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Design and synthesis of new cell penetrating peptides: Diffusion and distribution inside the cornea

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(Article begins on next page)

1 **Design and synthesis of new cell penetrating peptides: diffusion and**  
2 **distribution inside the cornea**

3

4

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8

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19

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21

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26 confocal microscopy  
27 corneal epithelium  
28 ocular drug delivery

29

## 30 1 Introduction

31 Cell penetrating peptides (CPPs) are short chain peptides (less than 30 amino acids) of natural,  
32 chimeric or synthetic origin and generally bearing a positive charge, able to penetrate cellular  
33 membranes <sup>1</sup>. The mechanism of cellular internalization is not completely understood: multiple  
34 pathways have been described, involving endocytosis (clatrin or caveolae mediated) or direct  
35 translocation through the formation of inverted micelles or transient trans-membrane pores <sup>1</sup>. The  
36 mechanism depends upon peptide sequence (i.e. amino acid units/residuals, hydrophobicity, total  
37 charge), experimental conditions and cell type <sup>2</sup>, and more than one mechanism is probably involved.  
38 Thanks to this intrinsic penetration ability and low toxicity, CPPs have been proposed as carriers to  
39 deliver different kinds of “cargo” to cells, where the cargo is represented by therapeutic molecules  
40 (small drugs, nucleic acids, proteins) or nanocarriers covalently or non-covalently bound <sup>2</sup>.  
41 CPPs have also been investigated to promote drug permeation across the skin <sup>3</sup> and across epithelial  
42 tissues such as nasal <sup>4</sup>, intestinal <sup>5</sup>, pulmonary <sup>6</sup> and across ocular tissues for the targeting of both  
43 anterior and posterior segments <sup>7 8 9</sup>.

44 The enhancement of trans-corneal transport for anterior segment targeting is a very important issue.  
45 In fact, the optimisation of drug transport could lead to higher drug availability and a lower instillation  
46 frequency with important outcomes on efficacy and patient compliance. Additionally, CCPs could  
47 give the opportunity to administer topically molecules with unfavourable penetration properties such  
48 as oligonucleotides and proteins. Johnson *et al.*, for instance, demonstrated in a murine model the  
49 capability of a penetrating peptide (POD) to carry green fluorescent protein in the corneal epithelium  
50 <sup>9</sup>.

51

52 The aim of the work was the design and synthesis of new CPPs and the comparison of their diffusion  
53 behaviour and distribution inside the cornea. Starting from the chemical structure of PEP-1 peptide,  
54 a well-known CPP <sup>10</sup> 6 mimotopic analogues were designed. The newly synthesized CPPs were  
55 labeled with carboxyfluorescein, to make them fluorescent. Using a validated *ex vivo* method  
56 (Pescina, Govoni et al. 2015) the ability of CPPs to diffuse through full thickness pig cornea was  
57 checked, while a confocal approach was set-up to study peptide localization in the different layers

58 of the viable epithelium. PEP-1 was used as reference compound while penetratin (PNT) was used  
59 as positive control, because of its ability to easily permeate corneal epithelium <sup>8</sup>. Newly synthesized  
60 peptides showed corneal accumulation similar or higher than PEP-1 and, interestingly, the tendency  
61 to diffuse by a paracellular route. On the contrary, while permeating to a higher extent through the  
62 cornea, PNT diffuses by intracellular route, evidencing a different accumulation between wings and  
63 basal epithelial cells, probably depending on the stage of cell development.

64  
65  
66

## 2 Materials and methods

### 67 2.1 Materials

68 N<sup>α</sup>-Fmoc-protected amino acids, Rink amide-resin, HOAt, HOBt, HBTU, DIEA, piperidine and  
69 trifluoroacetic acid were purchased from Iris Biotech (Germany). Rink Amide-ChemMatrix resin was  
70 purchased from Biotage AB (Sweden). Peptide synthesis solvents, reagents, as well as CH<sub>3</sub>CN for  
71 HPLC were reagent grade and were acquired from commercial sources and used without further  
72 purification unless otherwise noted. 5(6)-carboxyfluorescein (5-FAM; mixture of isomers, 97%; MW  
73 376.3 g/mol) was from Alfa Aesar (Karlsruhe, Germany); propidium iodide (PI; MW 668.4 g/mol) from  
74 Sigma Aldrich. Phosphate Buffered Saline (PBS) composition: 0.19 g/l KH<sub>2</sub>PO<sub>4</sub>, 2.37 g/l Na<sub>2</sub>HPO<sub>4</sub>,  
75 8.8 g/l NaCl; pH 7.4 by adding 85% H<sub>3</sub>PO<sub>4</sub>. All other materials were of analytical grade.

76

### 77 2.2 Peptide synthesis

78 The synthesis of PEP-1 mimotopic analogues (**2-7**) was performed according to the solid phase  
79 approach using standard Fmoc methodology in a manual reaction vessel <sup>11 12</sup>.

