Journal of Controlled Release 185 (2014) 88–98

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Safety, efficacy, and molecular mechanism of claudin-1-specific peptides to enhance blood–nerve–barrier permeability

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ARTICLE INFO

Article history:
Received 24 February 2014
Accepted 15 April 2014
Available online 26 April 2014

Keywords:
Drug delivery
Peripheral nerve
Peptide
Biocompatibility
In vivo test
In vitro test

ABSTRACT

The blood–nerve barrier consists of the perineurium and endoneurial vessels. The perineurial barrier is composed of a basal membrane and a layer of perineurial cells sealed by tight junction proteins preventing *e.g.* application of analgesics for selective regional pain control. One of the barrier-sealing proteins in the blood–nerve barrier is claudin-1. Therefore, the claudin-1-peptidomimetics (C1C2), derived from the first extracellular loop (ECL1) on claudin-1 was developed. In this study, we further evaluated the expression of tight junction proteins in the perineurium in Wistar rats and characterized the specificity, *in vivo* applicability, mechanism of action as well as the biocompatibility of C1C2. In the perineurium, claudin-19, tricellulin and ZO-1, but no claudin-2, 3, 8 and -11 were expressed. C1C2 specifically bound to the ECL1 of claudin-1 and fluorescent 5,6-carboxytetramethylrhodamine-C1C2 was rapidly internalized. Opening the perineurium with C1C2 reduced the mRNA and protein expression of claudin-1 and increased small and macromolecule permeability into the peripheral nerve. Application of C1C2 facilitated regional analgesia using μ-opioid receptor agonists like DAMGO or morphine without motor impairment in naïve rats as well as rats with hind paw inflammation. In contrast the control peptide C2C2 derived from ECL1 on claudin-2 did neither open the barrier nor facilitated opioid-mediated regional analgesia. C1C2 delivery was well tolerated and caused no morphological and functional nerve damage. C1C2 effects could be reversed by interference with the wnt-signal-transduction pathway, specifically the homeobox transcription factor cdx2, using a glycogen-synthase-kinase-3 inhibitor. In summary, we describe the composition of and a pathway to open the perineurial barrier employing a peptide to deliver hydrophilic substances to the peripheral nerve.

1. Introduction

The blood–nerve barrier (BNB) consists of the perineurium surrounding peripheral nerves and the endothelium of endoneurial blood vessels. The perineurium is composed of perineurial cells connected by tight junction proteins like occludin, claudin-1, claudin-3, and the intracellular scaffolding protein ZO-1 [1]. This barrier limits paracellular drug transport [2,3]. Different approaches exist to modulate the barrier (for review see [4]). Recently, we have shown that the perineurial barrier can be opened by perineurially injected hypertonic saline. Hypertonic saline triggers the release of metalloproteinase-9 (MMP-9), which binds to Low Density Lipoprotein Receptor-related Protein-1 (LRP-1) via its hemopexin domain and leads to the downregulation of claudin-1 in the perineurium [3,5]. Claudin-1 is essential for BNB sealing because siRNA targeting claudin-1 also opens the barrier for drug delivery [3,5].

Selective blockade of nociceptive nerve fibers in mixed peripheral nerves is a major experimental and clinical challenge. Co-injection of the transient receptor potential vanilloid-1 (TRPV1) channel agonist capsaicin and the quaternary amide local anesthetic QX-314 selectively targets nociceptive neurons [6]. However, capsaicin induces a brief, but intense hyperalgesia on injection and is, therefore, clinically not usable.

Abbreviations: BNB, blood–nerve–barrier; CFA, complete Freund's adjuvant; EBA, Evans blue-labeled albumin; ECL, extracellular loop; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol] enkephalin; GSK-3, glycogen synthase kinase 3; IDV, integrated density value; i.p, intraperitoneal; LRP-1, Low Density Lipoprotein Receptor-related Protein-1; MOR, μ-opioid receptor; NLX, naloxone; PBS, phosphate buffered saline; TAMRA, 5,6-carboxytetramethylrhodamine.

