Intestinal Epithelial Barrier Dysfunction in Disease and Possible Therapeutical Interventions

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Abstract: The intestinal epithelial monolayer constitutes a physical and functional barrier between the organism and the external environment. It regulates nutrients absorption, water and ion fluxes, and represents the first defensive barrier against toxins and enteric pathogens. Epithelial cells are linked together at the apical junctional complex by tight junctions that reduce the extracellular space and the passage of charge entities while forming a physical barrier to lipophilic molecules. Cultured intestinal epithelial cells have been extensively used to study intestinal absorption of newly synthesized drugs and the regulation of tight junctions structure and function. *In vitro* mild irritants, proinflammatory cytokines, toxins and pathogens, and adverse environmental conditions open tight junctions and increase paracellular permeability, an effect often accompanied by immune activation of the enterocytes. Conversely, inhibition of proinflammatory cytokines, exposure to growth factors and probiotics, among others, exert a protective effect. Impaired barrier function results from activation of signalling pathways that lead to alteration of junctional proteins expression and/or distribution. *In vivo*, intestinal barrier dysfunction is associated with various intestinal and non-intestinal disorders including inflammatory bowel disease, celiac disease, and diarrhoeal infection. This review will describe the current knowledge of the mechanisms regulating tight junctions and intestinal permeability, how these findings have lead to a better understanding of barrier alteration in human intestinal disorders, and what the emerging therapies to treat these pathologies are.

Keywords: Growth factors, inflammatory bowel disease, intestinal epithelial cells, myosin light chain kinase, NF-kB, probiotics, protease activated receptor-2, tight junction.

THE INTESTINAL EPITHELIAL BARRIER

The intestinal epithelium represents the initial formidable barrier between the external environment and the underlying host tissues. It is formed by a monolayer of differentiated epithelial cells linked together at the apical junctional complex (AJC) which establishes the cellular polarity and reduces the space between adjacent cells, therefore limiting access of pathogens, toxins and xenobiotics to host tissues and systemic circulation while supporting the growth of the commensal flora (Fig. **1**). While specific carrier proteins are responsible for the active or facilitated absorption process of nutrients and for regulating the transport of water and electrolytes, the majority of substances are able to cross the epithelial barrier essentially by diffusion either through the cell membranes *via* the transcellular route, in the case of lipophilic molecules, or through the intercellular space *via* the paracellular route, for small hydrophilic molecules. In physiological terms, the intercellular space is very restricted due to the presence of apical tight junctions (TJs) that make the paracellular route of transport negligible.

Structure of the Apical Junctional Complex

The AJC consists of two specialized multimolecular protein complexes, TJs and adherens junctions (AJs). TJ fibrils contained a complex of several integral and peripheral membrane proteins that interact strongly with the cytoskeleton [1-3]. Integral membrane proteins include occludin, junctional adhesion molecule (JAM), members of the claudin family of proteins, and tricellulin. Their extracellular loops provide the sites for intercellular interaction, while the cytoplasmic N- and C-terminal endings provide a binding site for the cytoplasmic plaque scaffolding proteins called Zonula occludens (ZO)-1, ZO-2, and ZO-3, whose role is to link the cytoplasmic component of the TJ to the actin-myosin cytoskeleton. Other components of the epithelial TJs include myosin IXB and zonulin, a releasable member of TJ protein that has been shown to increase paracellular permeability [4]. TJ are localized in membrane microdomains, detergent-insoluble glycolipid rafts with the role of facilitating recruitment of small signalling molecules implicated in TJs structure/organization.The main molecular component of the AJ is E-cadherin, a transmembrane protein that interacts extracellularly with its counterpart on adjacent cells by means of five extracellular domains and, intracellularly, with the cytoskeleton by means of β-catenin. AJC components, in particular the claudins, have either distinct or overlapping functions in regulating paracellular permeability. Some members of the claudin family, including claudin-1, claudin-4 and claudin-5, reduce paracellular diffusion by sealing neighbour cells leading to TJs strand continuity. Conversely, claudin-2 forms channels or pores selective for small cationic entities, thus contributing to epithelial leakiness and discontinuity. The characteristics of the epithelial TJ, in terms of resistance and paracellular permeability to both ionic species and non-charge molecules, will therefore be dictated by its composition. Changes in the relative expression, internalization and degradation of specific junctional proteins may lead to an increase in intestinal permeability and barrier defects in disease [2, 5]. Moreover, changes in the phosphorylation status of specific proteins, such as occludin or ZO-1, may affect their assembly to other components of the AJC and increase permeability. Activation and cross-talk of signalling pathways including protein kinase C (PKC), protein kinase A (PKA), phosphoinositide 3-kinase (PI3-kinase) and Rho signalling pathways influence epithelial barrier function [6]. Since the TJ protein complex is intimately related to the apical perijunctional actomyosin ring,

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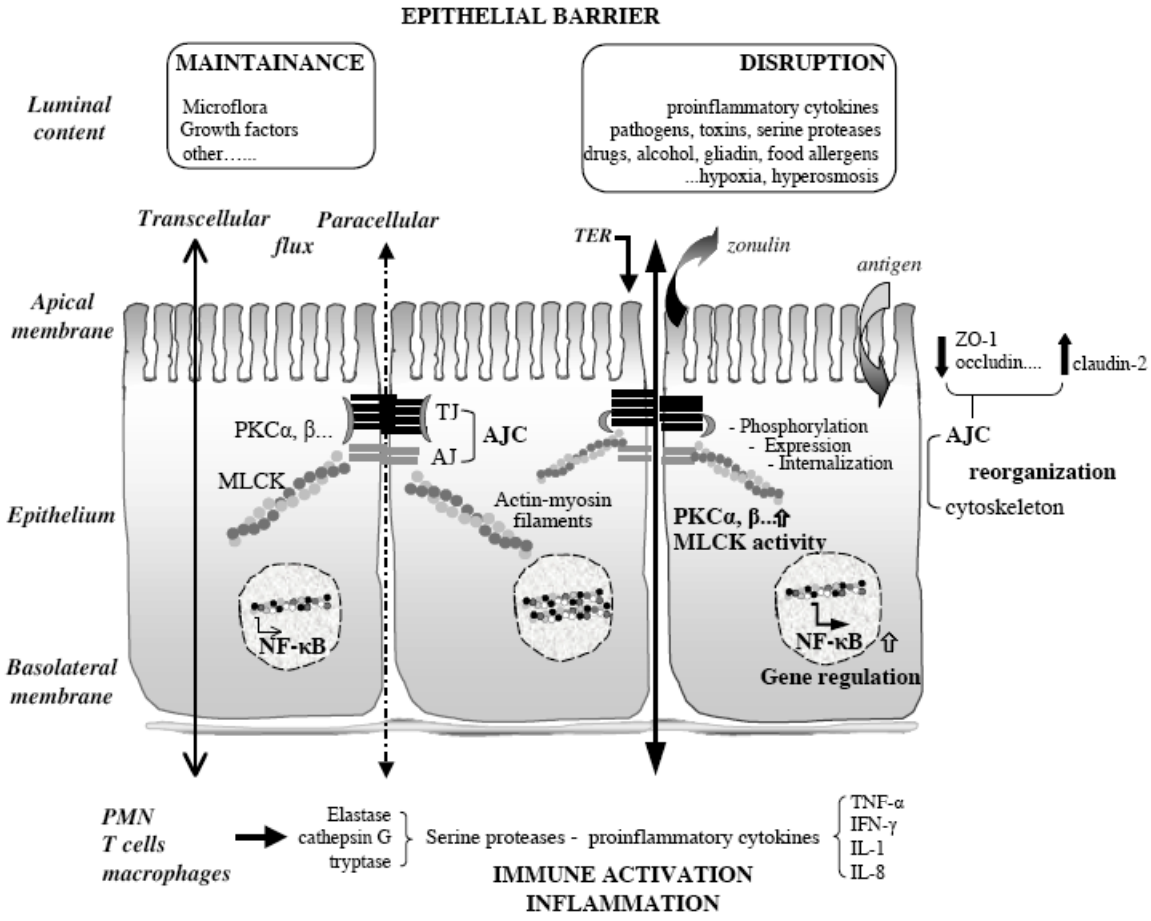


Fig. (1). The intestinal epithelial barrier.

The epithelial barrier consists of a monolayer of polarized differentiated cells tightly sealed by the AJC. Epithelial permeability is affected by both apical and basolateral factors, including proinflammatory cytokines, proteases, pathogens, food allergens. Modification of junctional protein expression and localization, and reorganization of the actin cytoskeleton lead to permeability increase and antigen uptake. Barrier dysfunction is common to several intestinal and non-intestinal inflammatory disorders and accompanied by activation of intracellular enzymes/pathways including MLCK and NF-κB.

initial alterations in either the cytoskeleton or specific TJ proteins produce structural and functional changes in the other. Indeed, MLC phosphorylation by myosin light chain kinase (MLCK) leads to contraction of myosin-based filaments and actin cytoskeleton reorganization, alteration of ZO-1 and occludin, but not claudin-1 and claudin-2, and distribution and size-selective increase in paracellular permeability to small molecules [7].

The AJC and epithelial permeability can be affected by a myriad of exogenous stimuli (e.g., bacterial attachment, bacterial toxins, and medications) and endogenous factors, particularly the neuro-endocrine and immune cells and their messenger molecules [8] (Fig. **1**). While some growth factors and nutrients are able to consolidate the intestinal barrier through increased expression of "sealing" TJ proteins, thus exerting a protective effect, viral and bacterial infections alter the gastrointestinal (GI) barrier through targeting TJs and provoke severe diarrhoea [9]. The Vibrio cholera enterotoxin, zonula occludens toxin (Zot), and its mammalian analogue zonulin, increase intestinal mucosal permeability by reversibly altering TJ structure in both the jejunum and the ileum [10].

Regulation of AJC by Endocytosis

The apical junctional complex is a dynamic structure undergoing intracellular trafficking in response to physiological and pathological stimuli. Junctional proteins are internalized through clathrin- and caveolar-mediated endocytosis, and macropinocytosis [11, 12]. Once internalized, junctional proteins may be degraded *via* late endosomes, or may return to the plasma membrane *via* recycling endosomes.

The consequences on intestinal barrier function depend on the number and nature of proteins affected by the endocytic process. During the normal turnover of AJC proteins, the internalization of individual proteins does not affect AJC integrity and function. By difference the selective internalization of TJ in response to proinflammatory cytokines, such as interferon(IFN)-γ and members of the tumour necrosis factor (TNF) family, pathogens and toxins cause paracellular permeability increase and barrier loss. Adverse environmental conditions like calcium depletion and oxidative stress cause clathrin mediated endocytosis of both adherens (E-cadherin) and TJ (claudin, occludin) proteins, leading to the loss of cell-cell contacts. Modification of cytoskeletal pro-

teins and contraction of the perijunctional actomyosin ring play a central role in the endocytic process of occludin and ZO-1 and alteration of the microfilaments is sufficient to trigger TJ protein internalization and affect epithelial permeability [12, 13]. Depending on the stimulus, either MLCK or Rho-associated kinase (ROCK) have been identified as intracellular signaling mediators of junctional proteins trafficking [12, 14]. Indeed, MLCK activation mediates TNF-induced endocytosis of occludin by caveolae-vesicles, both *in vitro* and *in vivo*. MLCK activity also controls the diffusion, from the TJ to the cytosol, of a slow exchange pool of ZO-1 proteins that is anchored to the actomyosin ring by an actin binding region [15]. The internalization of TJs by macropinocytosis and formation of vacuolar apical compartments in epithelial cells exposed to IFN-γ involves the Rho/ROCK signalling pathway and contraction of the actomyosin ring. Importantly, the three GTPases, Rho, Rac and Cdc42 play a critical role in the regulation of AJC assembly, maintenance and disassembly by influencing the cytoskeleton [16]. Distinct intracellular signalling pathways activate individual GTPases to regulate actin polymerization, contractility of the acto-myosin ring or both, and the status of both TJ and AL. Upstream activators of GTPases include PI3-kinase while downstream effectors include Rho-associated kinase. Finally, an integral membrane trypsin-like serine protease, matriptase, localized at the AJCs is necessary for the formation of AJC during epithelial differentiation and participates in their reassembly after barrier disruption [17]. The effect of matriptase is mediated by regulation of the turnover of intact claudin-2 through activation of an atypical PKCζ signalling pathway.

THE EPITHELIAL BARRIER IS COMPROMISED IN DISEASE

Decreased epithelial barrier function is a common denominator of a number of enteropathies, including inflammatory bowel disease (IBD) (Table **1**) [18]. Crohns disease (CD) and ulcerative colitis (UC), two chronic multifactorial inflammatory processes of the GI tract characterized by relapsing conditions, but also inflammatory bowel syndrome (IBS) and colitis, are linked to increased gut permeability and enhanced exposure of mucosa to luminal antigens. Perturbations in barrier function caused loss of fluid and electrolytes from the body (e.g., diarrhoea), bacteria translocation and transepithelial migration of neutrophils across the injured epithelium. Although the issue of whether the increase in epithelial permeability is a cause or a consequence of the disease is still matter of debate, its pathophysiological significance is well accepted since abnormal delivery of luminal antigens to the mucosal immune system and infiltration of inflammatory cells will amplify and perpetuate the host defence response, leading to chronicity of the intestinal barrier dysfunction and increasing disease severity.

Altered AJCs and Increased Permeability in IBD

CD and UC are both characterized by immune activation and inflammation that compromise GI function but are associated with different patterns of immunoregulatory cytokine production. In CD, a strong Th1 T cell–mediated immune response is induced, that includes an increased production of the key proinflammatory cytokine TNF-α, whereas in UC, the pattern of immune cell activation is more complex with an atypical Th2-mediated response and upregulation of IL-13 secretion.

UC patients have shown a drastic decrease in colonic epithelial electrical resistance, a concomitant increase in mucosal-to-serosal mannitol flux and a decreased number of TJ strands in the inflamed UC tissues [19]. The overall colonic resistance is in part compensated by a concomitant increase in subepithelial electrical resistance due to mucosal oedema, inflammatory cell infiltration, and increase in thickness of the muscularis mucosae. An altered intestinal permeability as indicated by reduction in epithelial resistance and abnormal passage of different marker substances has been observed in CD patients and in some of their healthy relatives. As observed in UC, increased epithelial permeability was associated with reduced and discontinuous TJ strands and was paralleled by an increase in subepithelial resistance [20]. Measurement of the increased intestinal permeability *in vivo* with the lactulose/mannitol test was useful for monitoring and identifying patients in apparent remission of disease but with a high risk of relapse [21]. In one case report, epithelial barrier dysfunction as evidenced by increased permeability to ^{51}Cr-EDTA, was documented 8 years before the diagnosis of CD in a subject at increased risk [22]. Decreased intestinal permeability was predictive of induction of remission in active CD with corticosteroid medication, and inversely, patients who subsequently relapsed displayed increased intestinal permeability [23]. Small intestinal permeability could

Table 1. Increased Intestinal Permeability Associated to Human Diseases/States

Intestinal Disorders	Non-Intestinal Disorders
Inflammatory Bowel Disease, CD & UC	Alcoholic liver disease
Irritable bowel syndrome	Nonalcoholic liver disease
Collagenous colitis	Total parenteral nutrition
Intestinal ischemia	Type 1 diabete
Chemotherapy-induced mucositis	Acute pancreatitis
NSAID-induced enteropathy	Chronic heart failure
Intestinal infections, sepsis	Emotional stress
Celiac disease and food allergy	Multiple organ dysfunction syndrome

also predict a more relapsing disease in children with UC [24]. IBD relapse was associated with a reduction of claudin-3 immunostaining at the colonic TJs and concomitant increased claudin-3 urine levels [25]. An impaired barrier function is present long before the onset of disease and has been demonstrated in the absence of intestinal inflammation.