80 The first amino acid, N<sup>α</sup>-Fmoc-Xaa-OH (Xaa = Trp(Boc), Gln(Trt), Lys(Boc), Val), was linked on  
81 to Rink amide resin (0.150 g, loading 0.59 mmol/g) previously deprotected by a 25% piperidine  
82 solution in DMF for 30 min. The following protected amino acids were then added on to the resin  
83 stepwise. Each coupling reaction was accomplished using a 3-fold excess of amino acid with  
84 HBTU and HOBt in the presence of DIEA (6 eq.). The N<sup>α</sup>-Fmoc protecting groups were removed  
85 by treating the protected peptide resin with a 25% solution of piperidine in DMF (1 × 5 min and 1  
86 × 25 min). The peptide resin was washed three times with DMF, and the next coupling step was

87 initiated in a stepwise manner. The peptide resin was washed with DCM (3×), DMF (3×), and  
88 DCM (3×), and the deprotection protocol was repeated after each coupling step. In addition, after  
89 each step of deprotection and after each coupling step, Kaiser test was performed to confirm the  
90 complete removal of the Fmoc protecting group, respectively, and to verify that complete coupling  
91 has occurred on all the free amines on the resin. The N-terminal Fmoc group was removed as  
92 described above and resin-bound peptides were reacted with 3 equiv of 5(6)-carboxyfluorescein,  
93 N,N'-diisopropyl carbodiimide, and 1-hydroxybenzotriazol, each in DMF for 16h in 10-mL  
94 syringes on a shaker at RT. Reactions were stopped by washing the resins three times each with  
95 DMF, methanol, dichloromethane, and diethylether. Completeness of N-terminal acylation was  
96 confirmed using the Kaiser test.

97 Finally the peptides were released from the resin with TFA/TIS/H<sub>2</sub>O (90:5:5) for 3 h. The resin  
98 was removed by filtration, and the crude peptide was recovered by precipitation with cold  
99 anhydrous ethyl ether to give a white powder and then lyophilized.

100

### 101 **2.2.1 Microwave peptides synthesis**

102 **PEP-1** and **PNT** were synthesized using an Automated Microwave Peptide Synthesizer from  
103 Biotage AB (Initiator + Alstra™). Peptides were synthesized on a Rink-Amide-ChemMatrix resin  
104 (0.150 g, loading 0.3 mmol/g).

105 The first amino acid, N<sup>α</sup>-Fmoc-Xaa-OH (Xaa = Val and Lys(Boc)), was linked on to the resin,  
106 using as coupling reagent HBTU (3eq, 0.6M), HOAt (3eq, 0.5M) and DIEA (6eq, 2M) in N-methyl-  
107 2-pyrrolidone (NMP)<sup>13</sup>. The N<sup>α</sup>-Fmoc protecting groups were removed by treating the protected  
108 peptide resin with a 25% solution of piperidine in DMF (1 × 3 min, 1 × 10 min) at room  
109 temperature. The resin was then washed with DMF (4 × 4.5 ml). The following protected amino  
110 acids were then added on to the resin stepwise.

111 Coupling reactions were performed using N<sup>α</sup>-Fmoc amino acids (3.0 eq., 0.5 M), using as  
112 coupling reagent HBTU (3eq, 0.6M), HOAt (3eq, 0.5M) and DIEA (6eq, 2M) in N-methyl-2-  
113 pyrrolidone (NMP). All couplings were achieved for 10 min at 75 °C (2x) and 2x45 min at RT for  
114 histidine and cysteine couplings to avoid the epimerization. After each coupling step, the Fmoc

115 protecting group was removed as described above. The resin was washed with DMF (4 × 4.5 ml)  
 116 after each coupling and deprotection step. Finally peptides were released as described above.

### 117 2.2.2 Purification and characterization

118 All crude peptides were purified by RP-HPLC on a preparative C18-bonded silica column  
 119 (Phenomenex Jupiter 100 proteo 90Å, 100 x 21.20mm, 10µm) using a Shimadzu SPD 10A  
 120 UV/VIS detector, with detection at 210 and 254 nm. The column was perfused at a flow rate of 3  
 121 ml/min with solvent A (10%, v/v, water in 0.1% aqueous TFA), and a linear gradient from 10 to  
 122 90% of solvent B (80%, v/v, acetonitrile in 0.1% aqueous TFA) over 40 min was adopted for  
 123 peptide elution. Analytical purity and retention time (tr) of each peptide were determined using  
 124 HPLC conditions in the above solvent system (solvents A and B) programmed at a flow rate of  
 125 1.500 ml/min using a linear gradient from 10 to 90% B over 15 min, fitted with C-18 column  
 126 Phenomenex, Aeris XB-C18 column (150 mm x 4.60, 3.6µm). All analogues showed >97% purity  
 127 when monitored at 215 nm. Homogeneous fractions, as established using analytical HPLC, were  
 128 pooled and lyophilized. Peptides molecular weights were determined by ESI mass spectrometry  
 129 and LC-MS in a LC-MS 2010 instrument fitted with Phenomenex, Aeris XB-C18 column (150 mm  
 130 x 4.60, 3.6µm), eluted with a linear gradient from 10% to 90% B over 15 min, at a flow rate of  
 131 1.000 mL/min. ESI-MS analysis in positive ion mode, were made using a Finnigan LCQ Deca ion  
 132 trap instrument, manufactured by Thermo Finnigan (San Jose, CA, USA), equipped with the  
 133 Excalibur software for processing the data acquired. The sample was dissolved in a mixture of  
 134 water and methanol (50/50) and injected directly into the electrospray source, using a syringe  
 135 pump, which maintains constant flow at 5 ml/min. The temperature of the capillary was set at  
 136 220°C.

