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Reversible opening of the blood-brain barrier by claudin-5-binding variants of *Clostridium perfringens* enterotoxin's claudin-binding domain

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ABSTRACT

The blood-brain barrier (BBB) prevents entry of neurotoxic substances but also that of drugs into the brain. Here, the paracellular barrier is formed by tight junctions (TJs) with claudin-5 (Cldn5) being the main sealing constituent. Transient BBB opening by targeting Cldn5 could improve paracellular drug delivery. The non-toxic C-terminal domain of *Clostridium perfringens* enterotoxin (cCPE) binds to a subset of claudins, e.g., Cldn3, -4. Structure-based mutagenesis was used to generate Cldn5-binding variants (cCPE-Y306W/S313H and cCPE-N218Q/Y306W/S313H). These cCPE-variants were tested for transient TJ opening using multiple *in vitro* BBB models: Primary porcine brain endothelial cells, coculture of primary rat brain endothelial cells with astrocytes and mouse cerebEND cells. cCPE-Y306W/S313H and cCPE-N218Q/Y306W/S313H but neither cCPE-wt nor cCPE-Y306A/L315A (not binding to claudins) decreased transendothelial electrical resistance in a concentration-dependent and reversible manner. Furthermore, permeability of carboxyfluorescein (with size of CNS drugs) was increased. cCPE-Y306W/S313H but neither cCPE-wt nor cCPE-Y306A/L315A bound to Cldn5-expressing brain endothelial cells. However, freeze-fracture EM showed that cCPE-Y306W/S313H did not cause drastic TJ breakdown. In sum, Cldn5-binding cCPE-variants enabled mild and transient opening of brain endothelial TJs. Using reliable *in vitro* BBB models, the results demonstrate that cCPE-based biologics designed to bind Cldn5 improve paracellular drug delivery across the BBB.

1. Introduction

The blood-brain barrier (BBB) prevents entry of neurotoxic substances into the brain. It is formed by an intimate contact between capillary endothelial cells, astrocytes and pericytes. Additionally it is influenced by neurons and microglia. However, the actual barrier is formed by the endothelial cells which contain efficient efflux transporters, metabolic enzymes and tight junctions (TJs) [1]. The TJs of the BBB represent one of the tightest paracellular barriers in the body [2,3]. The backbone of TJs is formed by the tetraspan transmembrane proteins of the claudin (Cldn) family, which are essential for regulation of paracellular permeability [4,5] and are expressed in a tissue-specific manner [6]. Claudins can be grouped according to the extent of their similarity in sequence and structure into classic and non-classic claudins [7,8]. Functionally, barrier- and channel-forming claudins, which form charge- and size-selective paracellular channels, can be distinguished [9]. The claudin composition of TJs mainly determines the paracellular permeability of tissue barriers in a given organ [9].

The main constituent of BBB TJs is Cldn5 [10], which seals the

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paracellular gap against molecules smaller than 800 Da *in vivo* [11]. Also *in vitro,* it was shown that Cldn5 forms a barrier restricting permeability for small solutes [12–14]. However, Cldn5 is not limited to tight endothelia of the BBB but also expressed in most other endothelial cells [15] and some epithelial cells [13,16]. It is assumed that in addition to Cldn5, presence of other factors, e.g. Cldn1, -3 and -12, occludin and tricellulin contribute to the very tight paracellular barrier of the BBB [11,17,18]. In particular, involvement of Cldn3 expression in induction and maintenance of BBB characteristics during development and loss of Cldn3 under pathological conditions was reported [19,20]. Copolymerization of Cldn3 and Cldn5 is sufficient for reconstitution of TJ-strands with an BBB-typical ultrastructure [21] and claudin-subtype specific *cis*-dimerization was suggested to contribute to this TJ morphology [22].

TJs restrict paracellular drug delivery across tissue barriers, like the BBB, the intestinal epithelium or the skin, limiting the clinical use of many hydrophilic bioactive compounds and biologics [23]. Diverse strategies were used to improve transcellular [24] or paracellular drug delivery [23,25]. For improvement of the latter across barriers in gut, brain or skin diverse TJ-modulators and absorption enhancers e.g. sodium caprate, chitosan or alkylglycerols were suggested [26–29]. In contrast to these absorption enhancers, claudin-binding peptides or proteins have the advantage to selectively and directly address structural components of TJs. Cldn1-derived peptides [30] were used to open the blood-nerve- and other endothelial and epithelial barriers [31,32].

A promising TJ-modulator is provided by *Clostridium perfringens* enterotoxin's (CPE) C-terminal claudin binding domain (cCPE). CPE forms cytotoxic pores in the membrane of enterocytes and causes *Clostridium perfringens* type A food poisoning [33–35]. In contrast, cCPE is not cytotoxic but still binds with nanomolar affinity to a subset of claudins (Cldn3, -4, -6, -7, -9) [36–39]. cCPE efficiently increases paracellular permeability for solutes up to 10 kDa by removing claudins from TJs in claudin-subtype- and in turn tissue-selective manner [35,39–41].