Both histochemical techniques and expression studies have shown that the composition of the AJC and the localization of individual components are affected in IBD and account for morphological and functional changes of the epithelial barrier. In pouchitis, epithelial resistance was markedly reduced compared to non-inflamed pouch and control ileum, the result of decreased expression of claudin-1 as opposed to a specular increase of claudin-2 [26]. The TJ proteins occludin and claudin-3/4/5/7 remained unchanged, as well as ZO-1, indicating no general perturbation of TJ integrity. A marked downregulation of several junctional proteins, including occludin, ZO-1, E-cadherin, and their respective mRNAs, was observed in the mucosa of actively inflamed CD or UC patients as compared to that of normal subjects or non-inflamed CD and UC epithelium, while β-catenin was decreased in UC only [27, 28]. Expression of junctional proteins was also reduced in inactively inflamed UC tissues, although to a lesser extent [27]. Downregulation of occludin expression in IBD was observed not only in inflamed mucosa where it may contribute to polymorphonuclear neutrophil (PMN) transepithelial migration but also in quiescent adjacent areas [28]. Non-inflamed ileal mucosa from CD patients was more reactive to the permeation enhancing effect of luminal sodium caprate as a result of a more pronounced disassembly of perijunctional F-actin and the presence of dilatations within the TJs [29]. Conversely, upregulation of claudin-18 was reported in patients with UC that did not however correlate to the histological severity of the disease, indicating that it could be a primary index of barrier dysfunction [30]. No change in occludin, ZO-1, claudin-1, JAM, β-catenin, and E-cadherin protein expression was found in colonic epithelium from patients with collagenous colitis, while other authors reported a decrease in occludin and claudin-4 expression without modification of claudin-1/3/5 and enhanced intestinal permeability that corresponded to the degree of the diarrhoea [31]. TJs proteins alteration was associated with a significant decrease in epithelial resistance but not with mucosal surface alterations or increased apoptosis rate while thickening of collagenous fibres produced an increase in subepithelial resistance.

Moreover, several studies reported that, in contrast to sealing claudins, the pore-forming claudin-2 was upregulated in human UC and CD, whereas absence or weak expression of claudin-2 have been documented in normal colonic epithelium. Overexpression of claudin-2 protein and mRNA was observed in the colonic crypt epithelium in CD and in both active and quiescent UC, while the expression of claudin-3 and –4 was poorly affected [32]. The increase in claudin-2 expression correlated with the severity of inflammation in UC. Increased permeability and upregulation of claudin 2 have been associated with downregulation and redistribution of occludin, claudin-5 and claudin-8 in patients with active CD [20] and with down-regulation of claudin-4 and claudin-7, protein and mRNA levels in active UC [33], in contrast to other claudins that were unchanged. Increased epithelial apoptosis index further contributed to impaired barrier function in both active UC and CD while TJ proteins and apoptosis were unaffected in CD remission. Elevated expression of both claudin-2 and claudin-1 was found in active IBD, adenomas, and IBD-associated dysplasia [34]. Claudin-4 expression was also enhanced in active IBD but only that of claudin-2 and claudin-1 correlated with the degree of inflammation. These data suggest a potential association between increased claudin-1 and claudin-2 expression and an increased risk of developing intestinal cancer at sites of chronic inflammation. Overall data show that down-regulation and/or upregulation of specific junctional proteins or subsets of proteins in IBD patients may account for changes in TJs architecture and impaired intestinal barrier function.

Genetic Origin of Barrier Defect in IBD

Recent studies indicate that a genetic defect may be the cause of enhanced intestinal permeability. MYO9B (Myosin IXB), PARD3 and MAGI2 genes have been analysed to link genetic polymorphisms and predisposition to disease. MYO9B is a motor protein containing a Rho-GTPase-activating domain that affects TJ assembly and paracellular permeability through remodelling the actin cytoskeleton while PARD3 and MAGI2 genes encode adaptor scaffolding proteins. Several investigations showed a significant association between single nucleotide polymorphisms in MYO9B and CD and UC alone, and IBD overall, although no clear correlation with abnormality of intestinal permeability was observed [35, 36]. MYO9B polymorphisms have been also associated with celiac disease and type 1 diabetes susceptibility. Both PARD3 and MAGI2 are genetically associated with celiac disease and a correlation between MAGI2 variants and IBD has been reported [37, 38]. These epidemiological findings support the idea that a common genetic background and intestinal epithelial barrier defect play a primary role in the pathogenesis of GI inflammatory disorders although other factors, including immune predisposition and environmental conditions, are of great importance.

Altered Luminal Content Contributes to Barrier Defect in IBD

In addition to abnormal junctional proteins expression and intestinal permeability, alteration of luminal and mucosal content are also common features of intestinal inflammatory diseases. CD and UC are associated with a high proportion of invasive colonic *Escherichia coli* (*E. coli*) strains capable of altering cytokine expression and barrier function, two pathological features of IBD [39, 40]. *E. coli* strains present in the inflamed mucosa of IBD patients displayed an exaggerated capacity to induce epithelial NF-kB activation and invade intestinal epithelial cells, although typical virulence genes for known pathogenic strains were not detected in these cells [40]. They induced TNF-α and interleukin (IL)-8 release from macrophages and epithelial cells respectively, and decreased transepithelial resistance (TER) that resulted from F-actin rearrangement, and displacement of ZO-1 and E-cadherin from the apical junctional complex [39]. Soluble mediators present in the intestinal lumen of IBS patients increased paracellular permeability *in vitro* as assessed by a

marked reduction of TER and a significant increased in dextran flux in differentiated intestinal epithelial cells [41]. Lower expression of ZO-1, but not occludin, was observed both *in vitro* and in colonic biopsies of IBS patients where it correlated with the severity of abdominal pain. Luminal serine protease activity increased in patients with diarrhoea-predominant IBS, UC and acute infectious diarrhoea [42, 43]. In IBS increased protease activity was not due to either enhanced pancreatic or inflammatory cell secretion. When applied to mice colonic mucosa, luminal content from IBS patients evoked visceral sensitivity to colorectal distension and increased paracellular permeability through epithelial MLC phosphorylation and ZO-1 redistribution, which were dependent on protease-activated receptor(PAR)-2 activation [42]. In UC and acute infectious diarrhoea, luminal PMN-elastase was increased as a result of inflammation and cell infiltration. Luminal cathepsin G activity also increased as a result of inflammatory cells recruitment and higher levels of cathepsin G and PAR-4 expression are found in colon biopsies of UC patients [43]. Luminal content from UC patients increased colonic paracellular permeability and induced inflammation in mice that was mediated by cathepsin G and activation of PAR-4 receptor.

The Epithelial Barrier in Animal Models of Colitis

The link between alterations of the intestinal barrier function, inflammation and development of GI disorders has been studied in animals which were exposed to various chemicals [44-47] or genetically modified [48-52] and in particular strains or conditions [53-57] (Table **2**). The SAMP1/YitFc mouse strain, a spontaneous model of IBD, developed earlier barrier dysfunction before the onset of inflammation, as shown by an evaluation of mucosal infiltration of inflammatory cells and epithelial injury, which correlated to an increase in claudin-2 in the ileum and a decrease in occludin in the colon and ileum [58]. In the dextran sodium sulfate (DSS) model of colitis, loss of ZO-1 and increased intestinal permeability preceded the development of significant inflammation and were not associated with apoptosis [59]. In JAM-A deficient mice, decreased TER and increased paracellular permeability resulted from induction of claudin-10 and –15 expression but not claudin-1/2/3 or occludin [49, 60]. Barrier dysfunction is not linked to inflammation and apoptosis in this model, where epithelial architecture and cytokine/chemokine levels are preserved. However, JAM-A knockout-mice display enhanced susceptibility to DSS-induced colitis with exacerbated production of colonic and serum inflammatory cytokines and chemokines, enhanced intestinal permeability and induction of apoptosis [60]. Disruption of barrier function in the absence of TJ disorganization is observed in transgenic mice expressing constitutively active MLCK within intestinal epithelium that resulted in mucosal immune cell recruitment and activation, and enhanced susceptibility to immune mediated colitis [50]. In the rat model of NSAID-induced enteropathy, both a preventive and curative administration of anti-TNFα antibody reduced the severity of the disease and intestinal permeability, indicating that the inflammatory process contributed to barrier dysfunction [61]. In this model, enhanced transcellular permeability coincided with intestinal inflammation and bacterial translocation, indicating mucosal damage while epithelial paracellular permeability in the jejunum and ileum were sustained during the quiescent phase of the disease. This suggests that a defect in the TJ barrier is responsible for chronicity [62].

Inflammation and TNF-α are also involved in barrier dysfunction in the trinitrobenzene sulfonic acid **(**TNBS) model of colitis. Indeed, induction of colitis is followed by early increase in circulating TNF-α levels and a rapid alteration of the TJs complex with downregulation of occludin and ZO-1 expression and induction of claudin-2 in the ileum [63]. Although these effects preceded macroscopic damage, anti-TNF-α treatment or genetic deletion of TNF-α receptor (TNFR) type 1 not only reduced macroscopic damage score

Table 2. Animal Models of Increased Intestinal Permeability

Chemically-Induced Bowel Disease	
- TNBS	hapten-induced chronic colitis similar to CD [44]
- oxazolone	hapten-induced chronic colitis similar to UC [45]
- DSS	epithelia injury related acute colitis [46]
- indomethacin	chronic recurrent intestinal inflammation [47]
Genetic Models	
- IL10 KO mice	chronic colitis similar to CD [48]
- JAM-A KO mice	increased susceptibility to DSS-induced colitis [49]
- epithelial MLCK TG mice	predisposition to immune-related colitis [50]
- MDR1 KO mice	spontaneous colitis development [51]
- epithelial NF-kB repressor TG mice	resistance to immune-mediated diarrhea [52]
Other Disease/States	
- SAMP1/YitFc mouse strain, spontaneous model of CD [53]	
- BB diabetic rats [54]	- *ob*/*ob* and *db*/*db* obese mice [55]
- total parenteral nutrition [56]	- acute and chronic stress [57]

but also counteracted TJ disruption. The multidrug resistance protein 1-deficient ($Mdr1a^{-/-}$) mice lacking the intestinal transporter P-glycoprotein developed intestinal barrier dysfunction related to increased colonic ion secretion, increased intestinal permeability, and decreased phosphorylation of TJ proteins, occludin and ZO-1, before the onset of diarrhoea [51]. However, altered gene expression, especially an increase in IFN-γ gene expression, higher basal chemokine secretion and increased responsiveness to lipopolysaccharide (LPS) were observed in mice with normal colonic structure and no signs of inflammation while colonic permeability increased later when signs of inflammation became evident [64]. These data showed that increased permeability is an early event not necessarily linked to inflammation and clinical signs of disease but immune activation may contribute to barrier dysfunction. Although epithelial paracellular permeability per se, in the absence of drastic alteration of the TJs, is not sufficient to induce colitis, it contributes to the pathogenesis and progression of the disease.

Epithelial Barrier Dysfunction in Celiac Disease and Diabetes

Epithelial barrier function is compromised in a variety of other intestinal and non-intestinal disorders including bacterial and viral infections, alcoholic and nonalcoholic liver disease, as well as celiac disease and type 1 diabetes (T1D), two models of immune-mediated small intestinal inflammation and damage (Tables **1**, **2**).

Celiac Disease

Celiac disease is a chronic disease triggered and maintained by gluten proteins from wheat and related cereals, and manifests mainly as inflammation, villous atrophy and crypt hyperplasia in the small intestine. It concerns genetically predisposed individuals characterized by the presence of the HLA class II molecules, HLA-DQ2 or HLA-DQ8, in antigen-presenting cells and the presence of circulating immunoglobulin (Ig)A auto-antibodies to transglutaminase, an ubiquitous enzyme associated with the extracellular matrix of the gut mucosa [65**]**. Deamidation by transglutaminase of gluten peptides increases their immunoreactivity and binding affinity to HLA class II molecules, thus generating a strong activation of $CD4^+$ T cells that secrete Th1 cytokines. Gluten peptides that traverse the intestinal epithelium also elicit an adaptive immune response and the production of Th2 cytokines and interleukin(IL)-15. Patients display various degrees of intestinal inflammation and clinical manifestations range from completely asymptomatic to global malabsorption. Celiac disease is frequently found in conjunction with (other) autoimmune diseases and if untreated, with an increased risk of developing cancer of the gastrointestinal tract. In celiac disease subjects without comorbidity, withdrawal of gluten from the diet is usually sufficient for reversing clinical manifestations of the disease, healing the damaged small intestinal mucosa and improving nutrient absorption.

In active symptomatic celiac disease, permeability is elevated in the gastroduodenum and small intestine as a result of an altered TJs ultrastructure but normalized by a long-term gluten-free diet. Moreover, zonulin expression was raised in intestinal tissues during the acute phase of celiac disease, a clinical condition in which TJs are opened and permeability is increased [10, 66]. Immunofluorescence studies showed a global under-expression of ZO-1 protein and messenger in the duodenal mucosa of active celiac disease patients associated with F-actin disorganization [67]. Gluten withdrawal from the diet allowed normalization of the ZO-1 expression in treated celiac disease patients suggesting that the regulatory mechanisms involved are fully reversible. Conversely, a more recent study showed that the molecular rearrangement of TJs and AJs proteins was consecutive to inappropriate levels of tyrosine phosphorylation and no difference in expression of ZO-1, β-catenin, occludin and-E-cadherin was found in the duodenum of patients with untreated celiac disease [68]. Drastic reductions of ZO-1 phosphorylation and excessive phosphorylation of β-catenin impaired the interaction of these proteins with their natural partners, occludin and E-cadherin, respectively, leading to disruption of AJCs and redistribution of components in cytoplasmic pools. Alterations of the ZO-1 and β-catenin phosphorylation status as well as AJC structure are reversed with a gluten-free diet.