137

138 Table 1. Sequence and analytical data of synthesized peptides.  
 139

Peptide	Sequence	HPLC	ESI MS	Calc net charge at pH 7
		k'	Calc.	
PEP-1	5FAM-KETWWETWWTEWSQPKKRKY	3.43	3205.28	+3
pep-2	5FAM-GGKETWWETW	3.27	1636.43	-1
pep-3	5FAM-GGWWETWWTE	3.43	1694.44	-2

<b>pep-4</b>	5FAM-GGTWWTEWSQ	3.59	1594.40	-1
<b>pep-5</b>	5FAM-GGTEWSQPCK	2.57	1474.62	+1
<b>pep-6</b>	5FAM-GGSQPCKKCRK	3.24	1470.36	+5
<b>pep-7</b>	5FAM-GGKKCRKV	2.22	1257.52	+5
<b>PNT</b>	5FAM-RQIKIWFQNRMRMKWCK	2.89	2602.65	+7

140  $k'$  [(peptide retention time – solvent retention time)/solvent retention time].  
 141 All peptides are amidated at C-terminal.

142

### 143 2.3 Permeation experiments

144 Permeation studies were carried out through isolated porcine cornea, as previously described <sup>14</sup>.  
 145 Briefly, porcine corneas were separated from fresh pig eyes (breed, Large White and Landrace;  
 146 weight: 145-190 kg; age: 10-11 months) and mounted on Franz-type diffusion cells (permeation area  
 147 of 0.2 cm<sup>2</sup>) with the epithelial side facing donor compartment. As donor solution, 200 µl of 5-FAM or  
 148 CPPs dissolved in PBS was used. Donor concentration for 5-FAM was 2 mg/ml, while in case of  
 149 CPPs different concentrations (between 0.14 and 0.41 mg/ml – see Table 2) were used. The  
 150 concentration selected was linked to the sensitivity of the analytical method and permeability of the  
 151 single CCPs.

152 The receptor compartment was filled with *approx* 4 ml of degassed PBS, constantly mixed and kept  
 153 at 37°C. Over the course of experiments (8 hours), 200 µl of the receptor fluid were collected and  
 154 immediately replaced with fresh PBS. Permeation samples were analysed using an Infinite F200  
 155 microplate reader (Tecan Trading AG, Switzerland) set at  $\lambda_{ex}$  485 nm and  $\lambda_{em}$  535 nm. Standard  
 156 solutions were prepared in PBS. Interval of calibration curves was 2.5-100 ng/ml for 5-FAM, while  
 157 50-1000 ng/ml for CPPs; RSD% (relative standard deviation %) and ER% (relative error %) resulted  
 158 lower than 5% and 10% respectively for all the concentrations tested.

159 The data was plotted as amount of 5-FAM or CPP permeated (µg/cm<sup>2</sup>) as a function of time (min);  
 160 the trans-corneal flux ( $J$ , µg/cm<sup>2</sup> min) was determined as the slope of the regression line at steady  
 161 state, while the apparent permeability coefficient ( $P_{app}$ , cm/s) was calculated at the steady state as:

$$162 P_{app} = J/C_D \text{ equation 1}$$

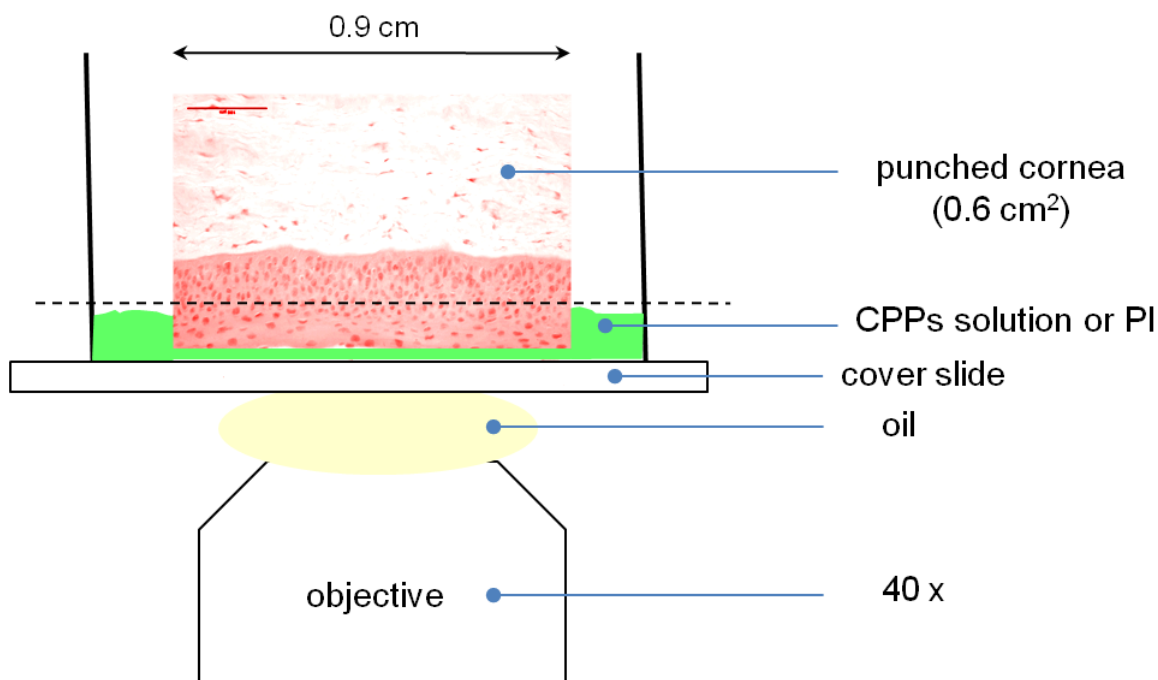
163 being  $C_D$  (µg/ml) the concentration of the donor solution.

