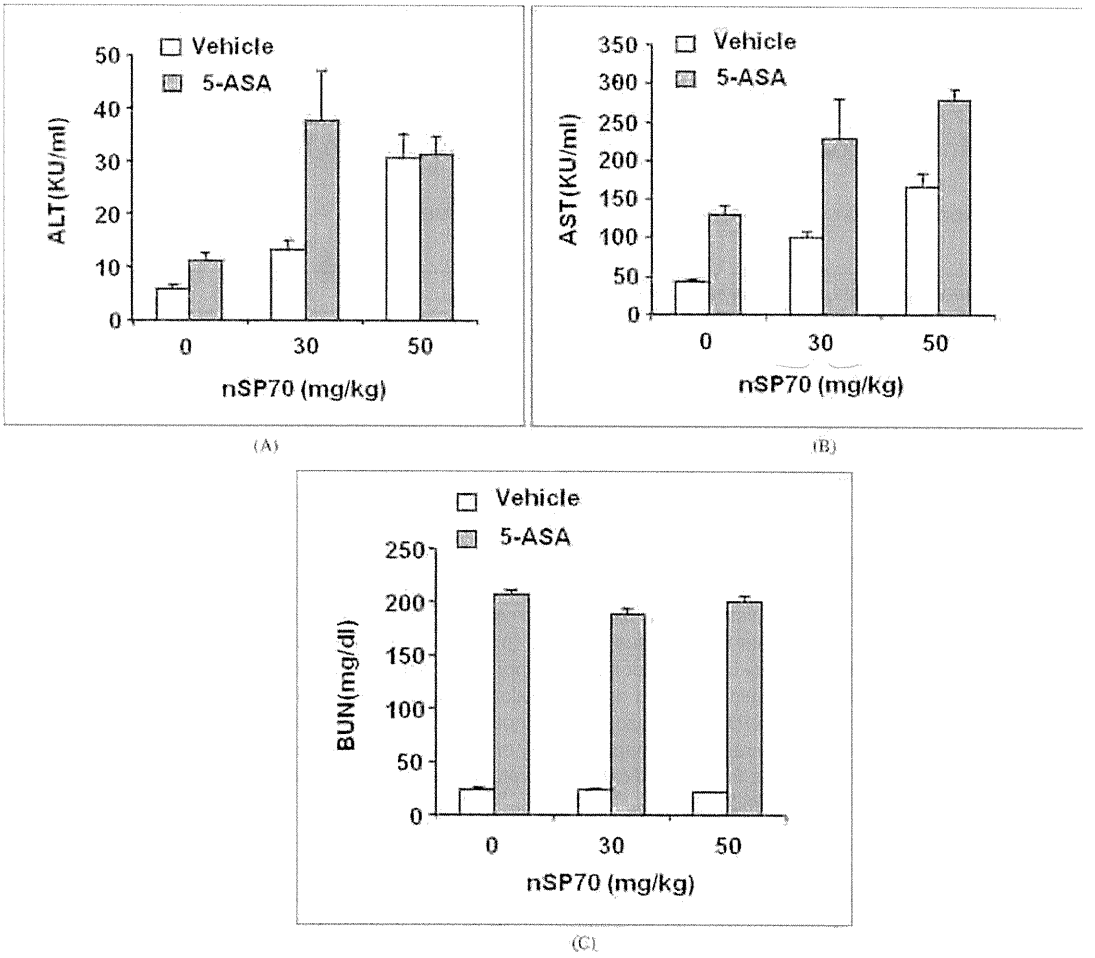

Fig. 1: Effect of nSP70 on 5-aminosalicylic acid (5-ASA)-induced toxicity Mice were injected intraperitoneally with 5-ASA at 0 (open column) or 500 mg/kg (gray column) and intravenously with nSP70 at the indicated doses. After 24 h, the serum was collected. Shown are the levels of ALT (A), AST (B), and BUN (C). Data are means ± SEM (n = 4)

tions between nSP70 and chemicals in their administration and absorption, nSP70 and chemicals were administered intravenously and intraperitoneally, respectively. Administration of nSP70 alone has been shown to cause liver injury but not kidney injury (Nishimori et al. 2009b). Also, in this study, nSP70 did not enhance kidney injury induced by 5-aminosalicylic acid or tetracycline, two drugs known to be nephrotoxic (Grisham et al. 1992; Kunin 1971). The renal toxicity of cisplatin, another nephrotoxic chemical, was unaffected by nSP70 (Nishimori et al. 2009a). Like 5-aminosalicylic acid, tetracycline, and acetaminophen (Chun et al. 2009; Herzog and Leuschner 1995; Kunin 1971), nSP70 is hepatotoxic (Nishimori et al. 2009b), and we showed here that its co-administration synergistically enhanced liver injury. These findings indicate that nSP70 may enhance the toxicity of certain chemicals. Therefore, it will be important to assess the tissue-specific risk of nano-sized materials.