Diabetes

Enhanced intestinal permeability, along with metabolic endotoxaemia and low-grade inflammation, are also a characteristic of obese and diabetic mice [54, 55]. A marked increase in small intestinal permeability is apparent in a model of spontaneous autoimmune diabetes, the Bio-breeding diabetic prone rat, before histological evidence of pancreatic islet destruction or overt manifestations of diabetes [54]. In the non-obese diabetic (NOD) mouse, intestinal permeability is also increased in the pre-diabetic state and increasing intestinal barrier permeability of the large intestine due to infection from enteric bacterial pathogens accelerates the development of insulitis, a necessary step for the onset of the diabetic state [69]. Two genetic models of obesity, *ob*/*ob* and *db*/*db* mice showed a significant increase in intestinal permeability, strong redistribution and decreased expression of the TJ proteins, ZO-1 and occludin, in the small intestine [55, 70]. A relationship was found between intestinal paracellular permeability, the development of systemic endotoxaemia and inflammation, and the exacerbated inflammatory response of hepatic stellate cells to bacteria endotoxins, oxidative stress and macrophage infiltration resulting in liver damage. In humans, altered intestinal permeability occurs in T1D before general manifestation of disease since subjects with islet autoimmunity showed an increase in intestinal permeability, irrespective of the disease status, which is still evident in the preclinical state [71]. A large subgroup (40%) of T1D patients showed higher serum zonulin levels and higher intestinal permeability than their relatives and control subjects [72]. A good correlation was found between serum zonulin levels and the increase in intestinal permeability, although no significant changes in gene expression were detected for claudin-1 and 2, myosin IX, occludin and ZO-1. Results from a retrospective pilot study showed that in most cases an increase in serum zonulin level precedes the onset of the disease. Similarly, Bio-breeding diabetic prone rats, but not their diabetes-resistant counterpart, showed an increase in both intraluminal and serum zonulin levels, paralleled by an increase in intestinal permeability in both the

jejunum and ileum, before clinical evidence of the disease including a significant increase in glycaemia [73]. Inhibition of zonulin prevented both the decrease in TER and the evolution of the disease indicating that zonulin mediated disruption of intestinal barrier integrity plays a central role in T1D pathogenesis in this model.

Increased Antigen Uptake in IBD and Celiac Disease

Disruption of AJCs affects active/facilitated transepithelial transport of ions and nutrients in both absorption and efflux direction since these processes require concentration gradients that are disturbed in the presence of a leaky epithelium. Most importantly, new findings show that AJC disruption and increased paracellular permeability in inflammatory diseases are associated with global impairment of the intestinal barrier function.

Increased permeability to macromolecules, bacterial components or products, have also been demonstrated in IBD and celiac disease [74]. Studies using the proteins ovalbumin and horseradish peroxidase (HRP) as markers of transcellular transport indicated that antigen uptake at the apical membrane *via* endocytosis and transcytosis is increased in these pathologies. Indeed the transport of HRP into endocytic compartments of enterocytes is increased in ileum tissue of CD relative to control, and correlates with the degree of TNF-α expression [75]. Antigen proteins preferentially accumulate in atypical less differentiated enterocytes characterized by lower sucrase isomaltase (SI) activity at the brush border membrane (BBM) and a reduced amount of apical villin and actin [76]. The number of these immature cells and the capacity to take up the antigen increase with the degree of mucosal inflammation in CD and UC. Similarly, the contact of intestinal epithelial cells with gluten peptides alters BBM-associated actin cytoskeleton and intracellular trafficking, leading to a drop in SI levels and activity in the BBM, while the cholesterol and sphingolipid content increases [77]. Both observations may explain carbohydrate malabsorption in CD and celiac disease. In CD and UC, antigens are initially carried in apical early endosomes before being delivered into multivesicular late endosomes where they become complexed to MHC I and MHC II molecules. Antigens-MHC complexes are exposed on the basolateral membranes of enterocytes or released by exosomes to initiate and propagate the immune response [78]. In celiac disease, gliadin peptides with different toxicity/immunogenecity follow separate endocytic pathways, since peptides AA56-68 and AA229-246, but not gliadin peptide AA31-49, are found in HLA-DR-positive late endosomes [79]. Consequently antigen delivery and presentation on the basolateral membrane and immune response activation will be different for these peptides. Indeed, gliadin peptides AA31-49 and a 33-mer (AA56-88) which are toxic/immunogenic in celiac disease patients, have been detected in endosomal compartments expressing CD71, the transferrin receptor, to which they bind by means of secretory IgA [80, 81]. Abnormal expression of the CD71 receptor at the apical membrane of enterocytes may be responsible for alternative endocytic pathway of some gliadin peptides in patients with active celiac disease.

Both acute and chronic stress cause an increase in both paracellular and transcellular permeability in the small and large intestine [57]. Chronic stress resulted in increased ion secretion and impairment of glucose absorption, enhanced adhesion and translocation of bacteria into the mucosa and infiltration of immune cells that cause inflammation. Antigen uptake has a key role in activating the immune system. However, the contribution of the transcellular pathway to the overall epithelial barrier dysfunction in IBD, celiac disease or stress that probably depends on the disease stage or the grade of inflammation, has to be further investigated to delineate a possible therapeutic target.

NUMEROUS FACTORS INCREASE INTESTINAL PERMEABILITY

Investigating the events underlying intestinal epithelial barrier dysfunction may help to define the mechanisms of protection and delineate potentially more effective treatment regimens for those disorders associated with intestinal hyperpermeability.

Proinflammatory Cytokines

The mucosal immune system and proinflammatory mediators such as IFN-γ, TNF-α, IL-1, and IL-8, not only play a central role in intestinal inflammation and injury but also produced important alterations in barrier function [82, 83]. IFN-γ induced a reversible decreased in TER in intestinal epithelial cells that is accompanied by a drastic reduction of ZO-1 expression levels and perturbation of actin cytoskeleton [84]. Membrane-associated phosphorylated occludin is decreased, leading to changes in subcellular localization, from the TJs to diffuse distribution in the cytoplasm [84, 85]. Barrier dysfunction was also associated with internalization of claudin-1 and redistribution of AJC proteins, E-cadherin and JAM-1, from "raft-like" membrane microdomains into soluble fraction [86]. The decrease in TER is paralleled by the selective increased permeability to large molecules allowing the passage of *E. coli* derived lipopolysaccharide [85]. TNF-α also caused a significant decrease in the epithelial resistance and increased permeability of the paracellular markers, mannitol and inulin, without affecting transcellular resistance, in colonic epithelial cells. In Caco-2 cells it was attributable to downregulation and discontinuous junctional localization of ZO-1 protein while in colonic HT-29/B6 cells, enhancement of claudin-2 gene expression has been reported [87, 88]. Moreover, epithelial barrier dysfunction can be induced *in vitro* by synergistic signalling between IFN-γ and TNF-α as observed in T84 cells where TNF-α potentiated the effect of IFN-γ on TER and paracellular permeability [86]. TNF-α and IFN-γ synergistically disrupted the structure of TJs by changing lipid composition in membrane microdomains, a modification that led to exclusion and internalization of occludin [89]. Although proapoptotic effects could be evidenced for both cytokines, inhibition of apoptosis by the caspase inhibitor ZVAD-fmk did not prevent the increased paracellular permeability induced by both cytokines, suggesting that apoptosis was not the mechanism involved in proinflammatory cytokines-induced TJ dysfunction [86, 89]. IFN-γ sensitized Caco-2 monolayers to low concentrations of TNF-α-induced decrease in TER and increase in paracellular permeability to 3kD fluorescein isothiocyanate-dextran [90, 91]. Even though both TNFR1 and

TNFR2 were upregulated in Caco-2 monolayers, only TNFR2 expression mediated IFN-γ synergistic effect on barrier dysfunction. IFN-γ and TNF-α caused redistribution of TJ proteins (ZO-1, occludin, and claudin-1) with decreased and irregular immunofluorescence staining at the membrane and increased intracellular pools of occludin and claudin-1 [90]. Overall epithelial integrity was not compromised and cytokines-induced paracellular permeability was apoptosis-independent at low but not high concentrations [87, 91]. Similarly LIGHT- and IL-1β-induced increase in Caco-2 TJ permeability was not due to induction of cell apoptosis or cell death. For IL-1β it resulted from the progressive down-regulation in occludin and upregulation in claudin-1 expression, while ZO-1 protein remained unaffected [92]. For LIGHT, barrier dysfunction was accompanied by ZO-1 disruption and internalization of TJ proteins, occludin and claudin-1, through caveolar endocytosis, and was dependent on IFN-γ pretreatment to induce lymphotoxin β receptor expression [93]. Proinflammatory cytokines are able to increase paracellular permeability, an effect that was independent from their proapoptotic properties and involved a global rearrangement of AJCs since the localization, phosphorylation state and expression of junctional proteins are modified. Moreover, a synergistic effect to reduce barrier function between TNF-α and IFN-γ has been observed.

Various Pathogenic Bacteria Strains Disrupt Epithelial Barrier *In Vitro* and *In Vivo*

Understanding how the interaction between host and pathogen regulates the TJ barrier is an important step in understanding intestinal diseases and the effect of intestinal infection by pathogenic *E. coli* strains has been extensively studied. Enteropathogenic *Escherichia coli* (EPEC) infection of T84 epithelial cells caused reversible dephosphorylation and dissociation of occludin from the TJ and a significant drop in TER [94]. At a molecular level, EPEC infection progressively disrupted the interactions between ZO-1, occludin and claudin-1, allowing changes in TJ proteins localization and ultrastructure [95]. These events are mediated by membrane translocation and activation of protein kinase C(PKC)-ζ [96]. In Caco-2 cells, EPEC-induced an increase in permeability that involved PKC-α phosphorylation and translocation to the membrane. Activated PKC-α associated with cadherins and caused the phosphorylation and dissociation of the cadherin/β-catenin complex and redistribution of β-catenin into the cytoplasm [97]. EPEC also activated the extracellular signal-regulated kinase (ERK) pathway to increase the phosphorylation and degradation of Inhibitory kappa B(IκBα), an important endogenous inhibitor of NF-kB, inducing IL-8 expression [98]. EPEC altered epithelial barrier function independently of host cell death or apoptosis since pharmacological prevention of apoptosis did not impair TER reduction. Moreover, a mutant EPEC deprived of its proapoptotic potential is still able to disrupt TJ occludin and increase permeability in human intestinal epithelial cells [99]. A recent study showed that EPEC penetrated into the host cell at the TJ through membrane microdomains as revealed by co-localization of EPEC with flotillin-1, a known lipid raft protein, and ZO-1 [100]. EPEC then produced the internalization of occludin, but not claudins from TJ membrane microdomains into the cytosolic compartment, and the progressive relocalization of both occludin and ZO-1 along the lateral membrane. Other *E. coli* strains have been reported to impair epithelial barrier function and to increase IL-8 secretion. Enteroinvasive *E. coli* (EIEC) strains caused F-actin rearrangement, downregulation and displacement of claudin-1, occludin, JAM-1, ZO-1 proteins and E-cadherin from the AJC [39, 101]. Enterohemorrhagic *Escherichia coli* (EHEC) altered the distribution of the cytoskeletal protein alpha-actinin and induced disruption of the junctional proteins, claudin-1, ZO-1 and claudin-4, while a non-pathogenic translocating *E. coli* provoked the internalization of the TJ protein claudin-1, but not that of ZO-1 or claudin-4 [102, 103]. Both PKC and mitogen-activated protein kinase (MAPK)-ERK signaling pathways, MLCK and MLC phosphorylation mediate EHEC-induced changes in T84 monolayers resistance. Also commensal *E. coli* caused significant increases in both paracellular and transcellular permeability, bacteria translocation and proinflammatory cytokine expression in intestinal epithelial cells previously exposed to dinitrophenol, an inducer of metabolic stress [104]. MAPK-ERK and nuclear transcription factor-kappa B (NF-κB) activation are involved in the inflammatory response and *E. coli* translocation but not the barrier defect, that was accentuated by the presence of TNF-α [105].

The capacity to trigger both barrier dysfunction and proinflammatory responses is not restricted to *E. coli* strains. *Campylobacter jejuni*-infection caused dephosphorylation and internalization of occludin accompanied by reorganization of lipid raft-associated claudin-1 and JAM-1 with increased and decreased levels respectively [106, 107]. Increased permeability was associated with enhanced NF-κB-dependent IL-8 secretion. Moreover *C. jejuni* exerted a synergistic effect on IFN-γ-induced rearrangement of occludin and F-actin cytoskeleton, and bacterial translocation that correlated with increased apoptotic index, indicating widespread breakdown of the epithelial barrier *via* by both junctional rearrangement and loss of cells [108]. In the case of *Clostridium difficile* toxin A, decreased TER and increased paracellular permeability are mediated by rapid ZO-1 translocation from TJ to the cytosolic compartment that was followed by extensive disorganization of F-actin and TJ disruption, and cell rounding. These effects were mediated by activation of phospholipase(PLC)γ and PKC signalling, mostly PKCα/β isoforms, while blockade of MAPK/ERK pathway and MLCK have no effect on toxin A-increased permeability [109]. The effect of toxin on permeability was moderate and reversible at low doses, while it became rapid and irreversible at high doses and associated to the secretion of the proinflammatory IL-8. The differential capacity of toxin A to disrupt barrier function was due to the dose-dependent biphasic production of TGF-β1, a protective factor that facilitated epithelial barrier recovery [110]. Overall data show that pathogenic bacteria activate distinct intracellular signalling pathways to affect junctional proteins regulation and inflammatory response. In the case of *Shigella flexneri*, the interaction between effector proteins and a single host cell signalling molecule, the RhoA specific guanine nucleotide exchange factor, GEF-H1, influences both actin cytoskeleton reorganization and immune response. GEF-H1 normaly interacts with the plaque protein cingulin to regulate paracellular permeability. Upon infection, GEF-

H1 is recruted by the *Shigella* effectors to mediate ROCK activation and NF-kB-induced gene expression through the nucleotide binding and oligomerization domain (NOD)-like receptors signalling pathway [111].

The effect of pathogens on barrier function has been also studied *in vivo*. Infection of C57BL/6J mice by EPEC is followed by a rapid decrease in both ileal and colonic TER and redistribution of occludin to a cytoplasmic pool [112]. Epithelial barrier breakdown was due to a direct effect of EPEC on TJ, while in the long term period it also involved immune activation and the release of inflammatory mediators, such as mucosal TNF-α. Redistribution of claudins-1/3/4/5/8 within the cytoplasm and upregulation of claudin-2 were observed in mice colon during polymicrobial sepsis [113]. Occludin and claudin displacement from lipid rafts to detergent soluble membrane microdomains explained disruption of the TJ structure and increased intestinal permeability. LPS themselves increased the colonic paracellular permeability, promoting bacterial translocation and neutrophils recruitment in the colonic tissues [114]. Moreover, LPS enhanced the luminal content in serine protease activity that also demonstrated the capacity to impair barrier integrity.