164

### 165 2.4 Confocal microscopy

166 For confocal microscopy studies, eye bulbs kept in a media for corneal storage (EUSOL-C, Alchimia,  
167 Padova, Italia) or PBS at 4°C, were used within 2 hours from enucleation. The cornea was dissected,  
168 a 0.6 cm<sup>2</sup> corneal disc was punched in the center of the cornea and incubated with PNT (25 µg/ml),  
169 PEP-1 (3 mg/ml) or pep-7 (2.4 mg/ml) at room temperature for 1h. The incubation was performed  
170 using a special flow chamber expressly designed for confocal observations <sup>15</sup>. 100 µl of CPPs  
171 solution was inserted at the bottom of the cell and the corneal disc was applied with the epithelial  
172 side facing the solution: with this apparatus (Figure 1) CPPs can diffuse across the epithelium, but  
173 can also penetrate via lateral side, allowing for a faster contact with the different epithelial layers.  
174 Analysis was performed using LSM 510 Meta confocal system scan integrated with Axiovert 200M  
175 inverted microscope (Carl Zeiss, Jena , Germany). Samples were observed through a 40x, 1.3 NA  
176 oil objective. 5-FAM and PI were excited with 488 nm argon and 543 nm He-Ne laser lines,  
177 respectively. Image acquisition was carried out with relevant beamsplitter and emission recorded  
178 through 505-530 BP filter for 5-FAM and 560 LP filter for PI. A series of x-y sections were acquired  
179 with a z-step of 1µm to cover the whole height of samples. For 3D reconstruction, stacks of digital  
180 images were processed employing the “transparency” algorithm of the “Inside 4D” module of  
181 Axiovision (release 4.5) software (Carl Zeiss, Jena Germany).

182 The set-up of the method was done using a formalin-fixed porcine cornea stained with propidium  
183 iodide (PI). Briefly, the tissue was fixed for 24 h in 10% buffered formalin and then permeabilized  
184 with 80% ethanol. Tissue was then stained with 1µg/ml PI solution for 30 minutes and then observed  
185 with confocal microscope. A sample of fresh cornea was stained immediately after dissection with  
186 the same solution to provide a test for tissue viability. Propidium iodide is a red fluorescent nuclear  
187 dye, which simultaneously provides a good fluorescent dye for nuclear morphology and a vital probe  
188 to assess cell viability.



**Figure 1.** Experimental set-up used for confocal microscopy studies.

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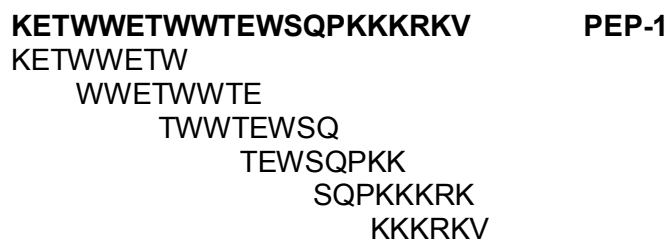
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### 192 3 Results and Discussion

#### 193 3.1 Design

194 Starting from PEP-1 we designed a small library of overlapping peptides, with a specific length and  
 195 specific offset, to cover the entire PEP-1 sequence as shown in Figure 2. The synthesis of seven  
 196 short fluorescein labeled peptides was attempted but (Table 1), unfortunately, we were not able to  
 197 obtain peptide 3 (pep-3) in sufficient yield to test. To avoid influence of fluorescein group on peptides  
 198 structure, a glycine spacer was inserted in the sequences. We also synthesized PEP-1 as reference  
 199 compound and Penetratin (PNT) as control.



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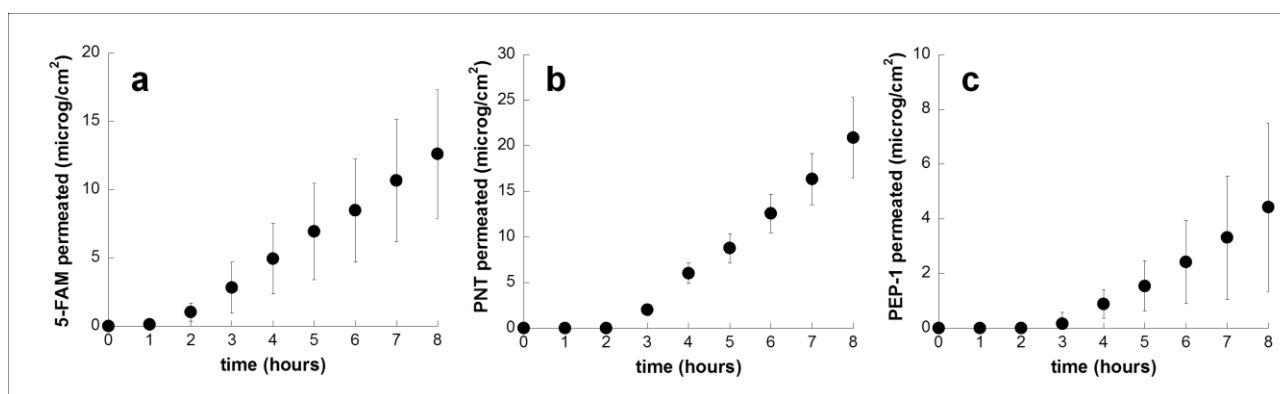
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203

**Figure 2.** Overlapping peptides library.

204 **3.2 Trans-corneal permeation**

205 The CPPs synthesised in the present work were labelled with 5-FAM, a low molecular weight (376.32  
 206 g/mol) highly hydrophilic and negatively charged molecule ( $\text{LogD}_{7.3} -3.15$ <sup>16</sup>, charge at pH 7.4 approx.  
 207  $-3$ <sup>17</sup>) to make them fluorescent; the trans-corneal permeation of the label 5-FAM was evaluated at  
 208 first. The permeation profile is reported in Figure 3 Panel a, and the permeability coefficient,  
 209 calculated using Equation 1, resulted *approx.*  $0.3 \cdot 10^{-6}$  cm/s (Table 2), value similar to that of  
 210 fluorescein<sup>18 14</sup>, molecule of comparable molecular weight.