The mechanism of cCPE-claudin interaction was analyzed in detail [8,35]. Mutagenesis studies defined Y306, Y310, Y312 and L315 in cCPE [42–44] and a motif within the extracellular loop 2 (ECL2) of claudins ($N^{(P-1)}$ P/$S^{(P)}$ L/M/V/I/$S^{(P+1)}$ V/$T^{(P+2)}$ P/A/N/$D^{(P+3)}$) to be responsible for the claudin-subtype-specific interaction [37,39,45]. In addition, the placement and orientation of ECL2 of Cldn3-/4 alongside the binding pocket of cCPE was revealed [39]. This was confirmed by complex crystal structures [46,47], which showed that apart of ECL2 also the ECL1 participates in the interaction with cCPE.

Structure-based mutagenesis was used to generate cCPE variants binding preferentially to Cldn3 (cCPE-L223A/D225A/R227A, cCPE-R227A), Cldn4 (cCPE-L254A/S256A/I258A/D284A) or binding strongly to Cldn5 (cCPE-Y306W/S313H) [39,45]. In this context it has to be noted that cCPE-wt does not bind to Cldn5. In addition, phage display was used to select a broad specificity claudin binder (cCPE-S305P/S307R/S313H) [48]. Such cCPE-variants have the potential to be applied as TJ-modulators e.g. addressing Cldn3 and Cldn4 in the gut, Cldn1 in the skin and Cldn5 in the BBB.

Knock-out of Cldn5 in mice opened the BBB for molecules smaller than 800 Da without total TJ breakdown and without edema formation [11]. siRNA targeting Cldn5 transiently increased BBB permeability to molecules up ~1 kDa in mice [49], reduced focal cerebral edema and improved cognitive function in mice after traumatic brain injury [50]. Hence, transient modulation of Cldn5 at the BBB *in vivo* is a promising therapeutic strategy, e.g. for, but not limited to, improvement of paracellular drug delivery to the brain.

Here we show that in contrast to cCPE-wt, the Cldn5-binding cCPE-Y306W/S313H reversibly increases paracellular permeability of small solutes across BBB models. Furthermore, with design and use of cCPE-N218Q/Y306W/S313H the Cldn5-binding properties were further optimized. We suggest application of cCPE-based biologics to improve drug delivery into the brain.

2. Material and methods

2.1. Plasmids

Plasmids encoding GST-CPE194-319 (GST-cCPE) fusion proteins cCPE-N218Q/Y306W/S313H, cCPE-N218E/Y306W/S313H, cCPE-L254Q/Y306W/S313H and cCPE-L254H/Y306W/S313H were generated by site-directed mutagenesis of pGEX-4T1-cCPE-wt, as described earlier [45].

2.2. Preparation of GST-cCPE fusion proteins

Transformation of expression vectors pGEX-4T1-cCPE-wt, pGEX-4T1-cCPE-S313H, pGEX-4T1-cCPE-Y306A/L315A, pGEX-4T1-cCPE-Y306W/S313H, pGEX-4T1-cCPE-N218Q/Y306W/S313H, pGEX-4T1-cCPE-N218E/Y306W/S313H, pGEX-4T1-cCPE-L254Q/Y306W/S313H, and pGEX-4T1-cCPE-L254H/Y306W/S313H for expression of the GST-cCPE fusion proteins in *E. coli* BL21 (DE3) and protein purification using glutathione-sepharose 4B beads (Amersham, Buckinghamshire, UK) was similar as described previously [39,45].

2.3. Cell culture for blood-brain barrier studies

Three different *in vitro* models of the BBB from three different species and with distinctly different barrier properties were used. Mouse cerebEND cells were a kind gift from Prof. Carola Förster and described in 2006 for the first time [51]. CerebENDs were cultivated as mono-culture in an incubator at 37 °C, 95% humidity and a 5% CO_2 and 95% air atmosphere as previously published [52]. In brief, cells were seeded with a density of 4×10^4 cells/cm^2 on collagen IV coated 12-well inserts and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 1% penicillin/streptomycin. Medium exchange was accomplished every other day, experiments with cCPE proteins started on day 13 after seeding, 100 nM hydrocortisone was supplied additionally at days 7–13. Primary rat brain microvascular endothelial cells (pRBMECs) as well as rat astrocytes (AST) were provided from Biopredic (Rennes, France) and grown as recently reported in detail [53]. For this model, 5×10^3 astrocytes per cm^2 were seeded on day 1 on the bottom of 12-wells and combined with freshly seeded pRBMECs (6.3×10^4 cells/cm^2) into the inserts on day 2. Every other day, half of the medium was renewed for the astrocytes and the total volume was changed for pRBMECs. Experimental start was between day 9–11 dependent on the transendothelial electrical resistance (TEER) of the coculture pRBMEC/AST which was aimed at 90 $\Omega \times$ cm^2. As third BBB model porcine brain microvascular endothelial cells (pPBMEC) were grown on inserts as previously described [54]. After seeding 2×10^5 cells/cm^2 on collagen G coated inserts, growth medium (M199 supplemented with 1% penicillin/streptomycin, 10% newborn calf serum, 10 mM Hepes, 0,8 mM L-glutamine) was changed to a serum-free cultivation medium (DMEM/Ham's F12 supplement with 1% penicillin/streptomycin, 2 mM L-glutamine, 25 mM Hepes, 550 nM hydrocortisone) on day 4. On day 5 the pPBMEC experiments with the cCPE proteins started. Before conduction of the cCPE studies, TEER was checked of every cell model with chopstick electrodes after 30–45 min temperature equilibration at RT after medium exchange as previously reported [55]. For the measurement of TEER progression, inserts