Gliadin Peptides and the Role of Zonulin

Gluten peptides that are responsible for gluten sensitive enteropathy, celiac disease, reach the lamina propria, *via* either vesicular transport or an increased epithelial tight junctional permeability. *In vitro* studies with human epithelial cells have shown that gliadin peptides caused a time-dependent, reversible increase in intracellular actin polymerisation and redistribution to the cell subcortical compartment as stress fibres leading to cell rounding [115]. Gliadin led to a significant reduction in transmural resistance when added to the luminal side of mice and rabbit intestinal segments mounted in Ussing chambers, and *in vivo*, it enhanced both gastroduodenal and small intestinal permeability tested using specific sugar probes (sucrose and lactulose/ mannitol, respectively) [115, 116]. The effect of gliadin on the cell cytoskeleton is PKC mediated and associated with zonulin release into the extracellular medium. Human zonulin is a ≈ 47 kDa protein greatly expressed in the adult human intestine and, like its homologue zonula occludens toxin, interacts with a specific intestinal epithelial surface receptor, and regulates TJ permeability [10]. Human zonulin has been recently identified as the precursor for haptoglobin-2 heterodimeric plasma glycoproteins, and its sequence includes an epidermal growth factor (EGF) motif necessary for EGF-like activity [116]. Zonulin reduced TER in both monkey jejunum and ileum, but not in the colon, as expected from receptor distribution within the intestine. A comparison of the amino acids sequence in the active Zot fragment and zonulin permitted identification of an N-terminal region determinant for receptor binding and activation to increase paracellular permeability. Gliadin-induced barrier dysfunction, actin polymerisation and reduction of epithelial resistance, but not zonulin release, were prevented by a zonulin antagonist. Gliadin also elicits a proinflammatory response since it increases murine macrophage proinflammatory gene expression and cytokine production. Gliadin is not a unique stimulus of zonulin release since both non-pathogenic *E. coli* and pathogen-induced drop in TER and increased paracellular flux in the rabbit small intestine, and in other species, correlated with zonulin secretion into the luminal side of the mucosa [117]. Zonulin secretion by gliadin and enteric microorganisms increased paracellular permeability through PKC-mediated cytoskeleton reorganisation. In addition to its effect on actin polymerisation, gliadin has been shown to decrease the expression of both phosphorylated and non-phosphorylated forms of occludin, the expression of ZO-1, and that of claudins-3/4, leading to redistribution of these TJ proteins into the cytosol [118]. As a result, gliadin altered barrier function by decreasing TER and increasing permeability to small molecules but not to macromolecules. Gliadin increased intestinal permeability in human duodenum by a similar mechanisms [119]. Moreover celiac patients displayed exacerbated responses that correlated to reduction of both ZO-1 and occludin gene expression. Zonulin expression in the intestinal mucosa was increased during active celiac disease while it was attenuated in a gluten free diet [116]. Gliadin effects are mediated through binding to the chemokine receptor, CXCR3, as shown by co-localization of CXCR3 and gliadin immunoreactivity, by competition of gliadin with the natural ligand, the chemokine CXCL11, and lack of gliadin effect on zonulin release and intestinal permeability in CXCR3 deficient mouse [120]. Interestingly CXCR3 expression in human mucosa is greatly increased both at the epithelial level and in the lamina propria during the active phase of celiac disease, while a gluten-free diet restored CXCR3 expression to a similar level to normal subjects. Gliadin interaction with the intestinal epithelium induced the release of zonulin which, through binding to the cell surface CXCR3, provoked disruption of TJs and cytoskeleton rearrangement leading to a paracellular permeability increase. This allowed for translocation of gliadin into the mucosal site where it initiated proinflammatory and immune responses.

Ethanol and its Metabolite, Acetaldehyde

The effects of ethanol on epithelial barrier integrity have been studied both *in vitro* and *in vivo*. In human intestinal epithelial cells, ethanol caused a dose-dependent increase in permeability to paracellular markers that correlated with the progressive decrease in epithelial resistance and perturbation of both TJ and cytoskeleton architecture [121]. Ethanol increased paracellular permeability without affecting transcellular and active transport, its effect being accompanied by prostaglandin E_2 production and it was reversible without causing long-term cytotoxicity [122]. Cyclooxygenase-2-stimulated prostaglandin E_2 release mediated the reversible effect of ethanol on TJs and simultaneously, contributed to cell survival. Ethanol treatment resulted in a progressive disruption of TJ ZO-1 proteins, disassembly of perijunctional myosin and actin filaments, and displacement of these proteins form peripheral membrane location to the cytoplasm. Ethanol-induced barrier disruption in Caco-2 cells is mediated by increased inducible nitric oxide synthase (iNOS) activation and (nitric oxide) NO production, increased oxidative stress and superoxide anion production that leads to nitration and oxidation of tubulin, and subsequent depolymerization and disassembly [123]. Indeed, exposure of intestinal epithelial monolayers to oxidative stress triggered disruption

of the microtubule cytoskeleton and altered barrier integrity [124]. Oxidants, iNOS activation and NO production also caused disruption of the actin cytoskeleton by inducing actin oxidation and depolymerisation. In an animal model of alcohol-induced liver injury, NO overproduction mediated ethanol-induced increases in intestinal hyperpermeability and oxidative tissue damage, contributing to endotoxemia, hepatic inflammation, and liver injury [125]. Ethanol was also reported to enhance the production of proinflammatory cytokines, including TNF-α, and both barrier dysfunction and inflammation were enhanced by the presence of *E. coli*.

Both ethanol and acetaldehyde, the product of oxidative metabolism, induced a reduction of TER and increased permeability of mannitol, sucrose and inulin in intestinal epithelial cells [126]. In Caco-2 cells, acetaldehyde-increased barrier permeability was due to dissociation of E-cadherin, β-catenin, occludin, and ZO-1 from cellular junctions, their redistribution from the membrane to an intracellular location and reorganization of actin cytoskeleton [127]. In the human colonic mucosa, a reduction of the detergent insoluble fractions of these junctional proteins and redistribution into the cytosol was observed [128]. These events were linked to inhibition of protein tyrosine phosphatase activity and increased tyrosine phosphorylation of occludin, E-cadherin and β-catenin. Moreover, ZO-1 protein levels, but not mRNA, decreased after exposure to ethanol and ZO-1 expression inversely correlated with barrier permeability and expression of the microRNA, miR-212, in both Caco-2 cells and the colonic mucosa from patients with alcoholic liver disease, giving new insight on the mechanisms of TJ barrier regulation [129]. When ethanol is orally administrated to rats, generation of acetaldehyde in the intestinal lumen by the colonic microflora increased colonic permeability to ^{51}Cr-EDTA and was associated with significant endotoxemia [130]. Mast cell activation and degranulation products mediated alteration of the epithelial barrier after ethanol intake. Acetaldehyde also induced a marked increase in oral bioavailability of low, but not high, molecular weight probes and of low permeability drugs [126]. The effects of both ethanol and acetaldehyde on epithelial barrier breakdown are reversible suggesting that opening of TJs is not associated with cellular cytotoxicity [121, 126]. These results are consistent with data showing that ethanol and acetaldehyde did not induce apoptosis or cause cell death in human intestinal epithelial cells at concentrations effective on cell permeability [121, 122, 128].

Miscellaneous

Cellular stress, including hypoxia, heat and hyperosmolarity, has been found to alter epithelial barrier integrity. Indeed, moderate temperature elevation produced a reversible decrease in TER and ZO-1 expression leading to increased paracellular permeability in the absence of cell apoptosis or necrosis [131, 132]. Hyperosmotic stress induced both expression of the proinflammatory chemokine IL-8 and epithelial barrier dysfunction [133, 134]. Activation of NF-κB along with the MAP kinase pathway contributed to IL-8 production while increased intestinal permeability by hyperosmotic conditions involved NF-κB-dependent upregulation of the ste20-like proline/alanine-rich kinase, both *in vitro* and *in vivo*. Interestingly, NF-κB activity and expression of ste20-like proline/alanine-rich kinase are elevated in colonic biopsies from CD patients [134]. Emotional stress is also an important trigger of epithelial barrier disruption. In rats, acute restraint stress transiently affected the distribution of the ileal junctional proteins, occludin and ZO-1, and TJ permeability without altering the overall TJ architecture, thus enabling barrier function recovery [135]. Conversely, in mice, alteration in ileal ZO-1, occludin and β-catenin localization was associated with increased apoptotic rate as a result of upregulation and downregulation of proapoptotic and antiapoptotic pathways, respectively. Acute stress also enhanced systemic and tissular TNF-α production that contributed to epithelial barrier dysfunction [136]. In a mouse model of acute mixed restraint and acoustic stress, impairment of the epithelial barrier was mediated by massive cholinergic-dependent release of trypsin activity within the colonic lumen and activation of the epithelial PAR-2 [137]. Repeated mixed restraint and acoustic stress induced progressive increase in colonic paracellular permeability that was mediated by the rapid and transient induction of IFN-γ expression [138]. No histological alterations were found in the colon of stressed animals, but chronic stress resulted in liver bacteria translocation, inflammation and damage. In mice subjected to chronic water avoidance stress epithelial barrier function is compromised in the jejunum, ileum and colon, with both paracellular and transcellular permeability being affected as indicated by increased conductance and transepithelial flux of macromolecules [139]. Altered permeability was accompanied by increased bacterial adhesion and translocation, and infiltration of mononuclear cells into the mucosa.

POTENTIAL THERAPIES TO RESTORE INTESTINAL BARRIER FUNCTION

Intestinal permeability plays an important role in pathologies such as IBD, T1D, celiac disease but actual therapies/interventions do not take this aspect into account. In addition, the chronic and relapsing course of IBD and IBS makes the diseases disabling and complex to treat, and many patients become refractory to conventional therapies during the course of the disease. Anti-TNF-α therapy (Infliximab monoclonal antibodies to TNF-α) is currently used for the treatment of corticosteroid-refractory CD, as well as refractory UC, with improvement of clinical symptoms and a single infusion of infliximab in patients with active CD was sufficient to normalize the entire gut permeability [140]. However, infliximab treatment is associated with 1) risk of clinical relapse, preceded by reactivation of the mucosal and systemic immune system, and 2) several distinct adverse effects leading to serious concerns about safety and long-term consequences**.** Among the therapies under investigation for GI disorders, growth factors, probiotics, zonulin receptor antagonists and NF-κB inhibitors may restore epithelial barrier function, however MLCK inhibitors and PAR-2 antagonists could provide additional solutions (Table **3**).

Biological Therapy Based on Growth Factor Receptor Activation

The beneficial effects of growth factors in experimental models of mucosal damage or atrophy are related to maintenance of mucosal integrity through regeneration, growth, and

Table 3. Investigational Drugs for Gastrointestinal Disorders that Could Prevent Barrier Disruption

Compound	Chemistry	Company Institution	Therapeutic Area	Development Status
GROWTH FACTOR				
Repifermin	KGF-2 protein recombinant	Human Genome Sciences	UC, CD	Discontinued
Velafermin	FGF-20 protein recombinant	CuraGen Corp	IBD	No development
Teduglutide	GLP-2 peptide	NPS Allelix Corp	CD, SBS, GID	Phase 3 clinical
ZP-1846	GLP-2 peptide	Zealand Pharma A/S	Diarrhea	Phase 1 clinical
ZP-1848	GLP-2 peptide	Zealand Pharma A/S	IBD	Phase 1 clinical
DAC:GLP-2	GLP-2 peptide	ConjuChemBiotec. Inc	CD	No development
AG-012	TFF bacteria based gene therapy	ActoGenix	UC	No development
PROBIOTICS				
PB6	Bacillus subtilis strain	Kemin Pharma Europe	IBD,IBS,diarrhea	Phase 2 clinical
Probactrix	M17 strain *E. Coli*	BioBalance Corp	Inflammation	No development
ZONULIN ANTAGONIST				
Larazotide	zonulin antagonist, peptide	Alba Therapeutics	Celiac disease, CD, IBS, T1D	Phase 2 clinical
NF-κB PATHWAY INHIBITORS				
DA-6034	Flavonoid derivative	Dong-A Pharmaceutical	IBD (CD, UC)	Phase 2 clinical
DIMS-0150	Oligonucleotide AS	Karolinska Institute*(InDexPharm)*	ID, UC	Phase 2 clinical
HE-3286	steroid	Hollis-Eden	ID, UC	Phase 2 clinical
HMPL-004	natural product	Hutchison Medipharma	IBD (CD, UC)	Phase 2 clinical
VGX-1027	Not specified	Ganial Immunotherapeutics	Colitis	Phase 1 clinical
ALTH-12S *5-ASA+NFkB inhibitor*	small molecule combination	Oklaoma University (*Althreus Therap)*	IBD, UC	Phase 1 clinical
IKK inhibitors	small molecule	Bristol-Myers Squibb	IBD, ID	discovery

repair of the intestinal epithelium, but other significant effects also include the demonstration of reduced permeability. EGF exerted a protective effect toward ethanol- and oxidants-induced barrier disruption in Caco-2 cells [123, 124]. Antioxidants, by preventing oxidative damage, and paclitaxel, by stabilizing microtubules, protected against ethanol-induced microtubules cytoskeleton disruption and loss of barrier function. An actin-stabilising agent, phalloidin, mimicked the protective action of EGF while cytochalasin-D, an inhibitor of actin assembly, not only caused actin disassembly and barrier dysfunction but also abolished the growth factor protective effect. EGF exerted a protective effect on acetaldehyde-induced protein tyrosine phosphorylation and reorganization of both TJs and AJs proteins, and prevented permeability increase in both intestinal epithelial cells and human colonic mucosa [127, 128]. Prevention of barrier dysfunction is not restricted to EGF since other growth factors normally present in the intestine, such as the hepatocyte growth factor, keratinocyte growth factor and insulin, alone or in combination, prevented the effect of ethanol and oxidant on paracellular permeability [122]. EGF induced rapid translocation and activation of PLCγ1 which mediated membrane translocation and increased activity of PKCβI and PKCε [141-143]. Similarly to EGF, overexpression of PLC-γ protected Caco-2 cells against oxidant induced paracellular permeability increase and inversely, dominant negative inhibition of endogenous PLC-γ counteracted EGF protection [142]. Moreover, stable antisense inactivation of native PKC-β1 isoform antagonized the protective effects of EGF barrier dysfunction and conversely, stable overexpression of PKC-β1 sensitized Caco-2 cells to low concentrations of EGF that per se did not exert a protective effect. Specific blockade of PKCβI and PKCε activity or impairment of membrane translocation prevented EGF-mediated protection of TJ from acetaldehyde [143]. The protective effect of EGF on epithelial barrier is mediated by activation of PLC-γ and distinct PKC isoforms. The protective effect of EGF is not restricted to ethanol or oxidants. Indeed, EGF protected intestinal epithelial cell monolayers from *Cryptosporidium andersoni* infection, by preserving the tight junctional organization of ZO-1 [144], and from *C. jejuni* invasion, by restoring normal expression and distribution of claudin-4 [107], thus impairing paracellular permeability increase. In a rat model of necrotizing enterocolitis, oral administration of EGF decreased ileal paracellular permeability, increased goblet cell density and mucin production, and normalized

expression of the TJ proteins [145]. EGF was also effective in peritonitis, a pathology associated with an increase in intestinal permeability and claudin-2 expression, decreased villus length and proliferation, and increased apoptosis and mortality [146]. Enterocyte-specific overexpression of EGF is sufficient to prevent intestinal barrier dysfunction and improve survival in peritonitis.