The nSP70 particles had a lethal effect when combined with tetracycline. The 50% lethal dose of tetracycline is 318 mg/kg by intraperitoneal injection in mice. A previous report showed that 100 mg/kg nSP70 is lethal in 100% of mice (Nishimori et al. 2009b). A single injection of tetracycline (100 mg/kg) or nSP70 (30 or 50 mg/kg) alone was not lethal in this study but a combination of the two was. Co-administration of cisplatin and nSP70 showed a similar synergistic lethal effect. This could be due to an interaction between nSP70 and serum albumin. Tetracycline in the bloodstream can bind to albumin (Popov et al. 1972; Powis 1974). Likewise, serum albumin adsorbs onto nano-sized silica particles (Dutta et al. 2007). When injected intravenously, 100-nm anionized albumin-modified liposomes are taken up by hepatic endothelial cells and Kupffer cells (Kamps et al. 1997), which normally clear chemically modified albumin (Jansen et al. 1991). Thus, tetracycline-bound serum albumin may adsorb onto nSP70, causing it to be taken up by the hepatic endothelial cells and Kupffer cells in the liver where it may accumulate and cause lethal liver damage.

Indirect interactions between chemicals and nano-sized particles mediated by serum albumin may be useful for estimating the toxicity of nano-sized materials. In this study, co-treatment of mice with nSP70 (50 mg/kg) and tetracycline decreased BUN levels compared to tetracycline alone or nSP70 (30 mg/kg) and tetracycline. A similar decrease in BUN levels

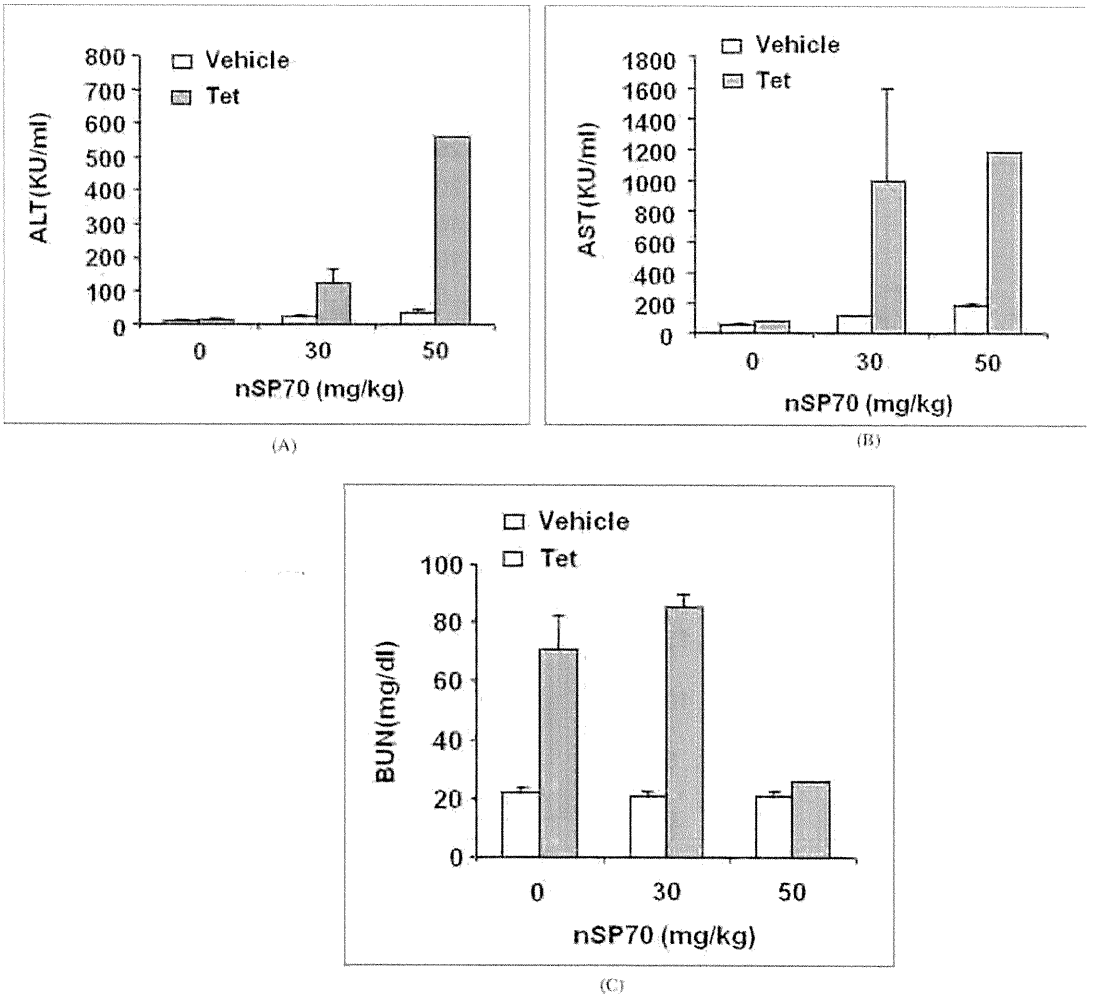

Fig. 2: Effect of nSP70 on tetracycline (Tet)-induced toxicity Mice were injected intraperitoneally with Tet at 0 (open column) or 100 mg/kg (gray column) and intravenously with nSP70 at the indicated doses. After 24 h, the serum was collected. Shown are the levels of ALT (A), AST (B), and BUN (C). One of 4 mice died when co-treated with nSP70 (30 mg/kg) and Tet (100 mg/kg), and 2 of 4 mice died when co-treated with nSP70 (50 mg/kg) and Tet (100 mg/kg). Data are means or means ± SEM (n = 2–4)

was also reported in mice co-treated with nSP70 and cisplatin (Nishimori et al. 2009a). However, the mechanism by which these decrease the BUN level remains to be determined.

