Construction of cCPE site-directed mutagen plasmids

This study involved the modification of the peptide sequence of cCPE in an attempt to improve its affinity and selectivity for the Claudin-4 target molecule. The DNA clone template used to produce the point-mutation forms was a cCPE-HIS plasmid (pETH10PER), comprising the COOH-terminal fragment of CPE (aa 184–319) linked to a HIS fusion peptide, generated and supplied by Yasuhiko Horiguchi (*1*).

First, we produced a 194-319 fragment, removing the N-terminal 10 amino-acids found not to be crucial for claudin-4 targeting, and to improve peptide monomer stability when synthesized in E coli (*2*), and further to result in a reduction in peptide size with the aim to produce more rapid pharmacokinetics and allow faster imaging. Additionally, a single cysteine residue was added to the sequence to allow site-specific conjugation of the peptide to a maleimide-DTPA chelator. Amplification of the truncated cCPE from the pETH10PER plasmid (*1*), using standard PCR methodology, was performed and the clone was validated by nucleotide sequencing (Source Bioscience, Oxford UK). Oligonucleotide primers were designed in-house and synthesized by Invitrogen. Twenty-five cycles of PCR amplification was performed using the reagents and protocols of the AccuPrime Taq DNA Polymerase System (# 12339-016, Invitrogen Thermo Fisher Scientific Waltham USA). Nhe1/Nde1 restriction digest of the PCR amplicon facilitated ligation, using T4 DNA ligase (New England Biolabs, Ipswich USA), into the original pETH10PER/pET16b vector. The ligated plasmid was transformed and propagated initially into Stbl-2 cells (Invitrogen).

From this 194-319 plasmid clone a number of variants were created using QuikChange II Site-Directed Mutagenesis (SDM) (kit #200523, Agilent Technologies Santa Clara USA), or Gene Synthesis (GenScript, Piscataway USA). The SDM process was performed using the

manufacturer's reagents and protocols. In brief, two oligonucleotide primers, both containing the desired mutation and complementary to opposite strands of the vector, were synthesized for each mutation required. PfuUltra HF DNA polymerase was used to extend the entire plasmid vector template. Treatment with Dpn I endonuclease digested the original parental DNA template, and the remaining nicked vector DNA containing the desired mutations was then transformed into XL1-Blue supercompetent cells. The mutated cCPE was then validated by nucleotide sequencing.

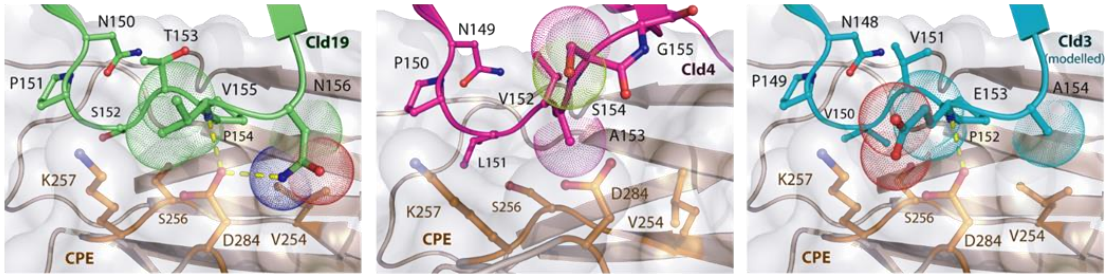
Clones S313A and S307A+N309A+S313A were created separately by SDM and Gene Synthesis, respectively – and were based on a previous report (*3*) in which peptide substitution experiments identified a number of forms exhibiting enhance affinity for claudin-4 in a LDH release assay: S313A exhibited the greatest single point mutation effect, and S307A+N309A+S313A were a combination of the greatest effects.

Clones L254F+K257D and D284A were separately generated by SDM, and were designed in-house with a view to improving cCPE specificity for claudin-4 over claudin-3. The mutated cCPE clones were all validated by nucleotide sequencing.