211 **Figure 3.** Permeation profiles across isolated porcine cornea of 5-FAM (donor 2 mg/ml) (a), PNT  
 212 (179.26  $\mu\text{g/ml}$ ) (b) and PEP-1 (359.51  $\mu\text{g/ml}$ ) (c) solutions. (Mean values $\pm$ sd)  
 213  
 214

215  
 216 PNT, despite the high molecular weight (2600 Dalton), considerable hydrophilicity (measured logP -  
 217  $2.1$ <sup>19</sup>) and positive charge, permeated the cornea easily (Figure 3, Panel b) and the permeability  
 218 coefficient resulted 20-fold higher than 5-FAM (Table 2).

219  
 220 Table 2. Apparent permeability coefficients of 5-FAM labelled CPPs and 5-FAM calculated using  
 221 Equation 1 (mean values $\pm$ sd)

	Permeant (all CPPs were 5-FAM labelled)	Donor concentration ( $\mu\text{g/ml}$ )	$P_{\text{app}} \cdot 10^{-6}$ (cm/s)
CPPs	<i>PNT</i>	179.26	$6.18 \pm 1.46$
	<i>PEP-1</i>	359.51	$0.75 \pm 0.56^{**}$
	<i>pep-2</i>	413.58	$1.52 \pm 1.36^*$
	<i>pep-4</i>	146.60	$2.00 \pm 1.41^*$
	<i>pep-5</i>	410.09	$0.66 \pm 0.29^{**}$
	<i>pep-6</i>	369.75	$0.51 \pm 0.06^*$
	<i>pep-7</i>	344.16	$0.71 \pm 0.28^{**}$
5-FAM		2000.00	$0.29 \pm 0.08^{**}$

\* statistically different from PNT,  $p < 0.05$  (Student's t test)  
\*\* statistically different from PNT,  $p < 0.01$  (Student's t test)

222

223 The permeation properties of 5-FAM-labelled PNT were previously investigated by Liu *et al.*<sup>8</sup> across  
224 isolated rabbit cornea and the permeability coefficient found ( $10.5 \pm 2.20$ )\*  $10^{-6}$  cm/s ) is in good  
225 agreement with the present data. The good penetration properties of PNT, the third helix of the  
226 Antennapedia homeodomain, can be attributed to its capability to interact with and penetrate across  
227 cell membranes<sup>20, 21 22</sup>.

228 Also PEP-1 is known to be able to penetrate into HeLa cells<sup>23</sup> although, unexpectedly, its  
229 permeability across the cornea was 8 fold lower than PNT (Table 2) and the permeation profile is  
230 characterised by a significantly longer lag time (Figure 3). The differences observed between PNT  
231 and PEP-1 reflect differences in the permeation across the epithelium: in fact, the permeability  
232 coefficient across de-epithelised cornea (i.e. stroma+endothelium) was similar for the two peptides,  
233  $17.7 \pm 8.5 \cdot 10^{-6}$  cm/s in case of PNT and  $12.9 \pm 3.4 \cdot 10^{-6}$  cm/s for PEP-1. These values are comparable  
234 to the ones found for the neutral 4.4 kDa dextran and a negatively charged 8 kDa ssDNA, across  
235 the same de-epithelized tissue<sup>24</sup>: the possible interaction of the peptides (both positively charged)  
236 with GAGs (negatively charged), and in particular with N-linked GAGs<sup>25</sup> highly represented in the  
237 extracellular matrix of the cornea<sup>26</sup>, did not markedly hinder CPPs diffusion across the corneal  
238 stroma.

239

240 The newly synthesised peptides (pep-2; pep-4; pep-5; pep-6; pep-7), were evaluated across the  
241 intact cornea and the permeability coefficient found resulted similar to the reference PEP-1 (Table  
242 2); the data are characterized by a high variability probably due to their propensity to aggregate in  
243 aqueous solution<sup>23, 27</sup>. In particular the highest variability was found for the negatively charged  
244 peptides pep-2 (CV% 90) and pep-3 (CV% 70).

245 No correlation was found between peptide permeability and molecular weight (Figure 4a), surface  
246 net charge (Figure 4b) nor charge/mass ratio (Figure 4c).

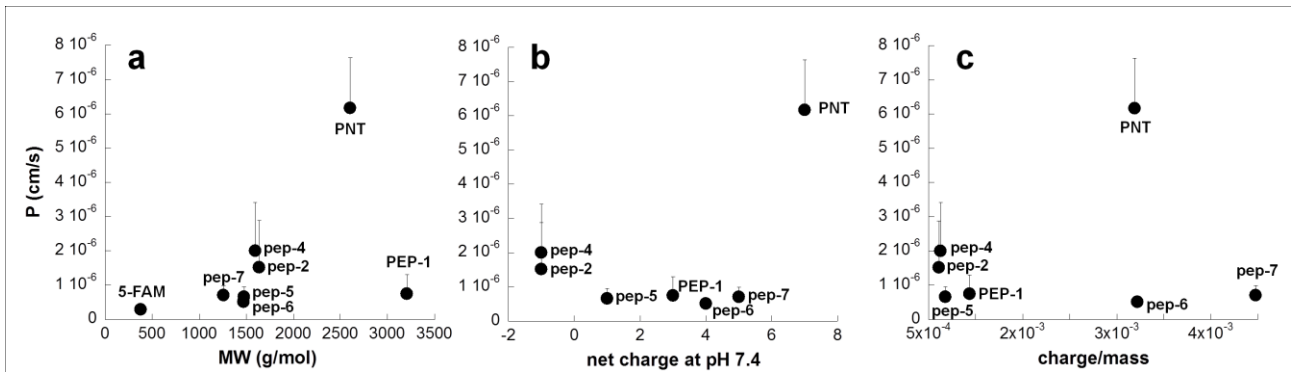


Figure 4. Permeability coefficients of peptides as function of MW (a), net charge (b) and charge/mass ratio (c). (Mean values+sd)