In conclusion, we found that nSP70 cause synergistic toxicity when combined with some clinically used drugs, although the synergistic effects differ between chemicals. One combination was lethal, and the others resulted in tissue injury. These studies suggest that evaluation of possible synergistic adverse effects with pharmaceutical drugs may be important for assessing the safety of nano-sized particles.

4. Experimental

4.1. Materials

The nSP70 nanoparticles were obtained from Micromod Partikeltechnologie GmnH (Rostock, Germany). The mean diameter of the particles, as analyzed by a Zetasizer (Sysmex Co., Kobe, Japan), was 55.7 nm, and the particles were spherical and nonporous. The particles were stored at 25 mg/ml as an aqueous suspension. The suspensions were thoroughly dispersed by sonication before use and diluted in water. An equal volume of solution was injected for each treatment. Acetaminophen, tetracycline, and trazodone were dissolved in saline solution, and 5-aminosalicylic acid was suspended in 1% sodium salt of carboxy methyl cellulose. All reagents were of research grade.

4.2. Animals

Eight-week-old BALB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). Mice were maintained in controlled environment (23 ± 1.5 °C; 12-h light/12-h dark cycle) with free access to standard rodent chow and water. The mice were given 1 week to adapt before experiments. All of the experimental protocols complied with the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

4.3. Biochemical analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN) were measured using commercially available kits according to the manufacturer's protocols (WAKO Pure Chemical, Osaka, Japan).

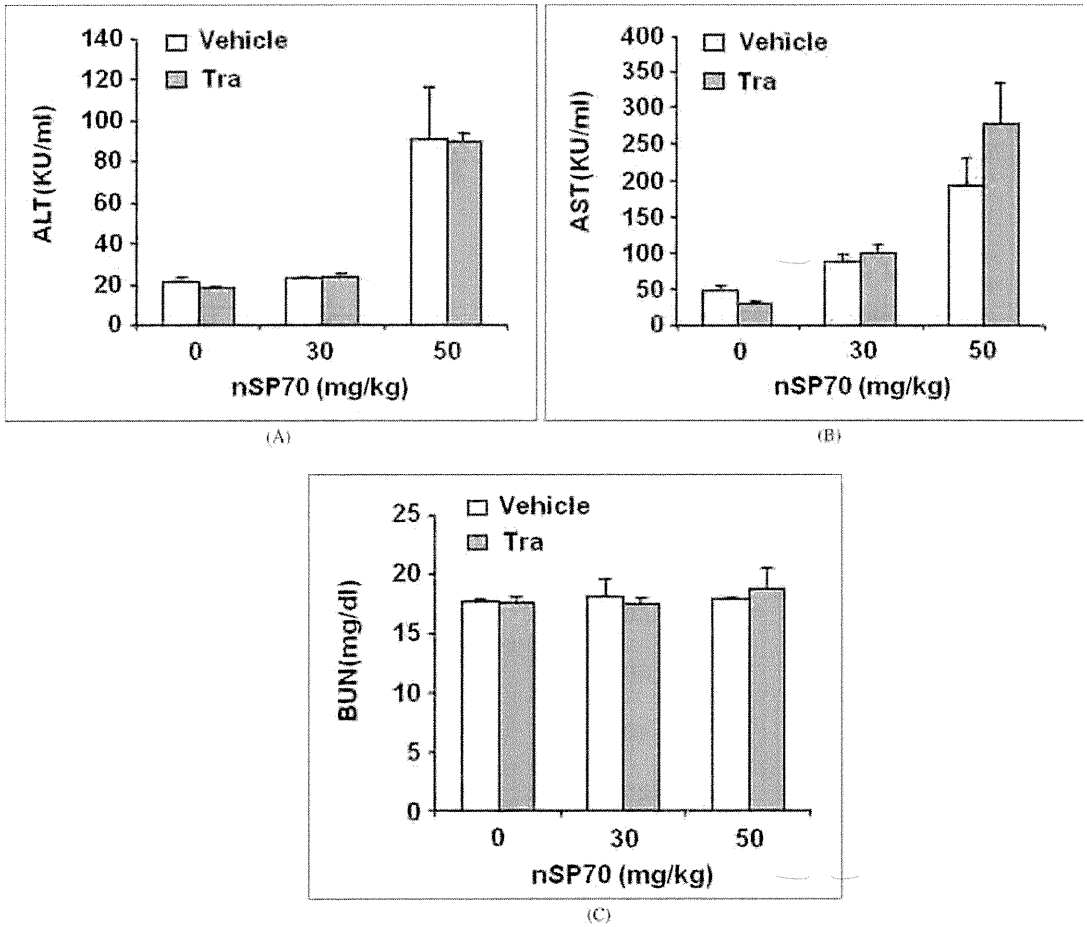

Fig. 3: Effect of nSP70 on trazodone (Tra)-induced toxicity Mice were injected intraperitoneally with Tra at 0 (open column) or 100 mg/kg (gray column) and intravenously with nSP70 at 30 or 50 mg/kg. After 24 h, the serum was collected. Shown are the levels of ALT (A), AST (B), and BUN (C). Data are means ± SEM (n = 4)