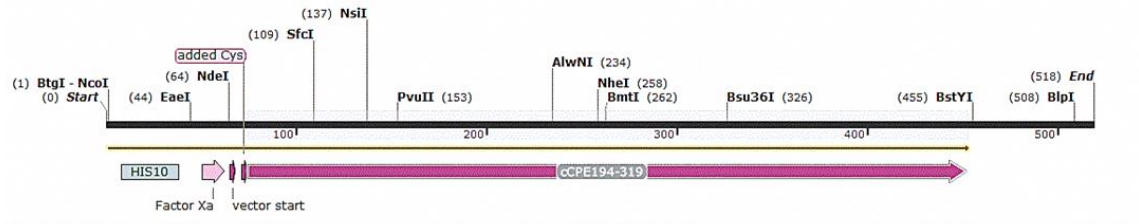
Supplemental Figures

Supplemental Figure 1:

A



B

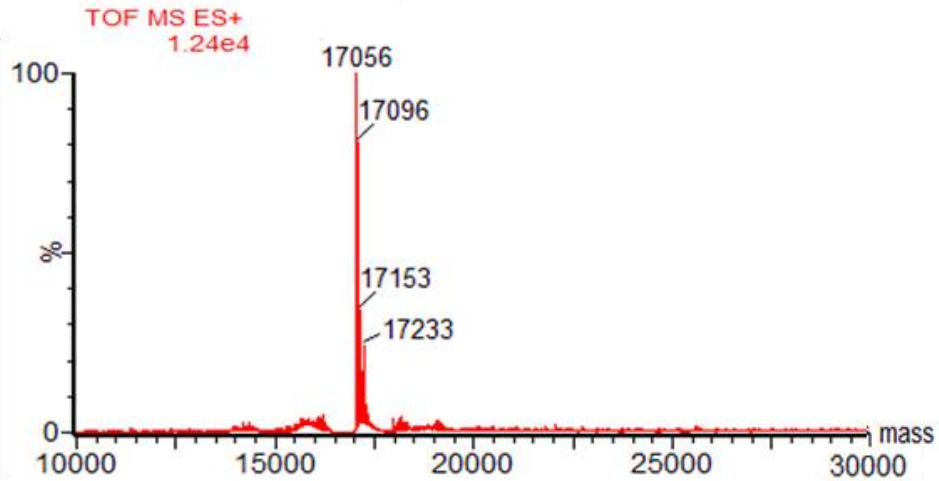


C

cCPE peptide	Sequence
$cCPE_{194-319}$	$(H_{10})cCPE_{194-249}$-KYRILASKIV DFNIYSNNFN NLVKLEQSLG DGVKDHYVDI SLDAGQYVLV MKANSSYSGNYPYSILFQKF
S313A	$(H_{10})cCPE_{194-249}$-KYRILASKIV DFNIYSNNFN NLVKLEQSLG DGVKDHYVDI SLDAGQYVLV MKANSSYSGNYPY**A**ILFQKF
S307A+N309A+S313A	$(H_{10})cCPE_{194-249}$-KYRILASKIV DFNIYSNNFN NLVKLEQSLG DGVKDHYVDI SLDAGQYVLV MKANSSY**A**G**A**YPY**A**ILFQKF
D284A	$(H_{10})cCPE_{194-249}$-KYRILASKIV DFNIYSNNFN NLVKLEQSLG DGVK**A**HYVDI SLDAGQYVLV MKANSSYSGNYPYSILFQKF
L254F+K257D	$(H_{10})cCPE_{194-249}$-KYRI**F**AS**D**IV DFNIYSNNFN NLVKLEQSLG DGVKDHYVDI SLDAGQYVLV MKANSSYSGNYPYSILFQKF