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248

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250

251

Even if the permeability of peps 1-7 is not as high as PNT, it is comparable to that of PEP-1.

252

Additionally, the permeability is comparable or in some cases higher (pep-7,  $p < 0.05$ ) to that of 5-

253

FAM, suggesting that the amphipathic nature and the lower negative charge of peptides

254

counterbalanced the increase in molecular weight.

255

256

### 3.3 Confocal microscopy studies on freshly enucleated pig eyes

257

Permeation experiments have demonstrated the ability of CPPs to cross the corneal epithelium; the

258

aim of confocal microscopy was then to evaluate the ability of CPPs to enter inside corneal cells.

259

This aspect is very important, since CPPs cell uptake is strictly linked not only to cell type, but also

260

to cell environment that can influence, on one side, the expression of glycosaminoglycans and lipids

261

on cell membranes<sup>2</sup> and, on the other side, the conformation and activity of CPPs<sup>28</sup>.

262

Corneal epithelium is a stratified, squamous, non keratinized tissue; the similarity between porcine

263

and human corneas, in terms of histology and permeability, has been recently demonstrated<sup>14</sup>.

264

Figure 5 shows 3D reconstruction of formalin-fixed (a) and vital (b) corneal epithelium. In the fixed

265

sample, nuclei of the entire epithelial layer (surface, wing, basal cells) are stained by propidium

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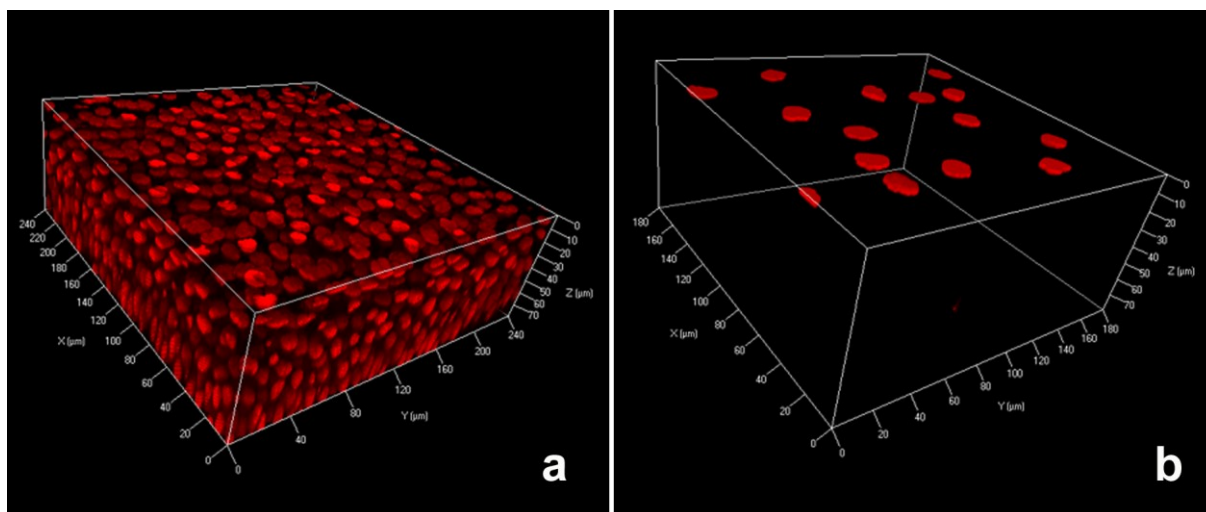
iodide, while in the viable tissue (b) only the superficial cell layer shows PI positive nuclei. PI is

267

excluded from competent plasma membrane (live cells), while is loaded from cells that have lost this

268

competence after cell death (apoptosis and/or necrosis or death after fixation).



269

270 **Figure 5.** 3D reconstruction of a fixed (**a**) and viable (**b**) corneal epithelium after nuclear staining with  
 271 PI. Panel **a**: nuclei of the entire epithelial layer (surface, wing and basal cells) are PI positive. Panel  
 272 **b**: Only superficial cell layer shows PI positive nuclei (dead cells). All underlying cells exclude PI thus  
 273 are still viable. (z axis represents vertical scanning axis and is *approx.* 80  $\mu\text{m}$ ; 0  $\mu\text{m}$  corresponds to  
 274 superficial layer).  
 275

276 The death of the most external layer can be attributed to the procedure of isolation and transport of  
 277 eye bulbs, since the epithelium natural turn-over generally occurs through the shedding of isolated  
 278 dead cells, replaced by underlying viable cells<sup>29</sup>. However, in response to superficial cell death, the  
 279 sub-surface cells start to develop tight-junctions within minutes; this has been demonstrated to occur  
 280 also *ex vivo* after the induction of a whole layer of devitalized cells<sup>30</sup>. It has been shown that this  
 281 process remains active even after keeping the excised cornea for three days in a nutrient medium  
 282<sup>30</sup>. These considerations support the reliability of the permeation data obtained in the present work,  
 283 taking also into account that they are comparable with data found in the literature for both low<sup>14</sup> and  
 284 high<sup>24</sup> molecular weight compounds.