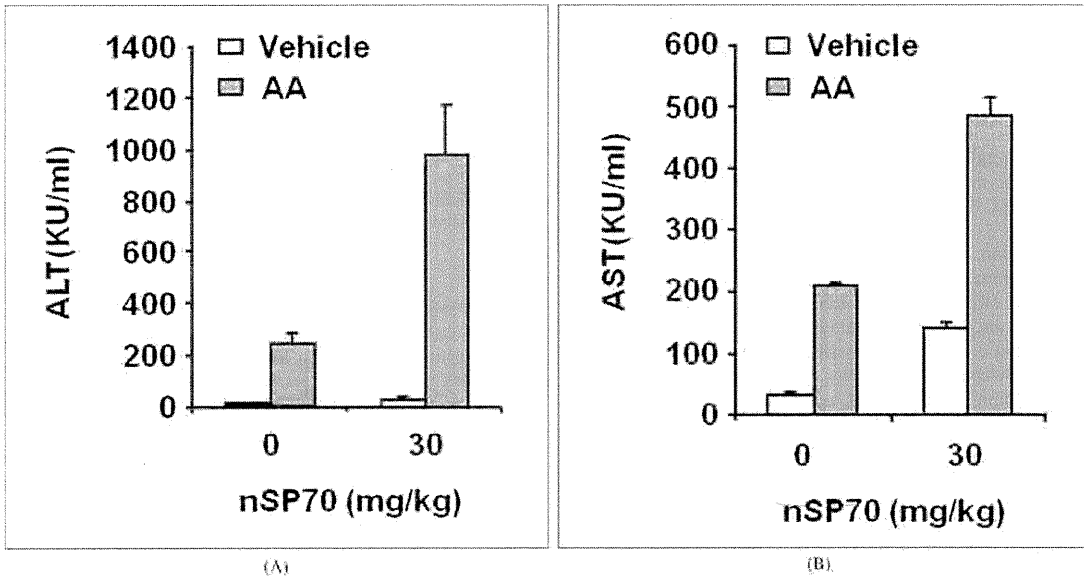

Fig. 4: Effect of nSP70 on acetaminophen (AA)-induced toxicity Mice were injected intraperitoneally with AA at 0 (open column) or 500 mg/kg (gray column) and intravenously with nSP70 (30 mg/kg). After 24 h, the serum was collected. Shown are the levels of ALT (A) and AST (B). Data are means ± SEM (n = 4)

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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.

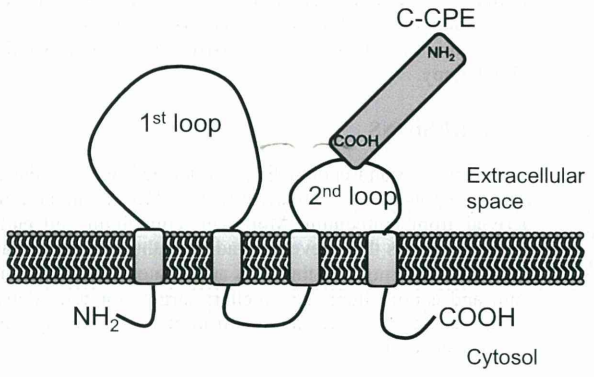

Fig. (1). Schematic illustration of interaction of C-CPE and claudin. Claudin is a tetra-transmembrane protein. C-CPE interacted with the 2nd loop region of claudin *via* its C-terminal domain [32, 76].

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, 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.

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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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These noninvasively administered drugs are expensive. Drugs administered orally without using specific equipment are the least expensive. Patients are often compliant in taking oral medication because this method of drug delivery is easy and pain-free. Therefore, orally administered drugs are ideal for drug development. However, the intestinal mucosa functions as a biological barrier, separating the outside environment from the inner body and preventing the free movement of solutes. Modulation of the epithelial barrier must be considered in the development of orally administered drugs.

The first report of mucosal absorption of drugs was published approximately 50 years ago [1]. Ethylenediaminetetraacetic acid (EDTA) was shown to enhance intestinal absorption of heparin and synthetic heparinoids, but the mode of action was unclear. Chelation of magnesium and calcium by EDTA was later shown to increase intestinal permeability to solutes, but the mechanism was not clarified [2]. EDTA was found to widen the intercellular junctional seal, enhancing intestinal permeability to solutes [3]. This was the first evidence that modulation of the intercellular seal in the mucosal epithelium may be a potent strategy for mucosal absorption of drugs.