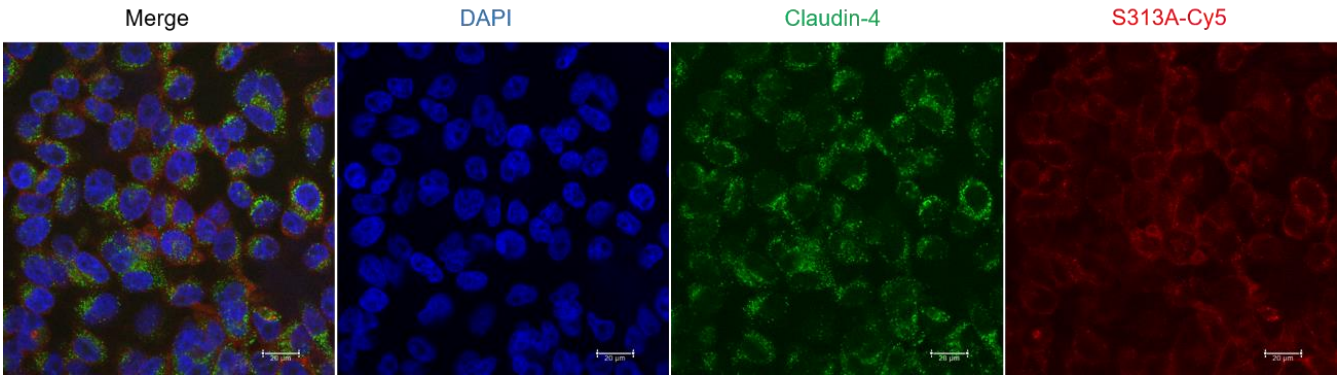
***Supplemental Figure 1*:** A) Modelling the interaction of claudin-19 (left), claudin-4 (middle) and claudin-3 (right) with wild type cCPE. B) Design of expression vector for cCPE peptides. C) cCPE mutant sequences [$(H_{10})cCPE_{194-249}$= MGHHHHHHHHHHSSGHIEGRHMLCDIEKEILDLAAATERLNLTDALNSNPAGNLYDWRSSNSYPWTQKLNLH LTITATGQ].

Supplemental Figure 2



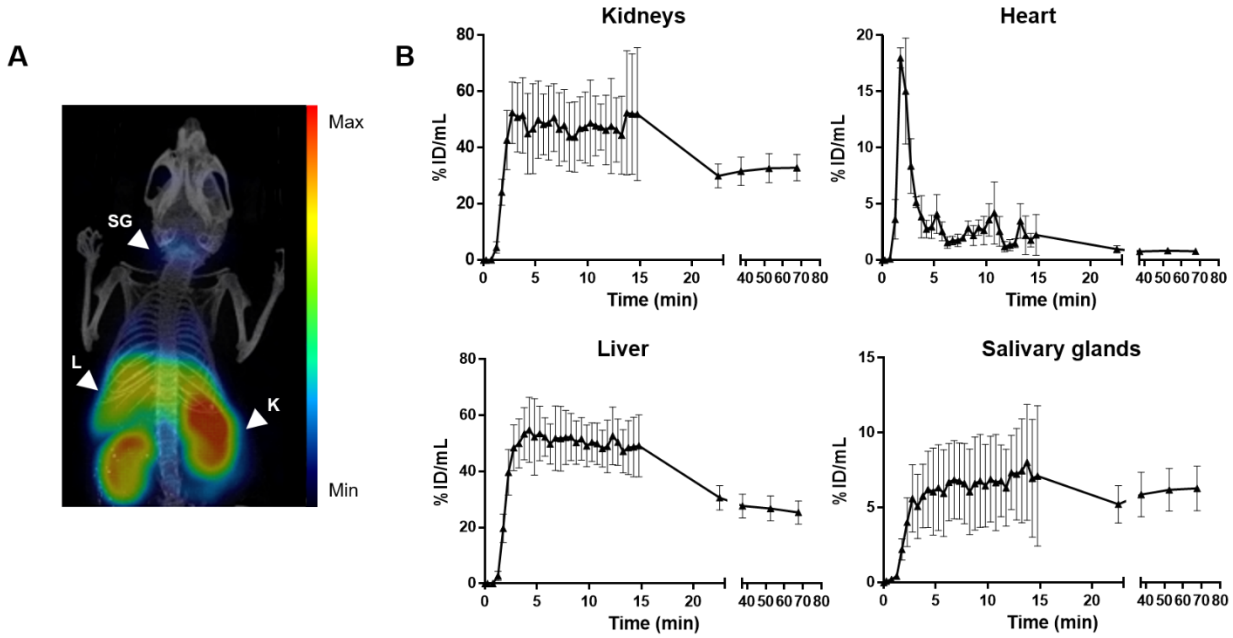
Supplemental Figure 2**:** Mass spectroscopy of cCPE^{S313A}.