There are limited but promising data on the beneficial activity of Glucagon-like peptide(GLP)-2 in various animal models of barrier dysfunction. GLP-2 and a dipeptidyl peptidase IV resistant GLP-2 analogue, h[Gly2]GLP-2, administered subcutaneously (s.c.) twice daily for up to 10 days, significantly improved the barrier property of normal murine small intestine, by affecting both paracellular and transcellular permeability [147]. Moreover, GLP-2 treatment significantly decreased intestinal permeability and bacterial translocation to mesenteric lymph nodes, pancreas, and peritoneum in acute pancreatitis [148]. A single injection of GLP-2 also exerted a protective effect in a murine model of food allergy since GLP-2 enhanced epithelial barrier function during both immediate and late phase of allergic response [149]. Indeed, GLP-2 decreased early antigen uptake and transport through the transcellular route and significantly attenuated the late-phase hypersensitivity reaction characterized by enhanced paracellular permeability and recruitment of inflammatory cells in the mucosa. Similarly, GLP-2 treatment of mice submitted to chronic water avoidance stress, improved barrier function by reducing ionic conductance in the jejunum and permeability to macromolecules in all regions of the gut [139]. Ion secretion, bacterial penetration and infiltration of inflammatory cells are also attenuated by GLP-2 treatment. In a recent study, GLP-2 was found to mediate the beneficial effects elicited by prebiotics on barrier function in ob/ob mice [70]. Prebiotic administration, through changes in the gut microbiota, reduced intestinal permeability by restoring ZO-1 and occludin expression and distribution, and ameliorated systemic and hepatic inflammation by inhibiting oxidative stress and macrophage infiltration. Chronic administration of a GLP-2 antagonist impaired the beneficial effects of prebiotic treatment and, inversely, chronic GLP-2 injected s.c. twice daily improved intestinal permeability, and reduced endotoxaemia and inflammation. GLP-2 also reduced the permeability and improved intestinal recovery in chemotherapy-induced enteritis.

Intestinal trefoil factor 3 (TFF3) is another growth factor that plays a role in maintaining epithelial barrier integrity. TFF3 protected intestinal epithelial cells from loss of barrier function and increased mannitol flux induced by various damaging agents including *C. difficile* toxin A [150]. Protection was enhanced by the co-presence of human colonic mucin glycoproteins. Induction of TFF3 mRNA and protein expression protected colonic epithelial cells from increased permeability induced by hypoxia [151]. Anti-TFF3 sera dose-dependently decreased TER in response to hypoxia and, inversely, recombinant protein added to hypoxia-sensitive cells normalized epithelial permeability. *In vivo*, hypoxia also increased TFF3 expression and production while TFF3 null mice exhibited an exacerbated increase in intestinal permeability upon exposure to hypoxia as compared to control animals. Mice lacking TFF3 are also more susceptible to DSS colitis and inversely mice overexpressing TFF3 in the intestine displayed increased resistance to intestinal damage and ulceration. TFF3 overexpression in transfected cells coincided with strong decrease in claudin-2 mRNA expression and protein at the TJ, while claudin-1 was increased, and development of a strong increase in TER indicating that the effect of TFF3 on claudin-1 and -2 expression may, at least in part, explain its protective effect on intestinal barrier function [152]. Interestingly, TNF-α, a key modulator of intestinal barrier function and inflammation, repressed TFF3 expression. Downregulation of TFF3 was mediated by NF-κB activation while the inhibitor IκB reversed it [153]. Moreover in the TNBS rat model of experimental colitis, colonic mucosal expression of NF-κB and TFF3 was regulated in an opposite manner during the course of the disease, with high expression of NF-κB and strong reduction of TFF3 during active disease, and inversely during recovery. Improvement of colitis symptoms by topical administration of 5-aminosalicylic acid (5-ASA) alone or in combination with sodium butyrate was associated with upregulation of TFF3 and downregulation of NF-κB expression [154] while daily intra-peritoneal injections of TFF3 starting one day after induction of colitis exerted a protective effect toward both microscopic and macroscopic colitis-induced mucosal injury [155]. TFF3 attenuated inflammation as shown by reduced inflammatory cells infiltration and reduced tissular TNF-α levels, and downregulated NF-κB mRNA and protein expression. TFF-secreting *Lactobacillus lactis* exerted both a preventive and therapeutical effect when administered in DSS-treated mice and also improved established chronic colitis in IL-$10^{-/-}$ mice [156]. TFF3 seemed to play a role also in celiac disease as indicated by reduced expression of TFF3 mRNA transcripts and protein in the distal duodenal mucosa of untreated patients with celiac disease that displayed intestinal hyperpermeability and restoration of normal expression on a gluten-free diet that re-establishes TJ barrier integrity [157].

Growth Factors in Development

Preclinical studies have shown that growth factors exerted a protective role against a variety of intestinal insults, preserved epithelial TJ integrity and reduced inflammatory indexes both *in vitro* and *in vivo*. Since prevention is not a property restricted to a specific growth factor or a specific insult, these data indicate that modulation of common intracellular signalling pathways is necessary to control AJC composition and function. Growth factors may therefore represent a possible therapeutic option for treating intestinal barrier dysfunction. A small number of growth factors have been tested in clinical trials (Table **3**). One trial showed that once-daily EGF enemas produced significant improvement in disease activity and induced clinical remission when administered in combination with oral mesalamine in patients with mild-to-moderate UC [158]. This clinical efficacy was not however confirmed. Indeed, results from a pilot study in paediatric patients with short bowel syndrome showed that enterally administered EGF was not associated with significant improvement in intestinal permeability, in the rate of weight gain or hepatic function [159]. Members of the FGF family have also progressed to being studied in clinical trials. Velafermin entered a phase I trial for the potential treatment of IBD but no development has been reported. Similarly,

repifermin initiated a phase II trials for the systemic injectable formulation in UC, CD and prevention of mucositis after chemotherapy. Development was discontinued because of the lack of significant benefits. ActoGenix was investigating AG-012, which consists of trefoil factor (TFF) delivered using genetically engineered microorganisms, for the potential treatment of UC. A phase I/II double-blind randomized trial showed that TFF-3 enemas were well tolerated in patients with mild-to-moderate left-sided colitis but did not provide additional benefit when given in combination with oral dose escalation therapy with mesalazine [160]. Development of TFF has been discontinued. Great interest has been demonstrated towards GLP-2. Indeed, a subcutaneous formulation of Teduglutide, a synthetic GLP-2 analogue (ALX-0600) is currently being evaluated by NPS Allelix for the potential treatment of GI diseases, in a phase II trial for moderate-to-severe CD and a confirmatory phase III trial for short-bowel syndrome. ALX-0600 is the Gly substituted form of GLP-2. The glycine substitution apparently renders the molecule resistant to enzymatic degradation and enhances binding to the receptor. Other substitutions demonstrate even greater increases in receptor affinity, suggesting that further refinements in potency of GLP-2 analogues may be possible. Efficacy studies have shown that teduglutide was well tolerated with no serious drug-related adverse events and significantly reduced the CD activity index inducing early remission [161].

Studies on the potential use of growth factors have given disappointing results and it is still unclear whether their efficacy might be improved. Further trials should consider increasing the duration of treatment, using the systemic route or combination enema therapy with other agents. Recent studies show that the therapeutic potential of gut commensal bacteria modified to produce human KGF-2 under the control of the xylanase promoter has been tested in the DSS mouse model of colitis [162]. This strategy allows oral administration of the growth factor, avoiding the inconvenience of systemic exposure. Moreover, lower amounts of the therapeutic molecule are used since it is delivered directly to the apical surface of the intestinal epithelium where production occurs in a controlled fashion when xylan is present in the diet.

Clearly, growth factors have been shown to promote growth of several tumour lines and their receptors have been closely linked to the invasiveness of tumours. Caution must be exercised with the use of these agents, particularly in the long-term and the potential effects of mucosal carcinogenesis need to be addressed in future studies.

Probiotic Bacteria to Fight Pathogens and Reconstitute a Protective Microflora

Both *in vitro* and *in vivo* observations have highlighted the capacity of commensal bacteria, probiotic bacterial strains and yeast to preserve epithelial barrier function while the pacific cohabitation between intestinal microflora and the epithelium barrier is altered in IBD patients.

The probiotic yeast *Saccharomyces boulardii* (*S. boulardii*) prevented epithelial barrier breakdown after EPEC infection by maintaining the TJ structure and ZO-1 distribution [163]. These effects were associated with inhibition of the ERK signalling pathway and protein tyrosine phosphorylation, reduction of bacteria penetration but not adhesion to the cell, and delayed apoptosis. *S. boulardii* also preserved the barrier function after EHEC infection by reducing MLC phosphorylation and also decreased IL-8 secretion by reducing IκBα phosphorylation and degradation and MAPK signalling [102]. *S. boulardii* did not affect bacteria attachment to epithelial cells. The probiotic combination, *Streptococcus thermophilus* plus *Lactobacillus acidophilus*, and *Lactobacillus plantarum* (*L. plantarum*) alone prevented EIEC induced decrease in TER and increased flux of paracellular probes [101, 164]. The protective effect of probiotic mixture is due in part, to limitation of EIEC adhesion and invasion, and in part, to maintenance (ZO-1) or enhancement (actinin, occludin) of junctional protein phosphorylation. On the other hand, the protective effect of *L. plantarum* was attributed to normalization of TJs proteins expression and distribution and to F-actin rearrangement. *L. plantarum* also prevented TNF-α-induced epithelial barrier alteration, inflammatory response and signal transduction as evidenced by inhibition of both ERK phosphorylation and IκB-α degradation [165]. Similarly, the probiotic *Lactobacillus rhamnosus* GG protected against both EHEC and hydrogen peroxide-induced drop in electrical resistance and increase in macromolecular permeability [166, 167]. Indeed, *Lactobacillus rhamnosus* GG reduced EHEC attachment and prevented ZO-1 redistribution and decreased protein expression. The protective effect against oxidant-induced barrier disruption was mediated by the release of soluble proteins that prevented the redistribution of the junctional proteins occludin, ZO-1, E-cadherin, and beta-catenin, through activation of PKCβI and PKCε isoforms and MAP kinase signalling pathway. In a similar manner, *Bifidobacterium infantis*, through the release of soluble factors, enhanced expression of the TJ proteins, claudin-4, ZO-1 and occludin, whereas claudin-2 levels decreased leading to improvement of barrier function [168]. It also counteracted the cytokine (TNF-α and IFN-γ)-induced TJ protein disruption and drop in TER. The protective effect of *B. infantis* on colonic permeability is maintained after oral administration in IL-10-deficient mice and prolonged treatment also attenuated colonic proinflammatory cytokines secretion and inflammation. Conversely, soluble protein(s) released from the probiotic mixture, VSL#3, prevented pathogen-induced ZO-1 redistribution, TER decrease and IL-8 secretion, but not the inflammatory response to IL-1β and TNF-α [169]. In the case of the commensal bacterium *Enterococcus hirae*, lipoteichoic acid, a lipid-related active component present in the cell wall, is indispensable for restoring ZO-1 expression and barrier function and inhibiting IL-8 secretion in cells exposed to TNF-α [170]. Another mechanisms by which some probiotic bacteria could exert a protective effect is the production of indole, an abundant secretion product of commensal *E. coli* [171]. Physiologically relevant amounts of indole regulated the expression of genes involved in epithelial barrier integrity and in inflammatory/immune response. Indeed, indole increased TER while it decreased TNF-α-mediated activation of NF-κB and IL-8 production, and EHEC adhesion to epithelial cells. The protective effect of probiotics is not restricted to pathogens, proinflammatory cytokines or mild irritants since *Bifidobacterium lactis* impaired gliadin-induced TER reduction, cy-

toskeleton rearrangement, and redistribution of TJ protein ZO-1, thus preventing cellular damage [172].

The beneficial effect of changing intestinal microflora with probiotics has been demonstrated *in vivo*. In a rat model of alcohol-induced steatohepatitis, *L. rhamnosus* attenuated alcohol-induced loss of intestinal barrier, ameliorated tissue (gut and liver) and systemic oxidative stress, and significantly reduced the severity of the disease, as shown by a decrease in hepatic fat content and signs of inflammation [173]. The probiotic mixture, Probiotics Ecologic® 641, was also effective as a preventive treatment in a rat model of acute pancreatitis which is associated with severe ileal mucosal barrier dysfunction and translocation of luminal bacteria that cause infectious complications [174, 175]. Pretreatment with probiotics antagonized the increase in both paracellular and transcellular permeability and tissue conductance, thus preventing *E. coli* translocation. The protective action of probiotics coincided with inhibition of oxidative stress, downregulation of occludin and claudin-1, upregulation of claudin-2, and reduction of mucosal injury. However, treatment at the time of acute pancreatitis induction was ineffective [175]. The probiotic mixture VSL#3 significantly ameliorated the disease activity index and reduced histological signs of inflammation in acute DSS colitis [176]. Colitis was associated with increased intestinal permeability, decreased distribution of occludin, ZO-1, claudin-1, -3, -4 and –5 from the apical junctional complex that was mostly due to decreased expression. VSL#3 completely restored the normal composition and structure of the TJs, preventing colonic permeability increase and epithelial apoptosis. VSL#3 also restored the colonic epithelial barrier integrity in IL-10 gene-deficient mice, normalized the mucosal immune response, as measured by basal and LPS stimulated TNF-α and IFN-γ production, and improved histological signs of disease [177]. Orally administered VSL#3 probiotics were also effective in a mouse model of sepsis, where maintenance of the epithelial barrier involved a PPARγ-dependent mechanism and correlated with inhibition of proinflammatory cytokines expression and secretion, and reduction of bacterial translocation and liver damage [178]. By contrast, VSL#3 prevented the onset of CD-like ileitis in SAMP mice however, since high doses of VSL#3 were required, the preventive effect in this model coincided with stimulation rather than suppression of the epithelial innate immune system, with enhanced TNF-α production and activation of NF-κB pathway [179].

Probiotics in Clinical Trials

Protective properties of probiotics concerned both permeability and inflammation and have been observed *in vitro* and in animal models of inflammatory diseases. Both single-strain probiotics and combinations of several organisms have been tested as novel therapies in randomized controlled trials, however limited, equivocal or negative data have been obtained [180].

The probiotic therapy VSL#3, administered orally once a day, is effective in patients with recurrent or refractory pouchitis (inflammation of a surgically created ileal pouch), since remission obtained by intense antibiotic treatment is maintained for at least one year [181]. VSL#3 is actually being tested as an alternative or supplementary therapy to 5-ASA and/or immunosuppressants in patients affected by relapsing mild-to-moderate UC [182-184]. Results are promising in terms of safety and efficacy, as they show clinical potential in the treatment of active UC and as maintenance therapy for patients in remission, although data from larger clinical trials are needed. *E. coli* Nissle 1917, a non-pathogenic bacteria initially isolated for its potential to protect from presumably infectious gastroenteritis, demonstrated antagonistic effects towards other members of the intestinal microbiota due to being able to modulate the immune response and reinforce the intestinal barrier function [185]. Nissle 1917 was found to be as effective as mesalamine in achieving remission in patients with UC, however mesalamine was used at sub-therapeutic doses. *S. boulardii* added to baseline medications improved intestinal permeability in patients with CD in remission, but complete normalization of the intestinal mucosal barrier was not achieved [186]. In patients with diarrhoea, predominant IBS associated with decreased expression of occludin and ZO-1, treatment for 4 weeks with probiotic fermented milk improved small bowel mucosal barrier function and disease scores but not colonic permeability [187]. *L. plantarum* enteral feeding in patients with acute pancreatitis resulted in decreased intestinal permeability, reduction of sepsis rate and diminution of pathogenic organisms [188]. Disease severity was attenuated with improved clinical outcomes. Conversely, in a randomized, placebo-controlled, multicenter trial in patients with predicted severe acute pancreatitis who developed intestinal barrier dysfunction early in the course of disease, Ecologic 641 probiotic mixture prophylaxis decreased bacterial translocation in those patients without organ failure but was not capable of preventing intestinal damage or an increase in permeability [189]. Most importantly, this probiotic mixture not only failed to show any beneficial effects on the occurrence of infectious complications but it also increased the mortality rate [190] indicating that administration of probiotics cannot be considered to be safe, especially in critically ill patients or patients with an extremely leaky intestine. Kemin Pharma is developing PB-6 (Inflammaban), a high dose oral antibacterial and anti-inflammatory natural *Bacillus subtilis* strain, for IBD, *C. difficile*-associated diarrhoea and antibiotic-associated diarrhoea. Preclinical results from a study conducted in a clindamycin-induced *C. difficile*-associated diarrhoea model have demonstrated the therapeutic efficacy of PB-6 in treating diarrhoea and increasing survival. PB-6 (Anaeban) is also developed at a low oral dosage for the potential treatment of severe hospital-acquired diarrhoea and stomach ulcers.

