Cell penetrating peptides in ocular drug delivery: State of the art

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Abstract

Despite the increasing number of effective therapeutics for eye diseases, their treatment is still challenging due to the presence of effective barriers protecting eye tissues. Cell Penetrating Peptides (CPPs), synthetic and natural short amino acid sequences able to cross cellular membrane thanks to a transduction domain, have been proposed as possible enhancing strategies for ophthalmic delivery. In this review, a general description of CPPs classes, design approaches and proposed cellular uptake mechanisms will be provided to the reader as an introduction to ocular CPPs application, together with an overview of the main problems related to ocular administration. The results obtained with CPPs for the treatment of anterior and posterior segment eye diseases will be then introduced, with a focus on non-invasive or minimally invasive administration, shifting from CPPs capability to obtain intracellular delivery to their ability to cross biological barriers. The problems related to *in vitro*, *ex*

vivo and *in vivo* models used to investigate CPPs mediated ocular delivery will be also addressed together with potential ocular toxicity issues.

List of Abbreviations

aa	amino acid
ACPP	activatable cell penetrating peptide
aFGF	acidic fibroblast growth factor
Aib	α-aminoisobutyric acid
AMD	age-related macular degeneration
apoA-I	human apolipoprotein
ARPE-19	adult human RPE
BN	Brown Norway (rats)
CAM	chorioallantoic membrane assay
CD	circular dichroism
CM	confocal microscopy
CN or CNV	choroidal neovascularization
CPP	cell penentrating peptide
CPPM	mimetics cell penetrating peptide
CPPos	cell penetrating peptoids
Dap	2,3-diaminopropionic acid
DP-9	primary human foreskin fibroblast
DSPC	1,2-Distearoyl-sn-glycero-3-phosphorylcholine
EB	entry blocker peptide
ELP	elastin like polypeptide
ERG	electroretinogramm
FAM	5-carboxyfluorescein
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FM	flow citometry
GAG	glycosaminoglycan
gC	HSV-1 glycoprotein C
GFP	green fluorescent protein
HA	hyaluronic acid
HCEC	human corneal epithelial cell
HCE-T	immortalized human corneal epithelial cell
hCF	human corneal fibroblast
HDL	high density lipoprotein
HeLa	immortal human cell line
HER	human embrionic retinal cell
HIV-1	human immunodeficency virus type 1
HLE-B3	human lens epithelial B3 cells
HSV-1	human *Herpes simplex* virus type 1
ICR	Institute of Cancer Research
IR	ischemia reperfusion
LSCD	limbal stem cell deficiency
MAP	model amphipathic peptide
MRR	membrane repair response
MW	molecular weight
NBP	nucleolin binding peptide

NHC	human conjunctival epithelial cells
NLS	nuclear localization sequence
NP	nanoparticle
OCT	optical coherence tomography
PAMAM	polyamidoamine dendrimer
PBS	phosphate buffered saline
PEG	polyethylene glycol
PEP-1	synthetic amphipathic CPP
PLGA	poly(lactic-co-glycolic acid)
PNT	penetratin
POD	peptide for ocular delivery
PPII	polyproline II
pRFP	red fluorescent protein plasmid
PTD	protein translocation domain
Pzp	pazopanib
RCEC	rabbit corneal endothelial cell
RGC	retinal ganglion cell
RGD	arginine-glycine-aspartic acid
RPE	retinal pigment epithelium
RRPC	rat retina microvascular endothelial cell
siCPD	cell penentrating poly(disulfide)
SV40	simian virus 40
TAT	HIV transactivator protein
TM-1	human trabecular meshwork cell line
UVEC	umbelical vein endothelial cell
VEGF	vascular endothelial growth factor
VP22	peptide from viral capsid protein of HSV-I
XG-102	brimapitide
VERO	kidney cell from African green monkey

Contents

1. Introduction

Different kind of diseases of inflammatory, infectious or degenerative nature can affect the eye with consequent discomfort, visual disturbance, vision impairment, or even blindness. Ocular diseases can be located both in the anterior (conjunctivitis, dry eye disease, keratitis, ulcer, cornea neovascularisation, keratoconus, cystinosis) and posterior eye segment (neovascular age-related macular degeneration AMD, diabetic retinopathy, glaucoma, hereditary retinal degenerations, retinitis, posterior uveitis, eye tumors) and represent a major challenge. Some of these pathologies are very frequent and are expected to grow in the next years due to population aging. Indeed, a 2010 estimate reports that 2.7 million adults in the U.S. were affected by glaucoma, with an expected increase to 6.3 million for 2050. In parallel, the number of U.S. adults older than 50 with AMD will increase from 2.07 million in 2010 to 5.44 million by 2050. Also lifestyle has a huge impact on ocular diseases: the increase of diabetes prevalence will bring diabetic retinopathy to rise among Americans (aged > 40) from 7.7 million (2010) to 14.6 million in 2050 [1]. The environmental pollution and the continuous use of digital devices are also predicted to greatly increase the prevalence of anterior segment eye diseases such as the dry eye syndrome, that presently affect from 8.7 to 30.1% of world population [2].