Supplemental Figure 3



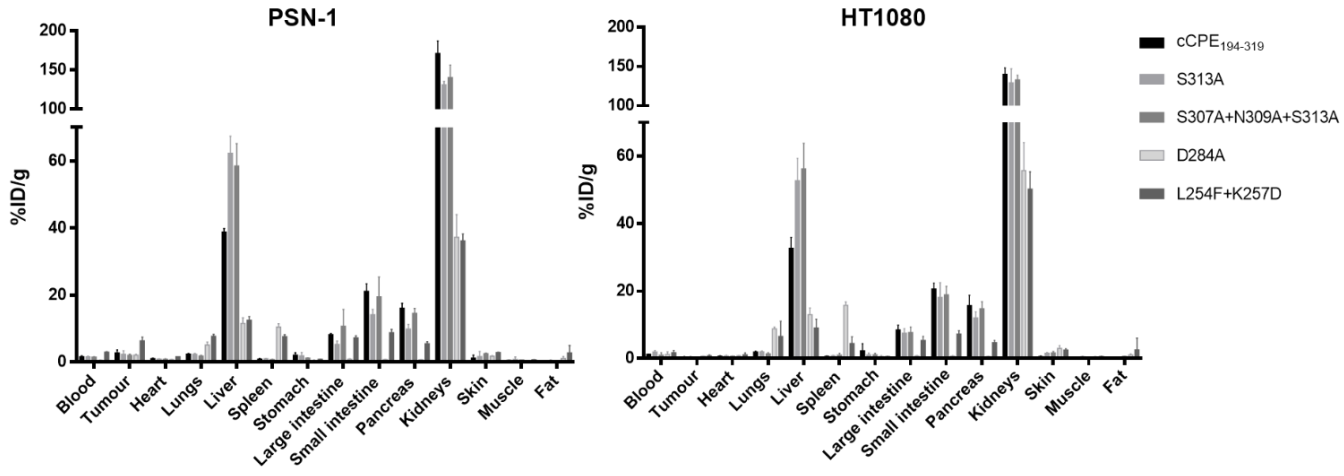
***Supplemental Figure 3*:** Confocal fluorescence microscopy images of PSN-1 cells stained with claudin-4 after incubation with S313A-Cy5 (100 nM, 30 min). Blue=DAPI; green=claudin-4; red=S313A-Cy5. Scale bar indicates 20 μm.

Supplemental Figure 4



***Supplemental Figure 4*:** A) Maximum intensity projection SPECT/CT image of wildtype mouse injected with ^{111}In-DTPA-cCPE$_{194-319}$ at 90 min post-injection of the tracer. SG= salivary glands; L= liver; K= kidneys. B) Time course of ^{111}In-DTPA-cCPE$_{194-319}$ accumulation over 90 min from time of injection in kidneys, heart, liver and salivary glands in wildtype mice (n=3).

Supplemental Figure 4



Supplemental Figure 4: Full *ex vivo* biodistribution of all ^{111}In-labelled cCPE peptides in mice bearing PSN-1 or HT1080 tumour xenografts.

References for supplemental information

1. Katahira J, Sugiyama H, Inoue N, Horiguchi Y, Matsuda M, Sugimoto N. Clostridium perfringens enterotoxin utilizes two structurally related membrane proteins as functional receptors in vivo. *J Biol Chem.* 1997;272:26652-26658.

2. Van Itallie CM, Betts L, Smedley JG, 3rd, McClane BA, Anderson JM. Structure of the claudin-binding domain of Clostridium perfringens enterotoxin. *J Biol Chem.* 2008;283:268-274.

3. Takahashi A, Komiya E, Kakutani H, et al. Domain mapping of a claudin-4 modulator, the C-terminal region of C-terminal fragment of Clostridium perfringens enterotoxin, by site-directed mutagenesis. *Biochem Pharmacol.* 2008;75:1639-1648.