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http://dx.doi.org/10.1016/j.jconrel.2014.04.029
0168-3659/

Alternatively, sensory and nociceptive fibers could be targeted by selective voltage gated sodium channel (Na_V 1.7) blockers like ProToxin-II or hydrophilic opioids such as [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) or morphine targeting μ-opioid receptors (MOR). Both classes of drugs however are ineffective when applied at the nerve because they do not cross the BNB [5].

To induce transient and selective permeability of the BNB, claudin-1 peptidomimetics were developed [7] as specific enhancers opposed to conventional absorption enhancers as caprate or chitosan [8–10]. One claudin-1 peptidomimetic derived from the first extracellular loop (ECL1) of murine claudin-1$^{53–80}$ compromises the intestinal barrier function both *in vitro* and *in vivo* [11]. This peptide, however, is difficult to be kept in solution. Therefore, we developed a modified peptide, C1C2 derived from claudin-1$^{53–81}$, which opens the barrier in Caco-2 cells and facilitates tetrodotoxin-induced analgesia *in vivo* [7]. However, *in vivo* function including the mechanism by which C1C2 opens the BNB as well as potential neurotoxicity is an unresolved issue.

A key intracellular regulator of tight junction protein expression and barrier function appears to be glycogen synthase kinase 3 (GSK-3) [12–14]. GSK-3 regulates transcription factor enhancers (*e.g.*, β-catenin or homeobox transcription factor cdx2) or suppressors (*e.g.* Snail [15]), which are involved in tight junction protein (including claudin-1) transcription and translation [16–18]. Inhibiting GSK-3 by siRNA or synthetic inhibitors modified claudin expression and barrier function [12,14,19].

This study here was designed to (i) characterize C1C2 in contrast to C2C2 (a modified sequence from the ECL1 of claudin-2) as a selective modifier of claudin-1-mediated barrier permeability, (ii) specifically target hydrophilic opioids to sensory and nociceptive neurons by C1C2 *in vivo* and thereby enhance analgesia, (iii) examine the biocompatibility and potential neurotoxic side-effects of C1C2, and (iv) to decipher the molecular mode of action of C1C2 *in vivo*.

2. Materials and methods

2.1. Peptide synthesis

Murine C1C2 modified peptide derived from claudin-1$^{53–81}$ in which Cys was replaced by Ser as underlined in the following sequence (SSVSQSTGQIQSKVFDSLLNLNSTLQATR-NH_2) and C2C2 (analogously modified sequence from the ECL1 of claudin-2) were assembled automatically (ACTIVO-P11, Activotec.com, Cambridge, UK) by the Fmoc solid-phase method in a batch-wise mode at elevated temperature (couplings for 10 min at 70 °C/Fmoc-removal for 7 min at 70 °C) on Peg-resins (SRAM, 0.2 mmol/g, Rapp-Polymere (Sigma-Aldrich, Germany)) as described before [7]. 5,6-Carboxytetramethylrhodamine (TAMRA) was introduced N-terminally after Fmoc-removal. Firstly, TAMRA (5 equiv. per Peg-resin, 0.1 mmole) was activated by 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (0.1 mmole) and *N*,*N*-diisopropylethylamine (0.2 mmole) and then coupled for 40 min in dimethylsulfoxide with the respective resin. The cleavage from the resin occurred with trifluoroacetic acid (TFA)/H_2O (9/1) for 3 h at room temperature. Then, peptides were precipitated with cold diethyl ether and purified by preparative reversed-phase high performance liquid chromatography (C-18 column) (Dionex, USA) with acetonitrile gradients in aqueous 0.1% TFA. Purified peptides were quantified and characterized by liquid chromatography (ACQUITY UPLC system, C18 column) electrospray time-of-flight mass spectrometry (LCT Premier, Waters, Germany) which showed a purity >95% (220 nm) and gave the expected masses. Molecular weights were 3108 Da for C1C2, 2987 Da for C2C2, 3520 Da for TAMRA-C1C2, and 3399 Da for TAMRA-C2C2. Peptides were dissolved in the necessary medium or Hank's buffered salt solution except for fluorescent TAMRA-C1C2 or TAMRA-C2C2, which were dissolved in a solution containing 0.1% dimethyl sulfoxide and/or pluronic ®F-127 (0.012%) (Sigma-Aldrich, Germany) [20].