Several new pharmacological strategies involving proteins (*i.e.* anti-VEGF monoclonal antibodies and growth factors), oligonucleotides and genes are in use or under clinical development to treat, delay the onset or slow down the progression of ocular diseases. Many of these drugs are biotech macromolecules whose efficacy is hampered by problems related to their administration to the eye. Indeed, ocular delivery, in particular to the posterior eye segment, is challenging due to the peculiar structure of this organ and the presence of static, dynamic and metabolic barriers, protecting the inner tissues. Among the different approaches that are under investigation to increase drug bioavailability to the ocular structures, Cell Penetrating Peptides (CPPs) represent one of the most recent and promising advancements [3]. Indeed, despite the relatively novelty of this delivery technology, a successful phase III clinical trial on an anti-inflammatory peptide conjugated with a CPP has just been reported [4]. Overall, the number of papers related to the use of cell penetrating peptides for ocular drug delivery is still relatively low, but it has seen an exponential increase in the last 5 years, demonstrating the growing interest and expectations in this specific field. In this review, after a general description of the Cell Penetrating Peptides classification, design approaches and mechanisms of cellular uptake, we will summarize the results reported in the literature on CPPs-enabled ocular delivery to the anterior and posterior eye segment, with a particular focus on non-invasive or minimally invasive administration, shifting from CPPs capability to obtain intracellular delivery to their aptitude to cross biological barriers. The problems related to their penetration pathways and to

the *in vitro*, *ex vivo* and *in vivo* models employed for their investigation will be also addressed together with the potential toxicity issues.

2. Cell Penetrating Peptides (CPPs)

CPPs are a family of various peptides, typically sequences of 5-40 amino acids (aa), also known as protein translocation domains (PTD), membrane translocation sequences or Trojan horse peptides [5]. CPPs can pass through tissues and membranes of mammalian, plant and bacterial cells, via energy-dependent or energy-independent mechanisms with no interaction with specific receptors [6]. Because in most cases CPPs show a low cytotoxicity and they are able to enhance the cellular internalization of the covalently or non-covalently conjugated cargoes, they have received considerable attention in drug delivery.

The history of CPPs dates back to the '90s. After the discovery of a receptor-independent activation of mast cells by substance P analogues by Bienert and his group in 1987 [7], new amphipathic model peptides, deriving from substance P sequences, were developed. Then, in 1988 was published the first proof-of-concept of protein transduction into cells, described independently by Frankel and Pabo [8] and by Green and Loewenstein [9]. Both groups discovered the cell membrane penetration activity of trans-acting activator of transcription (TAT) protein from human immunodeficiency virus 1 (HIV-1). A few years later, the homeodomain of Antennapedia, a homoprotein of *Drosophila melanogaster*, was also shown to enter cells of neuronal cultures [10]. The discovery of the capacity of these proteins to enter cells stimulated the investigation of their mechanism of action and of the minimal amino acid sequences able to enter the cell. In 1994 Derossi *et al.* described a 16 aa peptide, named penetratin, from residues of 43-58 of the third helix of *Drosophila* Antennapedia homeobox protein [11]. In 1997, Vives *et al.* [12] identified the minimal sequence of TAT that enabled cell internalization: they discovered that the domain responsible for cellular uptake is a basic region of 13 aa (TAT_{48-60}). Later on, it was demonstrated that the key motif for transduction could be further reduced (TAT_{49-57}) [13]. Since then, a number of CPPs have been identified from natural sources such as VP22 (from viral capsid protein of *Herpes simplex* virus type I), the above mentioned penetratin (Antennapedia homeobox protein), transportan (from a neuropeptide galanin and mastoparan) and from synthetic sources, such as polyarginine, polyhistidine, model amphipathic peptide and TP2 [14].