Whether the luminal microflora can be adequately manipulated to reproduce the preclinical potential effects on barrier function and yield a clinical benefit remains to be established. Contradictory results may be explained by the fact that the composition and pathogenic influence of the luminal microflora are different for each individual because of a different personal history. Definition of a probiotic regimen that will display efficacy in each individual seems difficult. Since several diseases states are chronic and usually intermittent, larger and longer term trials are required to determine whether probiotics are beneficial in GI diseases. An optimal specific intervention should be defined and research

should focus on the type, optimal dose of probiotic or combination of probiotics, and the subgroups of patients who are likely to benefit the most.

Blockade of the NF-κB Pathway to Prevent Inflammation and Hyperpermeability

IBD is associated with exaggerated activation of NF-κB pathway in macrophages and epithelial cells correlated with the degree of mucosal inflammation. CD patients who relapsed, displayed increased intestinal permeability associated with a high production of TNFα in the colonic mucosa, and increased translocation and activation of NF-κB p65 [23]. In recent years cumulative findings have shown that NF-κB plays a central role in both inflammation and epithelial barrier function in animal models and in humans since this pathway acts downstream from receptors of several proinflammatory cytokines.

Indeed, IL-1β induced disruption of Caco-2 epithelial barrier was paralleled by a rapid degradation of IκB-α and activation of NF-κB [92, 191]. Pharmacological inhibition of the NF-κB pathway and RNA silencing prevented cytokine-induced down-regulation of occludin expression and paracellular permeability increase. TNF-α also promoted IκB degradation, cytoplasmic-to-nuclear translocation of NF-κB p65 and increased binding to target genes [192]. NF-κB inhibitors, including curcumin, inhibited NF-κB activation but also a TNF-α-induced increase in paracellular permeability, decreased TER and downregulation of ZO-1 protein expression indicating that TJ disruption was mediated by the NF-κB signalling pathway [87]. In T84 cells, IFN-γ-induced NF-κB activation was secondary to the activation of the PI3-K/Akt [193]. Inhibition of PI3-K prevented NF-κB activity while inhibition of either PI3-K or NF-κB prevented the permeability increase and alteration in occludin expression, suggesting that IFN-γ induced barrier dysfunction involved the PI3-K pathway in activating NF-κB, which in turn modified TJ protein expression.

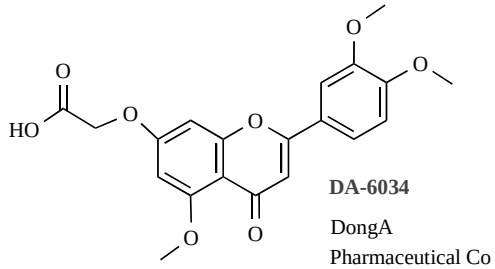
Involvement of NF-κB in irritant-induced barrier dysfunction has also been studied. Oxidant-induced loss of Caco-2 cells monolayer integrity and microtubule network disruption were associated with induction of NF-κB activation [194]. Blockade of the NF-κB pathway with inhibitors of either NF-κB, or IκBα and with a stable dominant negative mutant of IκBα, prevented the disruptive effects of oxidant on monolayer barrier integrity and the microtubule cytoskeleton. Moreover, the protective effect of EGF on epithelial barrier permeability coincided with inhibition of IκBα phosphorylation/degradation and NF-κB activity. Further investigations have shown that increasing activity or expression of either PKC-β1 or PLC-γ isoforms was associated with inhibition of IκBα phosphorylation and degradation, decreased NF-κB nuclear translocation and activation, stabilization of the actin cytoskeleton, and restoration of barrier function [141, 142]. Inversely, inactivation of PKC-β1 or PLC-γ isoforms antagonized the protective effects of EGF against NF-κB activation and barrier dysfunction indicating that epithelial barrier protection against oxidants is achieved by NF-κB inactivation downstream from the PKC-β1 – PLCγ signalling pathway. Such a protective mechanism is activated by EGF. The same authors showed that ethanol caused IκBα degradation, NF-κB nuclear translocation and activation, actin cytoskeleton disassembly, and barrier disruption [195]. Here also, the inhibition of NF-κB activation protected against ethanol-induced injury. Disruption of the actin cytoskeleton with agents such as cytochalasin D resulted in increased NF-κB activation, secondary to IκBα degradation, and increased gene expression and release of the NF-κB-dependent chemokine IL-8 [196]. Cytochalasin-D also caused barrier dysfunction, and antagonized the protective action of growth factors, suggesting a link between cytoskeletal disruption, NF-κB activation, and inflammation [124]. Similarly, both the anti-inflammatory property of *S. boulardii* and the protective effect on barrier function after infection with EHEC or exposure to cytokines, were correlated with the inhibition of IκBα degradation and NF-κB activation [102, 197].

In vivo studies have revealed that both preventive and therapeutic oral administration of agents capable of inhibiting the NF-κB pathway exerted positive effects on experimental models of IBD. A recent study showed that in hapten-induced colitis, epithelial specific repression of NF-kB inhibited the increase in intestinal epithelial conductance, transmucosal flux of both small molecular probes and macromolecules, and net water secretion associated with diarrhoea [52]. It also prevented internalization of TJ proteins (occludin, claudin-1 and ZO-1). Thus NFkB activation mediated diarrhoea and increased paracellular permeability. Moreover, deletion of the transcription factor FoxO4, that negatively regulated the transcriptional activity of NF-κB, increased intestinal epithelial permeability both *in vivo* and *in vitro*, with down-regulation of the TJ proteins, ZO-1 and claudin-1 [198]. In addition, deletion of FoxO4 increased susceptibility to TNBS–induced colitis in mice, by increasing T cell recruitment and inflammatory cytokines expression in the colon. Interestingly, the expression of FoxO4 is transiently down-regulated during TNBS-induced inflammation in mice and lower levels of FoxO4 expression are found in the inflamed colonic mucosa of UC patients. These observations suggest that upregulation of NF-κB activity could promote both epithelial barrier dysfunction and inflammation. NF-κB is a key mediator that links TJ and actin cytoskeleton disruption to hyperpermeability and inflammation in response to several mucosal damaging stimuli. Worth noting, commonly used drugs for the treatment of IBDs have been shown to block NF-κB activation in a number of different mechanisms: glucocorticoids increase the transcription of IκBα while sulphasalazine inhibits ATP binding to IκB kinases, thus preventing IκB phosphorylation and degradation. Drugs that directly or indirectly inhibit the NF-κB pathway are promising therapeutic tools in IBD, but also in those pathologies where barrier dysfunction is coupled to a minor inflammation component.

NF-kB Inhibitors in Development

A number of compounds, including small molecules, are currently being developed for inflammatory diseases, IBD and IBS, although no data on permeability and TJs structure are available (Table **3**, Fig. **2**). DA-6034 (7-carboxymethyloxy-3', 4', 5-trimethoxy flavone), a flavonoid derivative NF-κB modulator and mucus secretion enhancer, is in phase 2

for IBD, and is expected to enter phase 3 for gastritis. Preclinical studies have demonstrated the efficacy of DA-6034 in several animal models of IBD with therapeutic and preventive effects. Moreover, DA-6034 pre-treatment reduced gastric lesions induced by aspirin, indomethacin, stress or ethanol plus HCl. Phase I studies in healthy volunteers have shown DA-6034 to be safe and well-tolerated after single and multiple oral doses. HMPL-004, an extract from a Chinese herbal product, is being developed as an oral formulation for the potential treatment of CD and UC.



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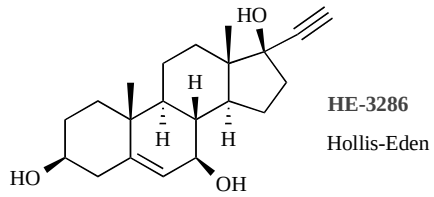

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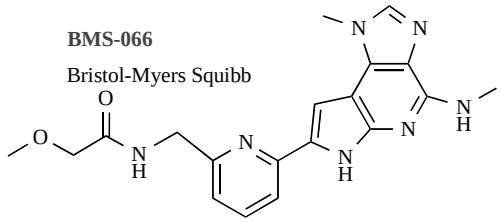

BMS-066

Bristol-Myers Squibb

Fig. (2). Chemical structure of NF-κB inhibitors developed for IBD.

HMPL-004 inhibited cytokine production and NF-κB activation, and demonstrated efficacy in phase 2 clinical trials by reducing the disease activity index and increasing the remission rate. HMPL-004 showed a favourable safety profile and no serious adverse events were reported. Other investigational drugs include DIMS-0150 (Kappaproct), a rectal formulation of an antisense NF-κB oligonucleotide, and ALTH-12S, a rectal formulation of 5-ASA associated with a selective NF-κB inhibitor, for the potential treatment of UC. Finally, Bristol-Myers Squibb is investigating a series of IκB kinase tricyclic inhibitors for the potential treatment of various inflammatory conditions, including IBD and arthritis. Lead optimization has given rise to the pyrrole-fused tricyclic compound, BMS-066. Compounds have been tested for their degree of selectivity versus IkappaB kinase(IKK)2 over IKK1 and versus other kinases, and for their efficacy in inhibiting LPS-induced TNF-α release from human peripheral blood mononuclear cells *in vitro* and serum TNF-α production in mice. The NF-κB family of transcription factors and the NF-κB pathway is known to mediate transcriptional regulation of numerous genes and to regulate a wide range of biological activities, including cellular proliferation, apoptosis and survival. An accurate evaluation of potential side effects of anti NF-κB therapy must be made.

Inhibition of MLCK, an Enzyme Activated by Several Stimuli

MLCK enhanced expression and activation are present in intestinal epithelia of IBD patients [199]. Several studies have shown that MLCK plays a central role in the regulation of intestinal epithelial barrier function. Indeed, inhibition of MLC phosphorylation by a membrane-permeant, peptide inhibitor of MLCK, improved paracellular permeability [200]. MLCK inhibition also preserved barrier function in Caco-2 epithelial cells infected by EPEC or exposed to TNF-α and IFN-γ. TNF-α- and LIGHT-induced barrier loss in epithelial cells was in fact associated with enhanced MLCK transcription and protein expression [87, 93, 201] while, conversely, inhibition of protein synthesis and transcription as well as MLCK inhibition reversed barrier loss by both cytokines. This suggested that cytokines increased MLCK transcription for enhancing MLCK activity and MLC phosphorylation and disrupting TJs in Caco-2 cells. Accordingly, the protective effect of the commensal bacteria *Enterococcus hirae* and cell wall fraction, lipoteichoic acid, on TNF-α-induced barrier dysfunction correlated with the recovery of ZO-1 expression and suppression of MLCK expression [170]. TNF-α synergized with IFN-γ to increase MLCK expression and MLC phosphorylation, an event that is necessary to promote redistribution of TJ proteins and paracellular permeability increase, while the clinically effective drug, sulfasalazine, at doses that did not inhibit NF-κB activation, exerted a protective effect by blocking MLCK up-regulation [90, 91]. That MLCK is a TNF-α inducible protein has recently been demonstrated. Data indicated that TNF-α through activation of the NF-κB signalling pathway increased MLCK promoter activity and gene transcription while the prevention of barrier dysfunction by NF-κB inhibitors is mediated by inhibition of MLCK gene transcription, protein expression and activity [192, 202]. Importantly, two NF-κB binding sites have been identified that modulated up-regulation and down-regulation of the promoter activity respectively. Indeed, prednisolone prevented the TNF-α-induced increase in paracellular permeability by inhibiting TNF-α-induced upregulation of MLCK promoter activity and MLCK expression [203]. The protective effect of prednisolone in antagonizing TNF-α-induced activation is mediated by association with its receptor and binding of the resulting complex to the MLCK promoter. The effect of IL-1β on epithelial barrier disruption was also mediated by the increased NF-κB pathway and enhanced MLCK expression. Indeed, blockade of MLCK activity /expression by specific inhibitors or RNA silencing prevented alteration of the epithelial barrier by IL-1β while knockdown of NF-κB prevented both MLCK expression and permeability increase [191]. MLCK activation mediated barrier dysfunction in response not only to cytokines but also to ethanol [121], EPEC and EHEC infection [96, 102], and heat stress. Both inhibition of MLC kinase activity and treatment with *S. boulardii* inhibited MLC-phosphorylation for preserving the barrier function in EHEC-infected cells [102] while specific inhibition of either PKC-ζ or MLCK protected against

EPEC-induced redistribution of occludin and barrier dysfunction [96]. Inhibition of MLCK did not prevent EPEC-induced PKC-ζ translocation or activation, suggesting that PKC–ζ controlled epithelial barrier through a distinct or upstream signalling pathway. As regards heat stress, intestinal epithelial barrier dysfunction was due to subsequent phosphorylation and activation of PKC and MLCK leading to upregulation of MLC phosphorylation [132].

MLCK also regulated the epithelial barrier *in vivo*. When injected in mice, TNF-α caused MLCK-dependent barrier dysfunction with increased paracellular permeability and internalization of the TJ protein occludin, protein leakage into the lumen and water secretion [204]. Intestinal epithelial MLC phosphorylation dependent on lymphotoxin β receptor activation mediated LIGHT-induced barrier dysfunction in mice [93]. In a murine model of immune-mediated colitis, systemic T cell activation caused induction of mucosal IFN-γ and TNF-α, intestinal inflammation and diarrhoea [205]. Epithelial barrier dysfunction is associated with increased epithelial MLCK expression and activity, and with increased intestinal paracellular permeability as a result of JAM-A and ZO-1 redistribution, and occludin internalization [91, 205]. Pharmacological inhibition and gene ablation of MLCK prevented barrier dysfunction, protecting from diarrhoea. In a rat model of sepsis, LPS administration triggered TNF-α and IFN-γ production in colonic mucosa and increased colonic paracellular permeability that was associated with enhanced MLC phosphorylation, bacterial translocation and visceral hyperalgesia, these effects being mediated by MLCK activation [206]. Chronic stress induced the rapid and transient induction of IFN-γ expression, colonic MLC phosphorylation, paracellular permeability increase and bacterial translocation [138]. Increased colonic permeability is dependent on both MLCK signalling pathways and IFN-γ production and lead to stress-induced liver inflammation and injury. A recent study showed that *in vivo* targeted expression of constitutively active MLCK within intestinal epithelium resulted in increased MLC phosphorylation and paracellular permeability to uncharged molecules in both jejunum and colon. Bacteria or endotoxin translocation did not occur and TJ architecture is maintained [50]. Transgenic animals did not display any signs of disease but were more susceptible to immune mediated colitis. These data confirmed previous *in vitro* studies showing that expression of constitutively active MLCK caused MLC phosphorylation, reorganization of junctional proteins, ZO-1 and occludin, and F-actin that led to reduced barrier function [7]. A recent study shows that *in vivo* epithelial MLCK activation has a more global effect on the epithelial barrier, since it induces IL-13 production which, in turns, mediates upregulation of claudin-2 and increases the permeability to cations [207].