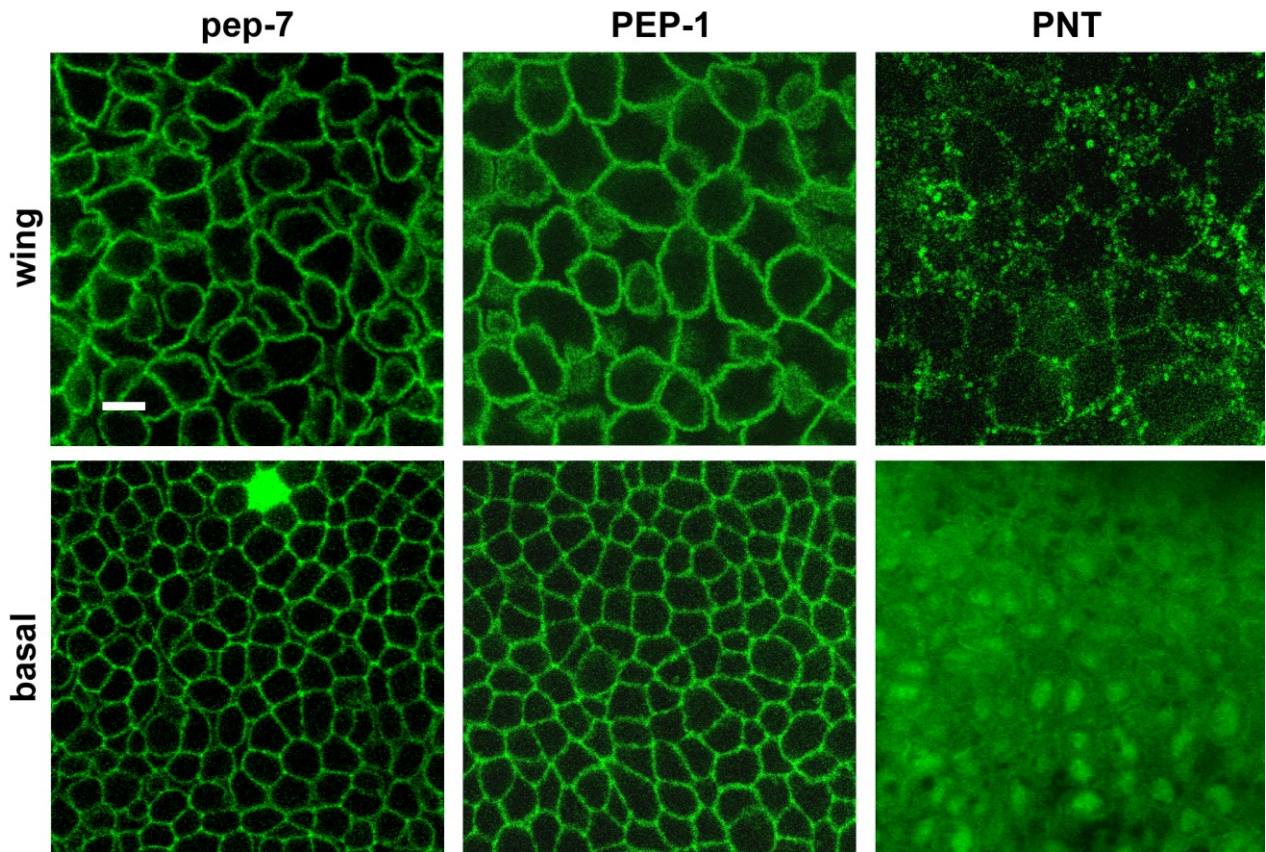
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286 Confocal analysis of freshly explanted porcine corneas was then performed after contact with either  
 287 5-FAM labelled pep-7, PEP-1 or PNT solutions. PNT was used as positive control CPP, PEP-1 as  
 288 the reference (net charge +3) while pep-7 (net charge +5) was chosen as representative, most  
 289 promising, peptide among those newly synthesised. The solutions of CPP were left in contact with  
 290 the cornea for 1 h before confocal analysis. Confocal sections and 3D reconstructed images are  
 291 reported in Figure 6 and 7, respectively.

292 Owing to the possibility of interaction (and artefact generation) of propidium iodide with the peptide  
293 bound 5-FAM, the marker for cell death was not used, and only the fluorescence of the peptides was  
294 observed.

295 Figure 6 shows confocal sections after 5-FAM-labelled CCPs contact with the corneal tissue. In the  
296 case of pep-7, fluorescence is localised only in the intercellular spaces both in wing and in basal  
297 epithelial cells, suggesting that the peptide diffuses across the corneal epithelium following the  
298 paracellular route. The paracellular route is probably the main diffusion pathway also for PEP-1,  
299 although in this case some diffusion across the cellular membrane cannot be completely excluded.  
300 In contrast, PEP-1 is reported as capable of entering the cell nucleus of different cells, including  
301 human fibroblasts, in less than 10 minutes<sup>31</sup>. In the case of pep-7, no literature data are present but  
302 its sequence is shorter and less hydrophobic than PEP-1, lacking of spacer domains as well as of  
303 the complete nuclear localization sequence (NLS), and this can contribute to explain its behaviour.  
304 Noticeably, promotion of ophthalmic active ingredients (especially peptides) trough the paracellular  
305 pathway has gained increasing interest in recent years. Intercellular spaces are, in fact, devoid of  
306 the massive intracellular metabolic activity, thereby minimizing the enzymatic penetration barrier<sup>32</sup>.  
307 Permeation of hydrophilic compounds is however limited to the small paracellular pores and  
308 regulated by the tight junctions. Thus, increasing molecular size of the permeant progressively  
309 decreases the rate of permeation<sup>33</sup>. Moreover, one of the main limit associated to the use of CPPs  
310 as absorption enhancer is represented by their sensitivity to proteolysis<sup>34</sup>. Limiting the contact with  
311 proteolytic enzymes could be a fruitful strategy in increasing their effectiveness.