Nowadays CPPs are used to improve cellular uptake of a number of cargos from small molecules such as taxol, methotrexate and doxorubicin for cancer therapy to macromolecules and nanocarriers [5, 15-17]. Peptides and proteins have particularly benefited from the use of CPPs because of their unfavorable physico-chemical properties. Proteins and peptides conjugates with CPPs are under investigation to treat cancer, ischemic events, asthma, allergic rhinitis and neurodegenerative diseases. The use of fluorescent peptide probes also allows for the study of the intracellular pathways

and to obtain fluorescent images of specific tissues. CPPs have been associated to DNA and plasmids for gene delivery. The surface modification of liposomes, micelles, polymeric and inorganic nanoparticles with CPPs allows to obtain efficient vectors for gene delivery, for specific tumor cells drug delivery and for central nervous system targeting via nasal route. Finally, CPPs have also proved to be useful in promoting the absorption of macromolecules across different tissues [14] such as the skin (cyclosporin A for the treatment of psoriasis), the intestine (insulin for the treatment of diabetes), the nasal mucosa for both vaccination and nose-to-brain delivery [18], and ocular tissues.

3. CPPs Classification

CPPs present a great variety in terms of amino acid composition and 3D structure, with examples of cationic, anionic, and neutral sequences showing different degrees of hydrophobicity and polarity [17]. A CPP database, established in 2012 and now updated to version 2.0 [19], is a useful source of information about the molecular diversity of this type of peptides [20, 21].

Although specific groups of CPPs show high sequence identity and common structural features, in general CPPs have no sequence homology. This structural diversity results in different mechanism for CPP internalization and uptake, which, beside their origin, makes their classification complex. In fact, no unified CPPs taxonomy exists so far. Parameters, such as origin, translocation mechanism, physico-chemical properties, among others, have been proposed [6, 16, 22-24].

In this chapter we present a classification of CPPs based on their **physico-chemical** properties, highlighting the key structural aspects influencing the internalization process and the basis for the rational design of more efficient CPPs. According to **physico-chemical** properties, CPPs, can be classified into three main groups: cationic, amphipathic and hydrophobic.

Table 1: Sequence, origin, physico-chemical properties and internalization mechanisms of some model CPPs [a]

Name	Sequence	Origin	Charge/ Structure	Internalization Mechanism
TAT	GRKKRRQRRRPPQ	HIV-1 TAT protein (48–60)	Cationic/ Unstructured	Endocytosis
Penetratin	RQIKIWFQNRRMKWKK	Antennapedia *Drosophila Melanogaster* (43–58)	Cationic/ Secondary amphipathic α helices or β-sheets	Direct translocation and endocytosis
Polyarginines	R_n	Synthesis	Cationic/ Flexible; unstructured; random coil	Direct translocation and endocytosis
DPV1047	VKRGLKLRHVRPRVTRMDV	Synthesis	Cationic	Endocytosis
pVEC	LLIILRRRIRKQAHAHSK	Vascular endothelial cadherin	Cationic/ Primary Amphipathic	Endocytosis-independent
M918	MVTVLFRRLRIRRACGPPRVRV	p14ARF protein	Cationic/ Primary Amphipathic	Endocytosis
M1073	MVRRFLVTLRIRRACGPPRVRV	p14ARF protein	Cationic/ Primary Amphipathic	Endocytosis
BPrPr (1-28)	MVKSKIGSWILVLFVAMWSDVGLCKKRP	Bovine prion protein	Cationic/ Primary Amphipathic	Macro pynocitosis
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	HIV glycoprotein 41/ SV40 T antigen NLS	Cationic/ Primary Amphipathic	Endocytosis-independent

Pep-1	KETWWETWWTEWSQPKKKRKV	Tryptophan-rich cluster/SV40 T antigen NLS	Cationic/ Primary Amphipathic	Endocytosis-independent
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Chimeric galanin– mastoparan	Secondary Amphipathic, α–Helical	Endocytosis
MAP	KLALKLALKALKAALKLA	Synthesis	Cationic, Secondary Amphipathic, α–Helical	Endocytosis
MAP12	LKTLTETLKELTKTLTEL	Synthesis	Anionic, Secondary Amphipathic, α–helical	Endocytosis-independent
MAP17	QLALQLALQALQAALQLA	Synthesis	Amphipathic, Secondary Amphipathic, α-helical	nd
GALA	WEAALAEALAEALAEHLAEALAEALEALAA	Synthesis	Anionic, Secondary Amphipathic	nd
p28	LSTAADMQGVVTDGMASGLDKDYLKPDD	Azurin	Anionic, Secondary Amphipathic	Caveolae-mediated
PreS2	PLSSIFSRIGDP	Hepatitis-B virus surface antigen	Amphipathic	nd
VT5	DPKGDPKGVTVTVTVTVTGKGDPKPD	Synthesis	Secondary Amphipathic; β-sheets	nd
Bac 7 [(Bac (1-24)]	RRIRPRPPRLPRPRPRPLPFPRPG	Bactenecin family of antimicrobial peptides	Cationic, Polyproline II helical	Endocytosis