The first breakthrough in the biology of the epithelial barrier was the identification of tight junctions (TJs) between adjacent epithelial cells, in 1963 [4]. This finding promoted the development of novel strategies for mucosal absorption of drugs by modulating the TJ seal. Development has progressed further with our understanding of the biology of the epithelial barrier.

Strategies for mucosal absorption of drugs are developing, accompanied by advancements in understanding of the biology of epithelial cells. This review describes the development of strategies for mucosal absorption of drugs as a result of the accumulation of knowledge of the epithelial barrier. We also describe the future directions in research on mucosal absorption of drugs.

2. First-generation mucosal absorption enhancers

The first advancement in the biology of the epithelial barrier was the discovery of the TJ (Table 1) [4]. High-resolution replica analysis revealed that TJs form a continuous band-like meshwork. Globular components bridge the width of the adjoining membranes linked together in the plane of the intercellular space [5]. However, it was unclear whether the TJ components are proteins or lipids. Membrane lipids, rather than membrane proteins, were thought to be the structural elements of TJ seals [6,7]. Therefore, the initial stage of the development of novel strategies for mucosal absorption of drugs was based on the knowledge that the intestinal mucosa is a barrier for drug absorption and that TJ components are responsible for intercellular sealing, but no molecular level information about TJ components was available.

The first advancements evoked the idea that disruption of the mucosal epithelial barrier or TJ seal would lead to the development of novel strategies for mucosal absorption of drugs (Table 2). Nonionic, anionic, and cationic surfactants are also intestinal absorption enhancers [8,9]. These surfactants were believed to disturb the integrity of the plasma membrane [9]. Surfactant–surfactant interactions occur at high surfactant concentrations in the plasma membrane and can result in dissolution of the plasma membrane into surfactant–membrane mixed micelles. Surfactants can also extract proteins from the plasma membrane. Surfactant-enhanced membrane permeability is generally assumed to be nonspecific and cytotoxic [9]. Some surfactants, such as polyoxyethylene esters and dodecylmaltoside, exhibit absorption-enhancing effects and toxic effects in the intestine [10–13].

EDTA, a calcium chelator, enhances mucosal absorption of drugs [1]. EDTA modulates TJ barrier integrity by opening intracellular TJ seals [14]. Some surfactants also enhance intestinal absorption by sequestering calcium ions [10].

Various fatty acids, including caprate, caprylate, and laurate, enhance membrane permeability [15]. Mucosal absorption of insulin and cefmetazole is increased with 1% caprate treatment, but only absorption of insulin is enhanced by treatment with 0.25% caprate. One possible explanation for the differential effects on mucosal absorption of drugs was that the electrically repulsive effects of the paracellular route might affect paracellular absorption of a neutral molecule, insulin, and an acidic molecule, such as cefmetazole [15]. This finding indirectly suggested that modulation of the paracellular route can lead to mucosal absorption of solutes in a solute-specific manner. A series of analyses aimed at determining the mode of action of sodium caprate indicated that the compound activates phospholipase C, elevates intercellular calcium levels, and subsequently stimulates contraction of calmodulin-dependent actin–myosin filaments, thereby opening TJ seals [16,17].

Cationic chitosan increases epithelial paracellular permeability [18]. Chitosans bind to the epithelial cell membrane through a charge-dependent interaction, resulting in F-actin depolymerization and separation of TJ components. This event triggers enhanced epithelial permeability. Polylysine also enhances epithelial paracellular permeability by opening TJ seals [19].

There are two issues in the development of absorption enhancers: the toxicity of these substances and the risk of opening TJ seals. The first-generation absorption enhancers disrupted the cell membrane and modulated the TJ seal. Transient modulation of TJ seals by EDTA, fatty acids, and polycations would be less toxic than disruption of the cell membrane by surfactants. Opening the intercellular TJ seal might lead to the influx of solutes other than drugs, including undigested food, metabolites of intestinal microorganisms, and bile salts. Mucosal

Table 1
Progressive elucidation of TJ biology.

Year	Event
1963	Identification of TJ [4]
1973	Identification of TJ strands [5]
1982	Membrane lipid hypothesis [6,7]
1986	Identification of ZO-1 [96]
1993	Identification of occludin[24]
1998	Identification of claudin [33]
1999 onward	Clarification of TJ barrier function of claudins [47] Identification of paracellular ion transport via claudins [94]
2011	Identification of transcellular transport coupled to claudin-based TJ strands [95]

TJ: tight junction.

Table 2
Progress in the development of absorption enhancers.

Category	Enhancer	Possible mode of action
First-generation absorption enhancers	EDTA	Sequestration of Ca^{2+} [14][a]
	Surfactants	Perturbation of the plasma membrane [8,9]
	Sodium caprate	Phospholipase C [16,17][b]
	Chitosan	Depolymerization of actin [19]
Second-generation absorption enhancers	Occludin peptide	Perturbation of occludin [26–29][c]
	C-CPE	Binding to claudin-4 [47,50]
	FSH-fused occludin peptide	Perturbation of occludin in BTB [30]
	Claudin peptide	Binding to claudins and occludin [77]

EDTA: ethylenediaminetetraacetic acid; FSH: follicle-stimulating hormone; BTB: blood–testis barrier.