2.2. Animals

Animal protocols were approved by the animal care committee of the provincial government of Wuerzburg and are in accordance with the International Association for the Study of Pain [21]. Male Wistar rats weighing 180–220 g were always injected under brief isoflurane anesthesia as described below. Experiments were conducted at indicated time points. For the inflammatory pain model, rats were injected intraplantarly with 150 μl complete Freund's adjuvant (CFA, Calbiochem/Merck, Darmstadt, Germany) in the right hind paw [5,22].

2.3. Perineurial injection at sciatic nerve

Under brief isoflurane anesthesia, the right sciatic nerve was located using a 22 G atraumatic needle (to avoid intraneural injection) connected to a nerve stimulator (Stimuplex Dig RC; Braun, Melsungen, Germany) as previously described [5,23]. The total injected volume was 300 μl maximum. Peptides were dissolved in 0.9% NaCl and injected perineurially. 400 μM C1C2 or C2C2 (=3.7 mg/300 μl), 30 μg DAMGO (30 μg/150 μl) and/or naloxone (NLX, 0.56 ng/150 μl, both Sigma Aldrich Chemical, St. Louis, MA, USA) were applied as outlined in the result section. In certain experiments rats were treated daily intraperitoneally (i.p.) with the GSK-3α/β inhibitors, SB216763 (0.06–0.6 mg/kg body weight) or SB415286 (0.1–1 mg/kg body weight, Sigma Aldrich). Doses were chosen by pilot experiments and references in the literature [5,24].

2.4. Western blotting

After indicated treatments sciatic nerves were taken and homogenized in lysis buffer for Triton X-100 soluble proteins (25 mM Tris pH 7.6, 120 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 25 mM NaF, 1% (*v*/*v*) Triton X 100) containing protease inhibitors (Complete, Roche Applied Science) [5]. Cytosol fractions were obtained by homogenization with minipistil and sonification (3 × 5 s/3 s break), followed by a centrifugation at 4500 ×*g* for 10 min and subsequent centrifugation of the remaining supernatant at 40,000 ×*g* for 30 min. The Triton X-100-insoluble pellet containing membrane fraction was resuspended in an equal volume of extraction buffer (25 mM Hepes pH 7.6, 2 mM EDTA, 25 mM NaF, 1% (*w*/*v*) SDS). Extracted protein was diluted in lysis buffer and incubated with BCA protein assay reagent (Pierce, Rockford, IL, USA) for quantification on a plate reader (Tecan, Grödig, Austria). Aliquots of protein were mixed with sodium dodecyl sulfate (SDS) containing buffer (Laemmli), denatured at 95 °C for 5 min, fractionated on SDS polyacrylamide gels and subsequently blotted onto PVDF membranes (PerkinElmer, Boston, MA, USA). Proteins were detected using specific antibodies rabbit polyclonal anti-claudin-1 (# 51-9000, 1:1000 Life Technologies, monoclonal mouse anti-claudin-2 (#32-5600, 1:1000, Life Technologies), polyclonal rabbit anti-claudin-3 (#34-1700, 1:1000, Life Technologies), oligoclonal rabbit anti-claudin-8 (#710223, 1:1000, clone 6HCLC, Life Technologies), polyclonal rabbit anti-claudin-11 (#36-4500, 1:1000, Life Technologies) polyclonal goat anti-claudin-19 (sc-162689, 1:500, Santa Cruz), rabbit polyclonal anti-ZO-1 (#61-7300, 1:200, Life Technologies), polyclonal rabbit anti-tricellulin (#488400, 1:200, Life Technologies), polyclonal rabbit anti-cdx2 (AB4123,1:500, Abcam) and as protein loading control β-actin (#A3854, 1:20,000, Sigma Aldrich). Peroxidase conjugated goat anti-rabbit IgG, goat anti-mouse IgG and the chemiluminescence detection system Lumi-LightPLUS Western blotting kit (Roche, Mannheim, Germany) were used to detect bound antibodies.

2.5. Immunofluorescence and confocal microscopy

Immunostaining of sciatic nerves was performed on control nerves, as well as 24 or 48 h after perisciatic injection of C1C2 or after treatment with C1C2 and a GSK-3 inhibitor. Frozen tissue was cut in sections, fixed,