PPR and PRR	$\underline{PP}R_n$ and $\underline{P}RR_n$, n =3,4,5, and 6	Synthesis	Cationic, Polyproline II helical	nd
SAP	VRLPPPVRLPPPVRLPPP	γ-zein	Amphipathic, Polyproline II helical	Endocytosis
SAP(E)	VELPPPVELPPPVELPPP	Synthesis	Anionic; Polyproline II helical	Endocytosis
CyLoP-1	CRWRWKCCKK	Crotamine crot(27–39)	Amphipathic, Disulfide-bridge	nd
gH 625	HGLASTLTRWAHYNALIRAF	*Herpes simplex* virus type I	Hydrophobic, α-helical	nd
CPP-C	PIEVCMYREP	FGF (140-149)	Hydrophobic	nd
C105Y	CSIPPEVKFNKPFVYLI	Synthesis	Hydrophobic	nd
Pep-7	SDLWEMMMVSLACQY	Phage-display	Hydrophobic	nd
SG3	RLSGMNEVLSFRW	Plasmid-display	Hydrophobic	nd

[a] The hydrophobic residues are underlined

nd: not determined

3.1. Cationic CPPs

The cationic class comprises peptides with highly positive net charges at physiological pH, primarily from arginine (Arg, R) and lysine (Lys, K) residues. Peptides belonging to this class include TAT-derived peptides [12, 13], penetratin [11], polyarginines [25], and Diatos peptide vector 1047 (DPV1047, Vectocel) [26] (Table 1). The majority of cationic CPPs derive from TAT and penetratin natural peptides and usually contain more than five positively charged amino acids [27]. Studies on arginine-based peptides (from R3 to R12) suggest that at least eight positive charges are needed for efficient uptake of cationic CPPs, and that their transduction ability increases with the number of consecutive arginine residues and the peptide concentration, with R8 and R9 performing overall best. Polylysine, in comparison, shows a much poorer uptake profile [28, 29]. In fact, the guanidine groups of the arginine side chain form bidentate hydrogen bonds with the negatively charged carboxylic, sulfate, and phosphate groups of cell membrane proteins, mucopolysaccharides and phospholipids, leading to cellular internalization of peptides under physiological conditions [30]. Beside arginine other amino acids have shown to efficiently mediate the translocation of cationic CPPs across cell membranes. The addition of four tryptophan residues (Trp, W) in the middle or evenly distributed along the TAT derived CPP sequence has shown to increase its cellular internalization [31, 32]. The elimination of one or two tryptophan residues, as in the case of mutated penetratin W48F and W48F/W56F, causes a reduction or complete lack of cellular uptake, respectively. Tryptophan has shown to be crucial for the interaction of CPPs with the phospholipid bilayer of plasma membrane [33] and also influence ocular distribution (see section 6.3).

3.2. Amphipathic CPPs

Amphipathic CPPs contain both polar (hydrophilic) and non-polar (hydrophobic) regions of amino acids. Besides lysine and arginine, which are distributed throughout the sequence, they are also rich in hydrophobic residues such as valine (Val, V), leucine (Leu, L), isoleucine (Ile, I), and alanine (Ala, A) [34]. Amphipathic CPPs are able to fold into α-helical and β-sheet-like structures and are generally classified into primary, secondary, and proline-rich CPPs [35]. These molecules are referred to as primary or secondary amphipathic CPPs, on the basis of their amphipathic primary sequence or the ability to assume amphipathic properties when folded, respectively [23]. Some of them fold only in the presence of membranes while others tend to assume secondary structures in solution, before interaction with biological surfaces [23].

Several primary amphipathic CPPs are fully derived from natural proteins (Table 1). Thus, pVEC contains 13 cytosolic and 5 trans-membrane residues from VE-cadherin [36], whereas ARF(1–22) is derived from the N-terminal domain of the tumor suppressor p14ARF protein [37], and BPrPr(1–28)

is based on the prion protein signal peptide followed by the KKRPKP motif, which corresponds to the N-terminus of the prion protein, once the signal peptide is cleaved [38].

Other interesting primary amphipathic CPPs are chimeric peptides designed to increase the efficiency of known CPPs by including fusion sequences containing specific functions of interest [39]. For example, the primary amphipathic CPPs MPG and PEP-1 are both generated by fusing the nuclear localization sequences (NLSs) of the simian virus 40 (SV40) large T antigen (KKKRKV) to the sequence of the HIV glycoprotein 41 (GALFLGFL- GAAGSTMGA) and a tryptophan-rich cluster (KETWWETWWTEW), respectively (Table 1). NLSs are a special group of short cationic CPPs containing polylysine, polyarginine or polyproline motifs which translocate to the nucleus through a multimeric complex containing 50–100 different proteins forming the nuclear pore [40]. The hydrophobic domains of both MPG and PEP-1, which are separated from the NLS through the WSQP linker, are required for efficient targeting of the cell membrane and for forming hydrophobic interactions with proteins [41, 42].