[a] Activation of protein kinase C is partly involved in modulation of the TJ barrier by chelation of Ca^{2+} [97,98].

[b] Activation of phospholipase C increases intracellular calcium levels, followed by contraction of calmodulin-dependent actin–myosin filaments and subsequent opening of the TJ-seal [16,17].

[c] Occludin peptides cause a decrease in the cellular content of occludin or perturbation of localization of occludin [26–28]. An occludin peptide interacts with occludin and claudin-1 [99].

absorption through the paracellular route requires opening of the intercellular seal, and most researchers believed (and may still think) that such a clinical application is impossible. Transcellular drug delivery via transporters has been widely investigated.

3. Second-generation mucosal absorption enhancers

3.1. Impact of occludin

3.1.1. Occludin

Mammalian cells have four types of intercellular junctions: adherens junctions (AJs), desmosomes (DSs), gap junctions (GPs), and TJs. Although specific types of integral membrane proteins – cadherins, desmogleins and desmocollins, and connexins – had been identified in AJs, DSs, and GPs, respectively [20–22], biochemical information about TJs remained sparse.

An electron microscopic analysis in 1973 revealed that TJs appear as a linear series of individual intramembranous particles [5]. Evidence later indicated that TJs might be pairs of inverted cylindrical lipid micelles [6,7]. Freeze fracture analysis revealed that TJ components are not exclusively lipids [23]. Progression in the understanding of TJ biology led to identification in 1993 of the integral membrane protein occludin located in TJs (Table 1) [24]. Furuse et al. isolated the AJ fraction from chicken livers and immunized rats with the fraction. They prepared monoclonal antibodies and found antibodies that recognized an approximately 65-kDa membrane protein enriched in the junctional region of endothelial and epithelial cells. The approximately 65-kDa protein containing four transmembrane domains was identified as occludin. Occludin homologs have been identified in humans, mice, dogs, and rat-kangaroos, and the development of drug delivery systems targeting occludin was proposed by Tsukita et al. in 1996 [25]. This was the first suggestion of a molecular TJ seal-based drug delivery system.

3.1.2. Occludin and drug delivery

The extracellular loop domains of occludin are rich in tyrosine and glycine residues [24]. The nonpolar nature of the extracellular domains and conservation of their sequences among humans, mice, dogs, chickens, and rat-kangaroos suggest that the extracellular domains have important functional roles in the formation of intercellular TJ seals. These findings suggest the possibility of modulating TJ seals by using a synthetic peptide corresponding to the extracellular domain of occludin (Table 2). The TJ seal is modulated by targeting occludin with a synthetic peptide corresponding to the second extracellular loop domain of occludin [24]. The TJ barrier in *Xenopus* kidney epithelial A6 cell lines is reduced by the occludin peptide but not by a peptide containing scrambled amino acid sequences [26]. The occludin peptide enhances the epithelial permeability of A6 monolayer cell sheets to mannitol, insulin, and 3- and 40-kDa dextran. Another synthetic peptide corresponding to the first loop domain of occludin decreases TJ barrier integrity and increases the permeability of human colon carcinoma Caco-2 cells to mannitol [27]. Treatment with a synthetic peptide corresponding to the first extracellular loop domain of occludin in human airway epithelial cells reversibly decreases the integrity of the TJ barrier. Permeability to 70-kDa dextran is transiently and reversibly increased in human airway epithelial cell sheets [28]. An occludin peptide also modulates TJ barrier integrity in rat Sertoli monolayer cell sheets [29]. Receptors for follicle-stimulating hormone (FSH) are expressed in Sertoli cells. An inactive FSH mutant was used as a ligand for the in vivo blood–testis barrier, and injection of the FSH mutant-fused occludin peptide led to reversible disruption of the blood–testis barrier [30]. Therefore, identification of occludin as a TJ component resulted in a shift in the mucosal absorption paradigm from a phenomenon- to a molecular-based approach. In addition, the discovery of occludin led to major progress in understanding of the biology of the epithelial barrier.

3.2. Impact of claudin

3.2.1. Claudin

Gene knockout analyses were used to clarify the roles of occludin in the TJ barrier. Occludin-deficient embryonic stem cells differentiate into polarized epithelial cells [31]. Paracellular influx of biotin is prevented in both wild-type and occludin-deficient epithelial cells. The morphology of the intestine, liver, and kidney in occludin-deficient mice is normal [32]. No dysfunction of the TJ barrier is observed in the intestinal epithelial cells of occludin-deficient mice. These findings indicate that occludin is not the only integral membrane protein component of TJs. Furuse et al. identified another integral membrane protein, claudin, by using occludin as a probe (Table 1) [33]. They isolated the junctional fraction containing occludin in chickens, removed the peripheral proteins, and obtained a fraction containing only integral membrane proteins by guanidine extraction. The isolated fraction was sonicated and fractionated by centrifugation in a stepwise discontinuous sucrose density gradient. The fraction containing occludin contained two novel integral membrane proteins, claudin-1 and -2, with no sequence homology with occludin. Claudin-1 and -2 are tetratransmembrane proteins with molecular masses of approximately 22-kDa. These proteins are directly incorporated into TJ strands [33]. Expression of claudins in L cells lacking TJs induced the formation of TJ networks and claudin polymerization in the plasma membrane [34]. These findings indicate that claudins are structural components of TJ seals. A genomic analysis revealed that claudins are a family containing at least 27 proteins [35–37].