Overall data showed that epithelial MLCK activation alone is sufficient to regulate TJ and is essential for intestinal barrier dysfunction by cytokines, pathogens and otherwise. Although increased epithelial paracellular permeability per se is not sufficient to induce colitis, it predisposes and con-

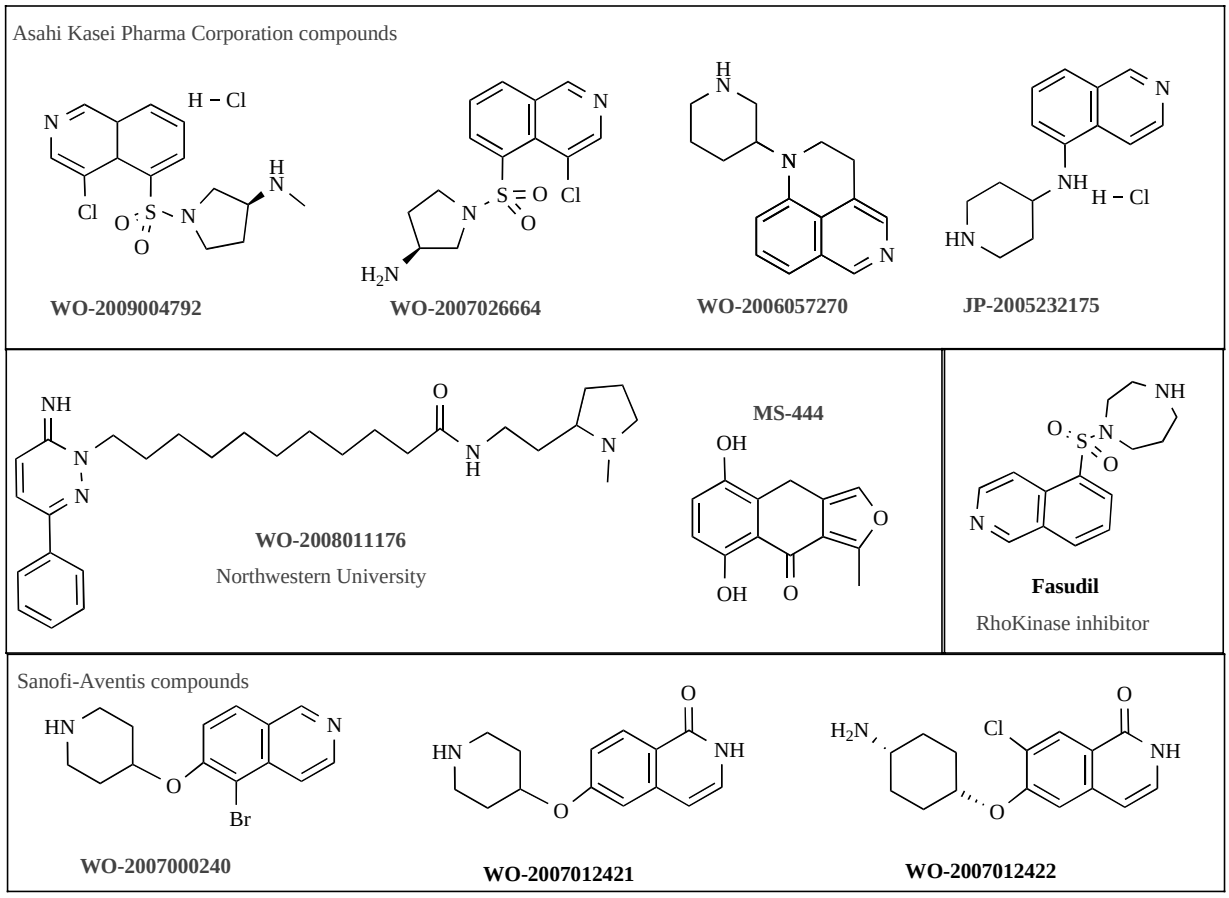

Fig. (3). Chemical structure of MLCK inhibitors.

tributes to pathogenesis and progression of the disease. Interestingly, CD ileum specimens, as well as colonic tissue involved in active UC, displayed increased epithelial MLCK expression and activity at the apical actomyosin ring that was correlated with the severity of the disease [199]. Inhibition of MLCK and MLC phosphorylation appears a promising approach to prevent intestinal epithelial barrier breakdown in disease.

MLCK Inhibitors

The primary challenge with the development of a MLCK kinase inhibitor is to define the degree of selectivity in order to limit the side effect profile of a potential drug due to off-target effects. This issue is particularly difficult because the kinome consists of more than 500 enzymes displaying a highly conserved structure within the kinase active site. More specifically, MLCK isoforms also share similar catalytic domains and regulatory domains since all are Ca^{2+}-calmodulin-dependent kinases. The vast majority of known kinase inhibitors bind to the ATP-binding site of the kinase catalytic domain while targeting the substrate binding site has not been sufficiently investigated until now. With several hundred kinases encoded in the human genome, almost every signal transduction process is influenced by interconnected phosphorylation events and the risk of side effects is high. Moreover, MLCK is expressed ubiquitously in almost all tissues, and is involved in many cellular activities, such as the regulation of endothelial permeability, migration, secretion, signalling pathways leading to proliferation and apoptosis. Therefore, the consequences of prolonged MLCK inhibition remain to be determined. Another challenge in targeting well conserved sites on the protein kinase is to design a structure differing from those of existing inhibitor chemotypes and from those claimed in the thousands of existing patents published which describe inhibitors of protein kinases.

Kyowa Hakko Kogyo started developing MS-444 (5,8-dihydroxy-3-methyl-(9H)-naphtho[2,3-c]furan-4-one) and MS-681 compounds isolated from the culture broth of a bacterial and fungal strain respectively, that inhibited MLCK activity with IC_{50} values of 10 μM and 0.095 μM (Fig. **3**), but not cyclic AMP- and cyclic GMP-dependent protein kinase and PKC at 100 μM for MS-681 compounds. However, no developments have been reported. Asahi Kasei has studied analogues of the Rho-associated kinase inhibitor, fasudil, launched for the therapy of cerebral vasospasm and ischemia, that included; 5-substituted isoquinoline derivatives [208], tricyclic fused isoquinoline derivatives [209], isoquinoline sulfonamide derivatives [210], and (S)-1-(4-Chloro-5-isoquinolinesulfonyl)-3(methylamino)pyrrolidine monohydrochloride [211]. These compounds are both MLCK and Rho kinase inhibitors, displaying IC_{50} less than 10 μM for inhibiting MLC phosphorylation. Micromolar concentrations of fasudil can also function as MLCK inhibitors. Sanofi-Aventis has claimed a series of compounds including cyclohexylamine-substituted isoquinolone [212], piperidinyl-substituted isoquinolone [213], and isoquinoline derivatives [214], all MLCK and Rho kinase inhibitors. Pyridazinylalkaneamide derivatives acted as MLCK inhibitors [215], inhibiting MLCK in the μM range, but selectivity toward Rho kinase is not reported. The design and development of a specific MLCK inhibitor is still an open issue.

PAR-2 Inactivation as a New Therapeutical Approach

Both *in vitro* and *in vivo* experiments provide cumulating findings for a role of proteases and PAR-2 in intestinal TJ barrier disruption. Indeed, trypsin and PAR-2 agonist peptides enhanced paracellular permeability in epithelial cells through activation of basolateral receptors and intracellular calcium mobilization [216, 217]. At a molecular level, PAR-2 agonists promoted ZO-1 and occludin, but not claudin-1 or E-cadherin, internalization and reorganization of F-actin. *In vivo*, intracolonic infusion of PAR-2-agonists induced receptor activation and internalization, upregulation of PAR-2 expression and iNOS activation in mouse colonic tissues [218, 219]. PAR-2 activation caused an increase of colonic paracellular permeability and bacterial translocation accompanied by elevation of proinflammatory cytokine expression (TNF-α, IL-1β, and IFN-γ), infiltration of inflammatory cells and tissue damage. PAR-2-deficiency prevented both inflammation and hyperpermeability, indicating that local activation of PAR-2 mediated these effects. Depletion of afferent fibres by capsaicin, blockade of either neurokinin 1 (NK_1) or calcitonin gene-related peptide receptor, and iNOS activity inhibitors, prevented the inflammatory process and attenuated paracellular permeability. Inversely, blockade of TJ opening did not prevent inflammation [219]. The inflammatory response but not perturbation of epithelial barrier by PAR-2-agonist peptide is also lost in IFN-γ-deficient mice or by decreasing the concentrations of peptide, indicating dissociation between inflammation and barrier function [216]. Activation of MLCK and MLC phosphorylation mediated increased paracellular permeability by low concentrations of PAR_2-agonist peptide, while NO-dependent release of neuromediators from capsaicin-sensitive afferent neurons mediated inflammation and IFN-γ production that contribute to barrier dysfunction at high concentrations. Similarly, increased permeability and hyperalgesia induced by intracolonic administration of a PAR-2-agonist in the rat may occur in the absence of inflammation [220]. The PAR-2 hyperalgesic effect was mediated by NK-1 receptor activation while dexamethasone treatment prevented visceral hypersensitivity and mast cells recruitment in colonic mucosa, but not colonic permeability.

Activation of PAR-2 by endogenous proteases also plays an important role in the regulation of the mucosal barrier since intracolonic infusion of protease inhibitors significantly reduced basal colonic paracellular permeability in mice [221]. Moreover, reduction of resident colonic bacteria by antibiotic treatment resulted in decreased luminal serine protease activity in the colon, lower PAR-2 mucosal expression, and reduced permeability increase in response to PAR-2 agonists. Proteases of different origins may regulate the intestinal epithelial barrier. Indeed, mast cell degranulation products including the serine proteinase, tryptase, activated PAR-2 on enterocytes and enhanced paracellular permeability by means of ERK-dependent process [217]. Increased epithelial permeability was strongly reduced, but not abolished, by tryptase inhibitors and inhibitors of the ERK signalling pathway, suggesting that mast cell mediators other

than tryptase may also regulate permeability. Similarly, the contact of PMN with the basolateral surfaces of human intestinal epithelial cells released serine proteases responsible for barrier dysfunction and transepithelial migration, events which are prevented by simultaneous knockdown of epithelial PAR-1 and -2 [222]. Inversely, the PMN serine proteases, elastase and proteinase-3, as well as PAR-1 and –2 agonist peptides, were able to alter epithelial permeability. Enhanced phosphorylation of MLCK and MLC was associated with PAR-1 and –2 mediated barrier dysfunction.

Proteases are involved in LPS-induced increase in colonic paracellular permeability and inflammation in rat colon [114]. Indeed, LPS administration was associated with enhanced luminal serine protease activity that displayed a permeability-enhancing effect when applied to rat proximal colon *in vitro*. Administration of a mast cell stabilizer prevented LPS effects *in vivo* while a mast cell degranulator reproduced its effects on permeability, inflammation and luminal content, demonstrating the key role of mast cells serine proteases in LPS-induced barrier dysfunction. Similarly, in a rat model of chronic stress, the colonic epithelial barrier defect was mediated by increased mast cell numbers and activation in the colonic mucosa [223]. The situation is somewhat different in a mouse model of acute stress where impairment of the epithelial barrier did not depend upon mast cell protease but rather, on pancreatic trypsin, although the colonic content and activity of both proteases were increased [137]. *In vitro*, the luminal content from stressed animals increased permeability across mouse colonic tissue that was abolished by PAR-2 antagonists. *In vivo*, blockade of the cholinergic pathways by atropine abolished colonic paracellular permeability and the release of trypsin activity but not mast cell protease within the colonic lumen. Therefore, acute stress impairs the epithelial barrier through a cholinergic-dependent release of trypsin and activation of PAR-2. Serine proteases and PAR-2 are involved in *C. difficile* toxin A-induced enteritis in mice [224]. Increased fluid secretion and oedema, infiltration of inflammatory cells and epithelial damage were attenuated by PAR-2 deletion and by inhibition of tryptase and trypsin. Inversely, direct activation of PAR-2 provoked ileitis. Furthermore, toxin A increased expression of PAR-2 in mice ileum and in human intestinal epithelial cells. PAR-2-mediated inflammation in toxin A-ileitis was dependent on a neurogenic pathway and NK1 receptor activation. Serine proteases and PAR-2 are involved in IBS. Indeed, the colonic luminal content from diarrhoea-predominant IBS (IBS-D) patients displayed higher amounts of serine protease activity and increased in paracellular permeability when applied to mice colonic mucosa [42]. Barrier dysfunction was associated to enhanced visceral sensitivity to colorectal distension, and both events are mediated by PAR-2 expression. IBS patients also showed higher transcript levels of trypsinogen IV, the inactive precursor of trypsin IV, a PAR-2 ligand [225]. Finally, a recent study shows the important role played by tryptase activity and PAR-2 activation in mediating the enhanced intestinal permeability to macromolecules in colonic mucosa of patients with diarrhoea-predominant IBS [226].

Both mast cell and neutrophil serine proteases as well as bacterial proteases and colonic digestive enzymes are potent activators of PAR-2 that play an important role in increased intestinal permeability during stress and IBS. PAR-2 is also expected to play a role in CD since colonic mucosa obtained from CD patients exhibited increased expression of this receptor [222] and in diseases associated with increased zonulin release, such as celiac disease, since the *in vitro* and *in vivo* permeability enhancing effect of human zonulin has been shown to be mediated by PAR-2-dependent transactivation of the EGF receptor [116].

PAR-2 Antagonists

Very little data are available on PAR-2 antagonists. EntreMed was investigating peptidomimetic inhibitors of PAR-2, including ENMD-1068, for the potential treatment of inflammatory diseases and cancer, and while preclinical data were presented but no further development was reported (Fig. **4**). Two structurally related peptidomimetic PAR-2 antagonist compounds, K-12940 and K-14585, have been identified by Kowa that demonstrate inhibitory effects on PAR-2-mediated responses [227]. Both compounds bind to human PAR-2 in a competitive manner, inhibiting the effect of PAR-2 agonist peptide on intracellular signalling pathways at both native and transfected receptors. Both compounds inhibit inflammatory responses, i.e. NF-kB activation and IL-8 production. K-14585 displayed some activity *in vivo*. In particular, it lowered plasma extravasation of Evans Blue in the dorsal skin of guinea pigs as an index of microvascular permeability, when administered intradermally. K-14585 was initially investigated for the potential treatment of inflammation, however no development has been published. Limitations of these compounds include the peptide structure, the low potency, and problems of solubility that prevent the use of higher concentrations. Moreover, K-14585 displayed antagonist properties at low μmolar concentrations and agonist properties at 30 μM as demonstrated by activation of p38 MAP kinase and the ERK pathway, upregulation of NF-kB reporter activity and IL-8 production [228].