Secondary amphipathic CPPs generally exhibit a common structural motif, a α-helical structure, in which hydrophilic and hydrophobic amino acids are grouped in separate faces of the helix [43, 44]. Secondary amphipathic α-helical CPPs have a highly hydrophobic patch on one face, as in the case of transportan [45] and MAP [46], whereas the other face can be cationic, anionic, or polar. Even though most amphipathic CPPs are cationic, evidence suggests that membrane translocation is a consequence of amphiphilicity and not of positive charges. Studies on MAP have shown that substituting lysine aa with other polar residues does not alter the membrane uptake when amphipathic properties are preserved: the anionic MAP12 [47] and the neutral MAP17 [48] and are both cell-penetrating. These studies on MAP also suggested that a minimum of four helix turns is essential for uptake efficiency. Other examples of anionic amphipathic CPPs are GALA [49] and p28, a peptide derived from the bacterial protein azurin [50]. On the basis of the Protein Data Bank structure of azurin (3N2J), p28 is a helical peptide with a stretch of hydrophobic amino acids clustered on one side of the helix. Owing to a lack of published structure-activity relationships data on p28, it is unclear whether the cell uptake and preferential targeting of cancer cells of p28 is driven by its amphipathic characteristics [51]. A peptide derived from the PreS2-domain of hepatitis-B virus surface antigens (PreS2) is another example of shorter amphipathic CPP with a null net charge and good uptake [52]. Alternatively, amphipathic CPPs can present their amino acid sequence in a β-sheet structure. Similarly to α-helical CPPs, amphipathic β-sheet peptides, such as VT5, contain one hydrophobic and one hydrophilic stretch of amino acids exposed to the solvent. The ability to form β-sheets is essential for their cellular internalization since the analogues containing single amino acid mutations, leading to the loss of beta-sheet conformation, have extremely poor cellular uptake [53, 54].

Proline-rich peptides (Pro, P) are a class of CPPs characterized by the presence of pyrrolidine rings from proline. When a large number of this rigid amino acid, unable to accept H-bonds like the secondary nitrogen of the other amino acids, are introduced in a peptide sequence a well-defined secondary structure, namely polyproline II (PPII), is obtained. Unlike the common α-helix, PPII is left-handed with 3.0 residues per turn [35]. Examples of proline-rich peptides are the Bac 1–24 residues of bactenecin-7 (Bac7), a 59-residue antimicrobial protein with four 14-residue repeats from the bactenecin family. Both cell permeability and antimicrobial activity of Bac 7 are due to the N-terminal 24 residues [55]. Several Pro-rich CPPs have been reported including fragments of Bac 7 [56], peptides based on a minimal cationic PPII motif (PPR_n and PRR_n where n = 3, 4, 5, and 6) [57], arginine-rich peptides based on the PPII helix of the avian pancreatic polypeptide [58] and polyproline-helix based peptides with various R-groups attached to the pyrrolidine ring [59, 60]. Additional examples are SAP, based on the γ-zein VHL(PPP)8 sequence, which adopts the PPII amphipathic structure and SAP(E) a mutant of SAP with a net negative charge $(VELPPP)_3$ [61, 62]. Theses amphipathic Pro-rich peptides are particularly effective, demonstrating efficient cellular uptake and lack of cytotoxicity. It has been shown that these CPPs are internalized via caveolae or lipid-rafts mediated endocytosis, which circumvents the lysosomal route of degradation [55]. A novel class of CPPs, the cysteine-rich (Cys, C) CPPs, has been derived from the snake venom toxin crotamine, containing two nuclear localization domains, crot (2–18) and crot (27–39). The decapeptide CyLoP-1 with enhanced internalization properties was identified through examination of various versions of crot (27–39) by systematic substitution and/or deletion of amino acid residues, coupled to structure–activity relationship studies [63]. The oxidation status of the cysteine residues is pivotal for these CPPs, since their cell penetrating efficacy is deeply dependent on the formation of intramolecular disulfide-bridges.