3.2.2. Claudin and the TJ barrier

Interestingly, the expression profiles and barrier functions of claudins differ among tissues. Paracellulin-1 (identical to claudin-16), which is expressed in the thick ascending limb of the loop of Henle, controls paracellular magnesium ion transport [38]. Claudin-19 expression in Schwann cells may be involved in the electrophysiological sealing function of Schwann cells [39]. Claudin-1-deficient mice exhibit dysfunction of the epidermal barrier [40]. A solute with a molecular mass of approximately 600 Da appears to pass through TJs in the epidermis of these mice. These mice possess normal morphological and biochemical structures in the epidermis, and claudin-1 can be specifically removed from the TJs of stratified epithelial cells without affecting TJ morphology. These findings indicate that claudin-based TJ strands are crucial for the barrier function of mammalian skin but not for maintenance of epidermal morphology. The cornified cell envelope and lipid lamellae in the stratum corneum were assumed to be the only epidermal barriers before the identification of claudin-1. Identification of claudin-1 allowed researchers to confirm that both the stratum corneum and stratified epithelial cells in the skin are epidermal barriers [41]. Therefore, modulation of the claudin-1 barrier could act as a potent strategy for epidermal absorption of drugs. Claudin-5-deficient mice exhibit a dysfunctional blood–brain barrier [42]. Small molecules, with molecular masses of <800 Da, pass through the blood–brain barrier of these mice. The mice exhibit normal blood vessel morphology and no bleeding or edema. TJs are often composed of more than two distinct species of claudin; the removal of one claudin species markedly changes the TJ barrier without affecting its continuous structural integrity. This suggests the possibility of improving drug delivery to the central nervous system by modulating claudin-5 [42].

Clostridium perfringens enterotoxin (CPE) is a 35-kDa polypeptide that causes food poisoning in humans [43]. The C-terminus of CPE is involved in binding to target cells, whereas the N-terminus is responsible for the cytotoxicity [44]. A receptor for CPE (CPE-R) was cloned from an expression library of CPE-sensitive cells [45]. Rat

ventral prostate-1 (RVP-1) possesses a sequence and function similar to those of CPE-R [46]. Morita et al. revealed CPE-R and RVP-1 to be claudin-4 and claudin-3, respectively [37]. CPE is cytotoxic to claudin-3- and -4-positive cells, but this effect is lost if its N-terminus is removed. Treatment of cells with the C-terminal fragment of CPE, corresponding to amino acids 184–319 (C-CPE184) reduces transepithelial electrical resistance, which is a typical indicator of the barrier function of TJs. TJ barrier integrity recovers after the removal of C-CPE184. Treatment of cells with C-CPE184 decreases the level of claudin-4 protein [47]. Claudin-4 levels recover after the removal of C-CPE184. C-CPE184 treatment does not affect claudin-1 protein levels. Claudin contains the clathrin-sorting signal, and endocytosis of claudin occurs during the remodeling of TJ strands [48,49]. C-CPE184-bound claudin-3/-4 may be taken up by endocytosis; this is followed by degradation of claudins. Two possible mechanisms were proposed to explain C-CPE184-induced modulation of the TJ-barrier [47]. C-CPE184 may bind directly to claudin-3/-4 within TJ strands and then evoke depolymerization of TJ strands. Alternatively, C-CPE184 may bind to claudin-3/-4 in the non-junctional area, and this binding to claudin may suppress the polymerization of TJ strands. Furthermore, C-CPE184 increases the paracellular permeability of 4- and 10-kDa dextran in epithelial cell sheets. Inhibition of the claudin-based TJ-barrier by using claudin binders would provide a new means to modulate the TJ barrier and improve the bioavailability of drugs to target organs.

3.2.3. Claudin-targeted drug delivery

The efficacy of claudin binders as a novel strategy for mucosal absorption of drugs was demonstrated 6 years after the idea was first proposed (Table 2). C-CPE184 dose-dependently enhances intestinal absorption of 4-kDa dextran [50]. The absorption-enhancing activity is more than 400 times that of capric acid, an enhancer used clinically. A C-CPE184 mutant lacking the claudin-3/-4-binding domain does not exhibit absorption-enhancing activity. C-CPE184 enhances intestinal absorption of 4-, 10-, and 20-kDa dextran but not 40-kDa dextran. There is no mucosal injury in the intestines of C-CPE184-treated animals. Therefore, the use of a claudin-3/-4 binder is a novel method to enhance mucosal absorption. CPE also binds to claudin-6, -7, -8 and -14, and CPE interacts with the second extracellular loop of claudins [51]. Electrostatic interaction could be involved in the interaction between CPE and the second extracellular loop of claudins [52]. Claudin-7 and -8 are also expressed in the intestine, but any interaction between C-CPE184 and claudin-7 and/or -8 in the intestine remains unclear [53].