312 The result obtained with PNT is completely different. In wing cell layer, the fluorescence is localised  
313 in the intercellular space and is characterized by the presence of spots (Figure 6) that, for size and  
314 localization, could be attributed to endocytic organelles. Basal cells are clearly fluorescent both  
315 inside cytoplasm and nucleus (Figure 6). Apparently, PNT behaves differently depending on the  
316 degree of cell maturation. This result is in line with the significant changes taking place in corneal  
317 epithelial cells, that from the basal membrane migrate toward the surface loosing proliferative  
318 capacity and acquiring features specific for each layers, differing for glycoconjugate composition<sup>30</sup>.

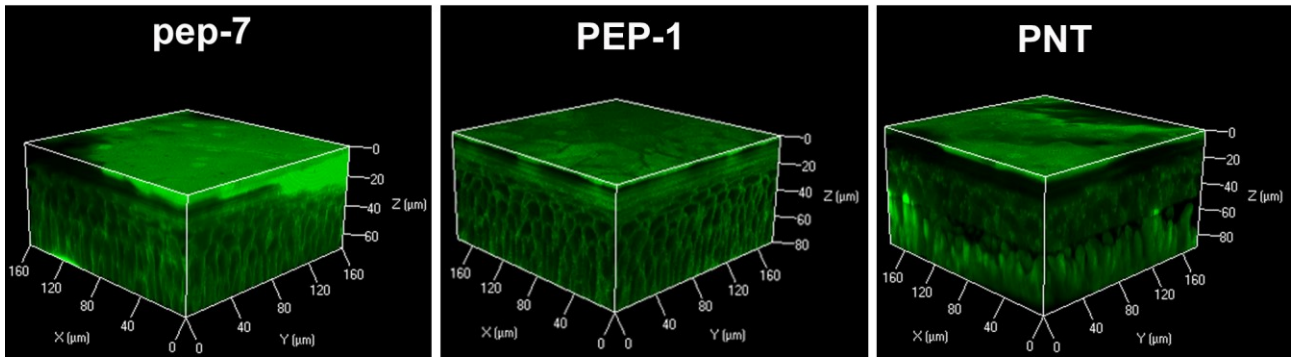


319

320 **Figure 6.** Confocal sections of vital corneal epithelium after incubation with pep-7, PEP-1 and PNT.  
 321 Images of the first line shows confocal planes acquired from wing layers (cells mean diameter  
 322 *approx.* 20  $\mu\text{m}$ ); images of the second line are planes from basal layers (mean cell diameter *approx*  
 323 10  $\mu\text{m}$ ). Pep-7 fluorescence distribution is mainly localised outside cells in both layers; pep-7 freely  
 324 diffuses across the membrane of one dead basal cell that appears green fluorescent. Distribution of  
 325 PEP-1 in wing and basal layers: fluorescence is mainly extracellular and surrounds cells, also if slight  
 326 intracellular areas are present in some cells in both layers. PNT shows a spot localization on plasma  
 327 membrane in wing layer, while localization in basal layers is diffused across cytoplasm and nucleus.  
 328 (Scale bar:10  $\mu\text{m}$ ).  
 329

330 Figure 7 shows 3D reconstruction obtained by processing the stacks of the digital images. The outer  
 331 layers (0-10  $\mu\text{m}$  depth) are fluorescent due to the loss of integrity of cellular membrane (as previously  
 332 discussed). 3D images of pep-7 and PEP-1 show clearly their intercellular localization. In the case  
 333 of PNT, the intermediate layers (containing some surface cells and all wing cells; depth 10-60  $\mu\text{m}$ )  
 334 show a light fluorescence, mainly localized within the extracellular space, while basal cells (60-80  
 335  $\mu\text{m}$  depth) are fluorescent.

336



337

338 **Figure 7.** 3D reconstruction of vital corneal epithelium after loading 5-FAM labelled PNT, PEP-1 and  
 339 pep-7.  
 340

340

#### 341 **4 Conclusion**

342 The mimotopic approach proposed in the present work for the design of novel CPPs proved to be a  
 343 suitable strategy allowing for the synthesis of shorter and more affordable sequences, with  
 344 penetration ability comparable to PEP-1, chosen as long-sequence reference peptide. Although  
 345 none of peptides was able to equal the performance of PNT, used as positive control, a preferential  
 346 paracellular penetration route was evidenced through the ocular tissue for PEP-1 and pep-7,  
 347 highlighting their potential application as specific absorption promoters for metabolic sensitive  
 348 ophthalmic drugs.

349 The confocal method developed, allowing for the localization the peptides in the different epithelium  
 350 layers by studying cell uptake on a 3D complex tissue, also revealed a clear cellular uptake in the  
 351 case of PNT. An interesting observation is also the different PNT behavior depending on the degree  
 352 of cell maturation: this result highlights the importance of studying CPPs uptake in conditions as  
 353 close as possible to the real in vivo condition.

354

355

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361



363 **5 References**

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