3.3. Hydrophobic CPPs

Hydrophobic CPPs predominantly contain non-polar amino acids, resulting in a low net charge and high affinity for the hydrophobic domains of cell membranes. It has been proposed that this family of peptides could translocate across lipid membranes in an energy-independent manner [64]. The secondary amphipathic transportan, which has a low net charge, could be considered a hydrophobic CPP. Recent examples of hydrophobic peptides possessing cell penetrating properties are gH 625, derived from *Herpes simplex* virus type I [65], the decapeptide CPP-C identified in the C-terminal region (residues 140-149) of the fibroblast-growth factor 12 (FGF12) [66], the synthetic C105Y, based on the amino acid sequence corresponding to residues 359-374 of alpha-1-antitrypsin, and its shorter C-terminal domain, PFVYLI [67]. Finally, Gao *et al.* reported the identification of two

different hydrophobic CPPs, Pep-7 and SG3, obtained from randomized peptide libraries using phage display- or plasmid display-based functional selection platform, respectively [68, 69].

4. General approaches in CPPs design

The examples of CPPs mentioned above reflect the enormous functional sequence space encompassed by these molecules. CPPs, with or without cargo, can enter cells actively (energy-dependent mechanism) or passively (energy-independent mechanism) depending on overall amphipathic properties and polycationic nature. In addition, the role of some features such as sequence length and conformation, is as important as the accumulation of positive charge in the internalization stage [22]. The application of distinct design strategies to these known **physico-chemical** properties of CPPs offers the opportunity to improve their penetration efficiency and/or internalization kinetics. However, peptide sequence and secondary structures alone are not enough to define and/or predict the mechanism of action as internalization is also determined by factors such as peptide concentration, membrane lipid composition, cargo, self-assembly state, folding properties of CPPs, response of the cell to the peptide, temperature and ionic strength, among others [22, 39]. In addition, it has not to be forgotten, that CPPs suffer shortcomings typical of pharmaceutical peptide or protein-based products, *i.e.* short duration of action and lack of oral bioavailability [70]. These considerations have led to more elaborate CPPs design addressing not only the internalization efficiency but also challenges such as endosomal escape, circulation times, specificity and selectivity (for cells, tissues, diseases), proteases stability and cytotoxicity [71]. Finally, it must be considered that the type of fluorophores used for CPPs labeling as well the histological features of the cell lines can deeply affect CPPs cellular distribution/uptake [72-74]. Although the first reports on CPPs were based on protein derivatives, rational design is now dominating research activity in the field [75]. Synthetic tools have paved the way to explore new approaches to improve the cell penetration of CPPs and both covalent and non-covalent CPP-therapeutic conjugates. The role of positive charge in cellular uptake of macromolecules is known from long time [76]. As described above, the primary structure–function relationship has been extensively studied for arginine-rich and other positively charged CPPs [77]. Recently, synthetic CPP mimetics (CPPMs) were developed to control the contribution of charge and π-electronics properties [78]. Using this strategy, Lein *et al.* [79], compared the transport ability of a lysine-rich CPP (PEP-1), against a guanidinium-rich 9-mer, bearing two guanidine aa per repeated unit. The results revealed a better performance of the guanidinium-rich carrier than PEP-1 in terms of transport efficiency.

The role of the hydrophobic residues has also been deeply investigated. The hydrophobic amino acids play a major role in the interaction with the membrane bilayer and are thought to enhance the peptide

translocation [80], if a correct balance between hydrophobicity and hydrophilicity is maintained [81]. Among CPPs backbones, tryptophan certainly plays the pivotal role. This amino acid, together with tyrosine (Tyr, Y), is predominant at the membrane surface of membrane proteins favoring non-covalent interactions of other aromatic π-electrons [82]. Ala-scan studies confirmed that the tryptophan residue is essential in penetratin both for membrane recognition and cellular uptake [83]. Moreover the number and the spatial distribution of tryptophan residues deeply affect the efficiency of CPPs and their nuclear localization [32, 84]. Another profitable strategy of CPPs primary structure modification is represented by the synthesis of peptoids and peptidomimetics. Cell-penetrating peptoids (CPPos), do not tend to assume secondary structures as the amine groups of the backbone are substituted and therefore are unable to participate in hydrogen bonding [85]. Finally, the use of cell-penetrating poly(disulfide)s (siCPDs) has been recently introduced as a conceptually innovative approach for cell delivery of substrates [86-88].

The improvement in hydrophobicity is another profitable approach in CPPs design. N-terminal acylation of known CPPs represents the early strategy used to enhance hydrophobic interactions with the membrane and, consequently, selective targeting, uptake efficiency and safety [89]. Hydrophobicity can also be modulated by incorporating amino acids with alkyl side chains into the peptide sequence as for the use of silaprolines (γ-dimethylsilaproline) [90]. Alternatively, hydrophobicity has been achieved by partial charge neutralization by hydrophobic counterions [91].