Recent progress in genomic and proteomic technologies has yielded some new biologics, including peptides, proteins, and nucleic acids, as pharmaceutical candidates. Most of these compounds are hydrophilic molecules that are poorly absorbed by the mucosa. Although injection is a compelling route for the administration of biologics, a transmucosal delivery system would be an ideal route because it is noninvasive and therefore would enable easy, pain-free administration by patients. C-CPE184 enhances nasal, but not jejunal and pulmonary, absorption of a peptide drug [54]. The solubility of C-CPE184 is <0.3 mg/ml. C-CPE194 is a derivative of C-CPE184 with a deletion of 10 N-terminal amino acids. C-CPE194 has greater solubility (10 mg/ml) than C-CPE184. C-CPE194 is also a claudin-3/-4 binder that enhances jejunal and pulmonary absorption of a peptide drug [54]. These findings support the idea, proposed by Sonoda et al. in 1999, that claudin binders may improve drug bioavailability. Modulation of the claudin barrier could be useful for development of non-invasive administration of biologics, most of which must be injected in patients.

3.2.4. Development of claudin binders

A series of studies using C-CPEs have revealed the possibility of developing claudin-targeted drug absorption [50,54]. The claudin family has at least 27 members. Several claudins are expressed in the duodenum, jejunum, ileum, and colon, and the expression profiles of claudins differ throughout the segments of the intestinal tract (see review in [53]). Development of claudin binders with broad and narrow specificity for claudins will be needed for the development of oral drug delivery systems based on modulation of the claudin barrier. However, claudins have four transmembrane domains, are hydrophobic, and have small extracellular loop domains. Therefore, preparation of recombinant proteins and antibodies to these extracellular domains is difficult. Only claudin-4 protein has been purified [55], and the development of specific claudin binders has been delayed. Claudin binders are classified into three categories: C-CPE derivatives, antibodies, and claudin peptides.

3.2.5. C-CPE derivatives

C-CPE184 is the first claudin binder and the only one discovered before 2005. C-CPE184 is a toxin fragment, and overcoming its antigenicity is critical for its clinical application [56]. Two approaches have been utilized to develop a claudin binder by using C-CPE184. One approach is the development of a binder by using C-CPE184 as a prototype. A deletion analysis revealed that the C-terminal segment, corresponding to 30 or 16 amino acids, is essential for the interaction of C-CPE184 with claudin-4 [57,58]. Synthetic peptides, corresponding to the 30 amino acids, bind to claudin-4 and modulate the TJ barrier in human intestinal cell lines [59]. Deletion of a portion of the N-terminal segment of C-CPE184 improves its solubility, and the improved C-CPE enhances mucosal absorption of a bioactive peptide drug [54]. Therefore, the deletion approach is one method of developing claudin binders, but there are no reports of a synthetic short peptide that can enhance mucosal absorption. Random mutagenesis of C-CPE184 is another approach. A site-directed mutagenesis analysis identified the functional amino acids of C-CPE184, and a C-CPE mutant library was formed by randomly mutating functional residues into other amino acid residues [60,61]. Recombinant claudin proteins are needed for the selection of claudin binders. It is generally difficult to obtain recombinant membrane proteins, including claudins, with an intact structure, and a protocol has been established only for recombinant claudin-4 [55]. Functional membrane proteins are heterogeneously expressed on the budded baculovirus, and interactions between membrane proteins can be detected by using receptor- and ligand-displaying budded baculoviruses [62–64]. Very recent studies suggest that claudins on budded baculovirus possess the native form and the claudin-displaying baculovirus functions as a screening system for claudin binders [60]. Indeed, claudin binders with broad specificity for claudins were isolated from the C-CPE mutant library by using the baculoviral display system, and the binders enhance mucosal absorption (our unpublished data). Chemical claudin binders are ideal for clinical application in terms of costs and antigenicity. Structural information about claudins and C-CPEs can provide the theoretical basis for claudin binders, but the three-dimensional structure of claudins has never been identified. An X-ray diffraction analysis revealed that C-CPE194 contains nine beta-sheets and one alpha helix and that the loop between beta-sheets 8 and 9 is a binding site of C-CPE194 to claudin-3 and -4 [65]. CPE interacts with the second loop of claudin-3 [51,66]. A series of analyses of the interaction between CPE and claudins indicated that the negatively charged cleft of C-CPE surrounded by Tyr306, Tyr310, Tyr312, and Leu315 might interact with the positively charged CPE-sensitive region [52]. Future determination of complex interactions between claudins and C-CPEs will facilitate the development of chemical claudin binders.

3.2.6. Antibodies and claudin peptides

The first antibodies to the extracellular domains of claudins were prepared by immunizing chickens with synthetic peptides corresponding to the extracellular domains [67]. Some of the polyclonal antibodies bound to claudin-3 and -4. Single-chain Fv fragments that bound to claudin-3 were isolated through selection from a human recombinant