The major concern of PAR-2 antagonists is the difference in antagonist potency between the activating serine protease and cognate activating peptide. Indeed, potent, selective, non-peptide competitive antagonists should be developed which can effectively inhibit both agonist peptide-activated and the proteolytically activated receptor. Studies on the structure-activity relationship are necessary to identify the structural requirements for PAR-2 antagonism that includes a common site of receptor interaction for the PAR peptide and tethered ligand and leads to future development of high affinity antagonists. As an alternative approach, Amgen Inc is investigating isolated antibodies (human, monoclonal and chimeric) and fragments capable of binding to both intact and ligand-cleaved PAR-2 and antagonizing the receptor activation as a consequence of proteolytic cleavage [229, 230].

Development of Zonulin Antagonist, Larazotide

Alba Therapeutics is developing larazotide acetate (AT-1001), an oral peptide zonulin receptor antagonist, for the potential treatment of celiac disease and other autoimmune diseases, including CD (Table **3**, Fig. **5**). AT-1001 is a syn-

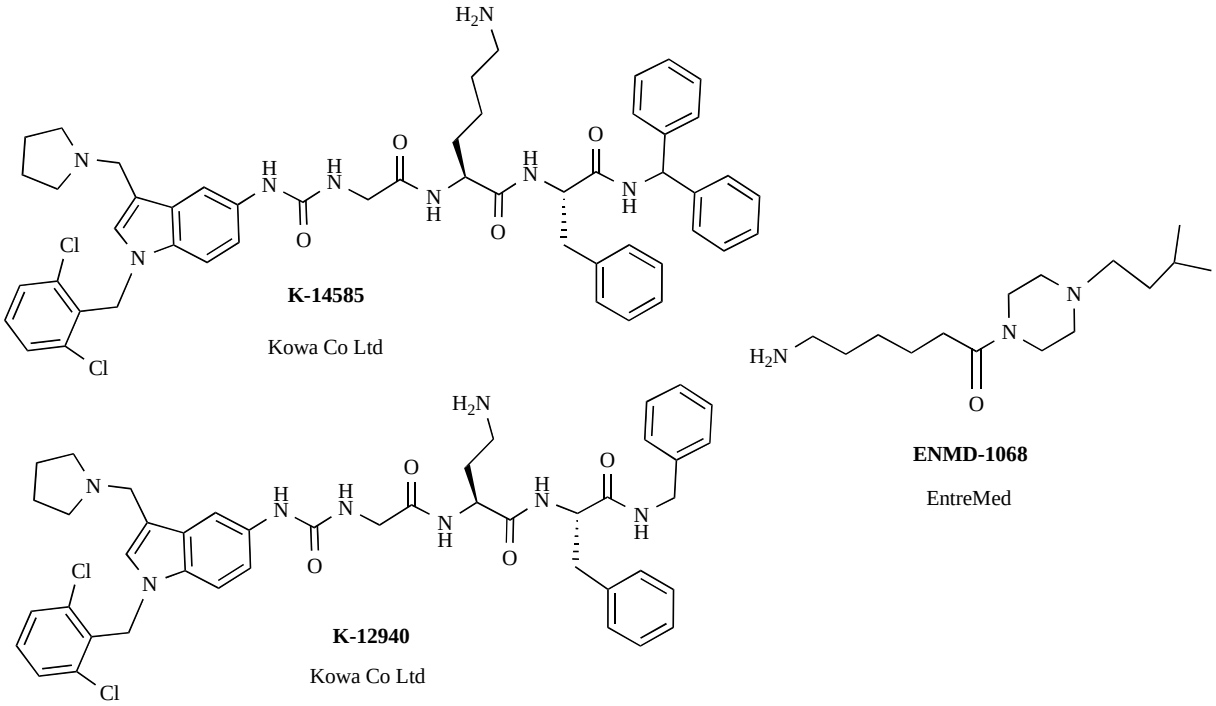

Fig. (4). Chemical structure of PAR-2 antagonists.

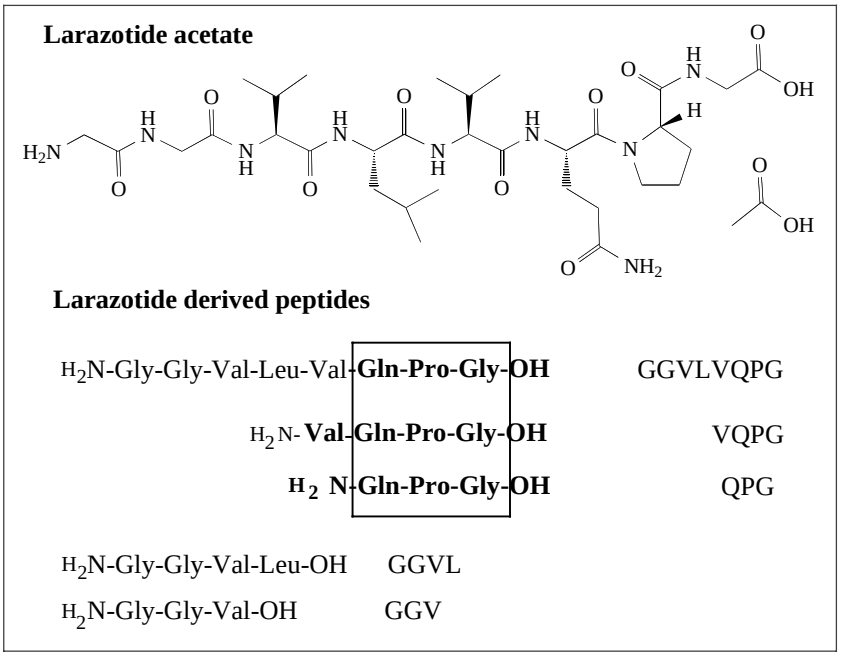

Fig. (5). Structure of zonulin antagonist, larazotide, and derived peptides.

thetic zonulin octapeptide inhibitor whose sequence corresponds to that of the zonulin receptor-binding domain.

This peptide binds to the zonulin receptor present on the apical membrane of enterocytes in a competitive manner, preventing its activation by zonulin and subsequent intracellular signalling leading to opening of TJs. Since the ZOT/zonulin receptor is expressed in the jejunum and distal ileum, the effect of AT-1001 will be restricted to the small intestine. AT-1001 was able to counteract any increase in small intestinal permeability and TER reduction produced by administration of a zonulin agonist. Larazotide also antagonized actin cytoskeleton rearrangement that resulted from exposure to gliadin.

In a pilot study, AT-1001 was well tolerated with no increase in adverse events compared to placebo, the majority of adverse events being GI disorders, mostly frequenty diarrhoea [231]. A trend to reduce intestinal barrier permeability, IFN-γ production and GI symptoms after acute gluten exposure was observed although this must be confirmed. Randomized, double blind, placebo-controlled, phase II trials

were begun in patients with celiac disease to evaluate the safety, tolerability and efficacy of dose ranging of larazotide during a gluten challenge. In the first trial, the primary endpoint (a significant decrease of the lactulose to mannitol ratio vs. the placebo group) was the efficacy of multiple doses of larazotide acetate (1, 4 and 8 mg daily) in preventing intestinal permeability changes induced by a 6-week gluten challenge. Although the primary endpoint was not reached, AT-1001 treated patients showed a significant improvement in symptoms. Larazotide was considered to be safe and well-tolerated in these patients. Other clinical trials are ongoing but the primary endpoints are response to gluten and variations in villous height to crypt depth. Larazotide acetate was listed as in phase I for CD and IBS. Preclinical studies showed that when administered orally in IL10$^{-/-}$ mouse, a model of CD, the compound induced a marked reduction in small intestinal permeability, followed by a significant reduction of colonic mucosal permeability, prevention of cytokine production, and a significant reduction of histological signs of disease [232]. These observations suggest that prevention of the barrier defect can attenuate the disease, implying that intestinal permeability is a central event in the pathogenesis of IBD. It is important to note that colitis was ameliorated but not resolved by the zonulin inhibitor.

Alba Therapeutics started developing larazotide acetate for type I diabetes, and a phase I development was claimed. However, type I diabetes indication has been withdrawn from Alba's pipeline. This may be due to the fact that only a few diabetics patients with high serum levels of zonulin are potentially responsive to a zonulin antagonist therapy. Moreover, clinical utility of intestinal permeability assessment must still be determined.

The next step in the development of a zonulin antagonist will be the design and synthesis of small nonpeptide compounds. Alba Therapeutics is currently investigating novel shorter peptides inhibitors of TJ opening, deriving from the zonulin antagonist larazotide [233, 234]. Tetra- and tripeptides can in fact be obtained from hydrolysis of larazotide by gastric and intestinal fluids. The tripeptide, QPG, still maintained some protective activity against TER reduction, increased paracellular permeability and actin network disorganization. Structure activity relationship studies showed that the glutamine residue cannot be substituted by proline, threonine or aspartic acid but glutamine - pyroglutamic acid substitution is possible while proline or glycine substitutions lead to loss of activity.

CONCLUSIONS

Barrier loss as a consequence of reduced TJ and AJ proteins expression and/or altered distribution is a common feature of several intestinal and nonintestinal diseases. It is however not yet clear if inflammation causes barrier defect or inversely. Several extracellular stimuli, like cytokines, pathogens and proteases, and intracellular mediators includ-

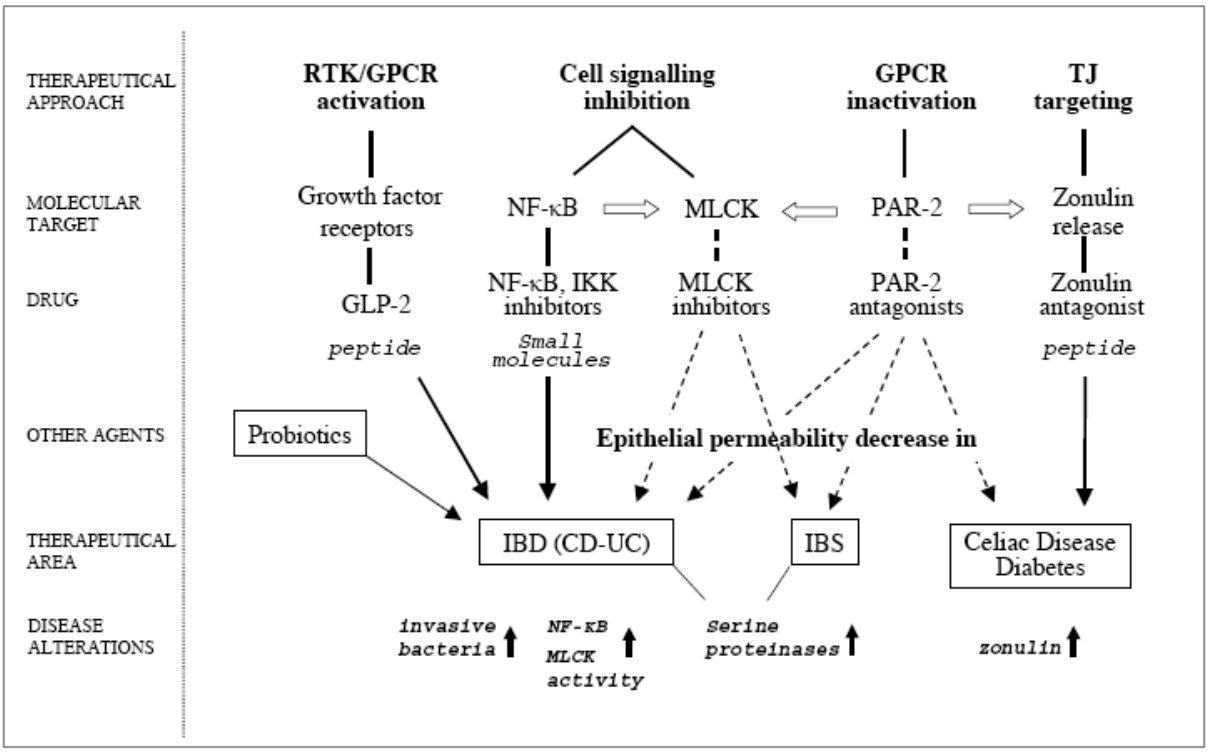

Fig. (6). Possible therapeutical approach for reducing intestinal permeability in disease.

Activation of growth factor receptors, including the GLP-2 receptor, inactivation of PAR-2, and probiotics reduce epithelial permeability in preclinical studies. Other molecular targets identified are key intracellular mediators (MLCK and NF-kB) and zonulin. Some compounds are currently in clinical development for treating IBD and celiac disease while MLCK inhibitors and PAR-2 antagonists are not available. White arrows show stimulatory interaction between pathways, black arrows indicate the drugs in clinical development, dashed arrows the drugs that need to be developed.

ing NF-κB- and MLCK–dependent processes have a direct influence on barrier function. However, the epithelial protective effect of growth factors, probiotics and inhibitors of intracellular signalling molecules is not dependent on the nature of the insult and potential targets for therapeutical intervention have been identified that are capable of producing similar effects as conventional medication in animal models (Fig. **6**).

Since NF-κB activation is also an index of inflammation, inhibitors of this signalling pathway show promising use as monotherapy in diseases with barrier dysfunction and an important inflammatory component like IBD. The zonulin antagonist larazotide, by directly targeting the cause of TJ opening, is a promising therapy for celiac disease while PAR-2 antagonists will have a great influence in IBS where emotional stress greatly affects the course of the disease. Conversely, probiotics warrant further study as potential curative drugs in IBD, IBS or diarrhoea, however they could represent a good therapeutic option for preventing relapse. Although the blocking of the intestinal epithelial barrier alone could be a limited approach in multifactorial recurrent diseases, however, its combination with other treatments appears promising. Future trials to determine the effects of monotherapy versus combination therapy will be important in guiding this strategy in order to evaluate the potential reduction of concomitant therapies and their long-term side effects, and to increase maintenance of remission.

Considering the high prevalence of resistance and recurrence in IBD and the lack of effective therapies in IBS and celiac disease, even a slight reduction in symptoms could have positive public health consequences. Identification of compounds that effectively decrease TJ permeability is an attractive area for drug discovery and development.

ABBREVIATIONS

CD = Crohn's Disease

DSS = Dextran sodium sulfate

EHEC = Enterohemorrhagic *Escherichia coli*

EPEC = Enteropathogenic *Escherichia coli*

IBD = Inflammatory Bowel Disease

IBS = Inflammatory Bowel Syndrome

IFN-γ = Interferon-gamma

IκB = Inhibitory kappa B

IKK = IkappaB kinase

JAM = Junctional adhesion molecule

MLC = Myosin light chain

MLCK = Myosin light chain kinase

NF-κB = Nuclear factor kappa B

PAR = Protease-activated receptor

TER = Transepithelial resistance

TJ = Tight Junction

TNBS = Trinitrobenzene sulfonic acid

TNF-α = Tumour necrosis factor-alpha

UC = Ulcerative Colitis

ZO = Zonula occludens

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