The susceptibility of conventional CPPs based on natural L-amino acids to proteases is a major drawback for their application *in vivo*. Thus, the replacement of L-amino acids with their non-natural D-stereoisomer and, more recently, their combined use has been described as a profitable strategy to increase stability. Ma and co-workers showed that the number of D-arginine residues in the peptide sequence affects the internalization efficiency [92]. Verdurmen and coworkers observed that the uptake of L- versus D-amino acid CPPs is cell-dependent. In fact, they explained that chirality has an effect on the initiation of internalization in selected cell types, thereby providing useful knowledge for potential future stereochemistry-dependent targeting [93].

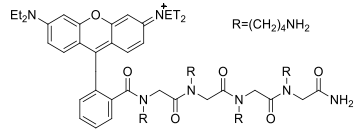
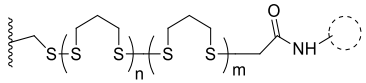
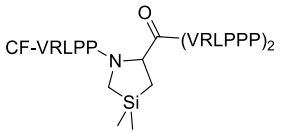
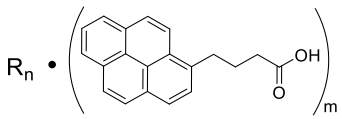
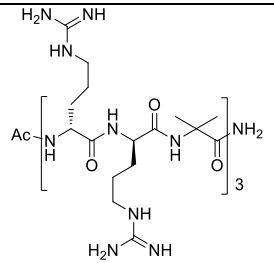
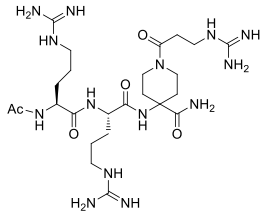
Unlike the examples based on unstructured CPPs that adopt a bioactive conformation when in contact with cell surfaces, helicity can be obtained through the rational design of primary sequences. The natural non-coded α-aminoisobutyric acid (Aib) is, for instance, one of the widely described helix inducers and is often used to stabilize peptide helical structures [94]. Alternatively, non-arginine analogues containing L-proline or the modified guanidinyl-Pro have been designed, achieving a much greater cell-penetrating ability and also plasmid DNA transport properties into HeLa cells [95]. Alternatively, stabilization of the helical structure has been attained by the crosslinking of N-terminal aspartic acid with the amino group of the 2,3-diaminopropionic acid (Dap) residue [96]. CPPs

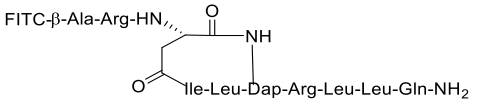
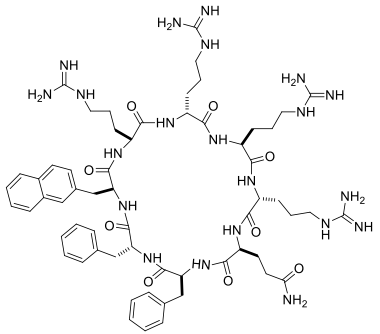
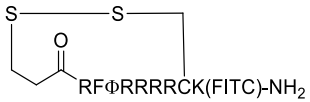
cyclization has also been accounted for resistance to proteolysis [97-99]. Thus, increase in CPPs rigidity is an emerging concept in CPPs design [71, 100]. Qian and co-workers synthesized cyclic CPPs using varying design parameters, such as sequence lengths, stereochemistry and the use of non-natural amino acids [98]. Compound cyclo(FΦRRRRQ) (cFΦR4, where Φ stands for L-2-naphthylalanine), containing two aromatic and three arginine residues binds directly to plasma membrane phospholipids, enters cells via multiple endocytic mechanisms, and efficiently escapes from the early endosomes. This compound has a cytosolic/nuclear delivery efficiency 4-12 times higher than R9, TAT and penetratin [97]. Similarly, the use of disulfide bridges represents the latest trend in the design and development of cyclic CPPs. In comparison to the classical N-to-C cyclization, the advantage of the disulfide bridge strategy is the reversibility of the cyclization in a reductive environment and the resulting increased proteolytic stability. Disulfide bridging of arginine rich CPPs and TAT resulted in improved uptake kinetics when compared to the linear counterparts [100], while Mandal and co-workers reported a study on 11 amphipathic cyclic peptides composed of hydrophobic (W, F, L) and charged residues (K, R, E) to obtain an optimal amphipathic CPP able to undergo intramolecular interactions [101]. In order to improve the results obtained with the cFΦR4 CPPs [98, 102], Qian and collaborators proposed the cyclization of arginine rich sequences through introduction of two cysteine residues to obtain intramolecular disulfide bond [99]. The results showed that the disulfide bridged CPP (DS CPPs) was also efficiently internalized by HeLa cells and was able to escape endosomes.

Moreover, disease-specific targeting moieties such as RGD or homing peptide sequences were added CPPs in order to achieve cell and tissue targeting (*e.g.,* cancer cells) [103]. A new direction comprises the development of activable CPPs where either a pH- or an enzyme-responsive moiety is added to the design [104, 105]. This approach makes the peptides *stimuli*-responsive to the tumor microenvironment, a property that can result in increased selectivity [106]. Alternatively, multivalency of covalent dimers (primary) and supramolecular structures (quaternary) can be used to improve internalization and provide endosomal escape pathways [39, 107, 108].

A brief overview of the concepts and strategies through which the rational design of CPPs can be pursued to enhance their efficiency is reported in Table 2.

Table 2. Main approaches in CPPs rational design with exemplary structures

Design approach		Exemplary structures	Ref
Synthesis of peptoids (CPPos)	Cationic side chains introduced on the peptide bonds		[85]
Synthesis of poly(disulfide)s (CPDs)	Replacement of the peptide backbone		[87]
Improvements in hydrophobicity	N-acylation	CF-(Val-Arg-Pro-Pro-Pro)$_2$Val-Lys(CO(CH$_2$)$_4$CH$_3$)-Leu-Pro-Pro	[89]
	Use of silaprolines		[90]
	Partial charge neutralization by aromatic counterions		[91]
Secondary structure stabilization and helicity induction	α-Aminoisobutyric acid (Aib)		[94]
	Guanidinyl-Proline		[95]

	N-Terminal crosslinked aspartic acids		[96]
Cyclization	Head-to-tail		[97]
	Disulfide bridge		[99]

5. Mechanisms of cellular uptake of CPPs

As reported before, CPPs have different characteristics, and their classification tries in some ways to identify common features that are determinant for their uptake. Anyway, even among CPPs sharing many similarities, these mechanisms vary considerably and rather a landscape of overwhelming mechanisms should be considered. The difficulty in understanding the mechanisms involved in the internalization, even of a single CPP, mainly derives from the fact that the greatest part of their biological properties are determined by variable factors. These factors include the local peptide concentration, its secondary structure, cell surface sugars, local lipid composition, peptide-to-lipid ratio, response of the cell to the peptide and, most of all, type and size of the cargo [22]. Studies of CPP internalization are mainly performed with the help of fluorophores highly different in terms of size, charge and physical properties, such as, for example, pH sensitivity. A consistent matter of discussion about the mechanisms of uptake of CPPs actually centres on the adequacy of the method used for the determination of the pathways involved. Some artefacts were reported in the past and many limits still persist today due, on one side, to the sensitivity of tags associated to CPPs to environmental factors, and on the other to the active role they play as cargos and permeability enhancers. Anyway, while looking for more reliable measurement methods, a general agreement on the mechanisms involved in the uptake of CPPs has been reached, that could be eventually updated and tuned on the basis of new experimental data.

Whatever the type of CPP or target cell, the uptake of CPPs with a cargo can be divided in three different steps: the first contact with membrane, the actual internalization and the release of the drug to its target.

The first step is driven by a combination of electrostatic and hydrophobic interactions. The electrostatic component is predominant for CPPs bearing a positive charge, that establish their first contact with cell membranes by means of anionic membrane lipids and negatively charged glycosaminoglycans (such as heparin, heparan sulphate and chondroitin sulphate) and proteoglycans exposed on cell surface. Hydrophobic contacts are important as well for the interaction of CPPs with membranes, both for amphipathic and hydrophobic peptides: for this ability Wimley [109] also defined CPPs as “interfacially active peptides”, possessing the ability to bind at the bilayer-water interface and perturb membrane structure by selectively activating some small GTPases that induce the remodelling of actin network and the formation of lamellipodia [110].

Once the contact is established, the fate of CPPs can follow different routes, that can be divided at least into two major categories, namely passive (or cell-energy independent) or active (energy-dependent) uptake that differ in terms of efficiency of accumulation [16]. The energy-independent

uptake is not simple passive diffusion across the membrane but is driven by plasma membrane potential [111] and is tightly related to the contact step: it consists in the direct and spontaneous membrane translocation of small molecules through the lipid bilayer, without significant membrane disruption, and is defined as adaptive translocation. This is the mechanism reported for TP2. Spontaneous translocation is generally referred to monomeric peptides: when peptides self-assemble into higher complexes, two different mechanisms of direct translocation can occur, transient pore formation or, if membrane is perturbed, transient plasma membrane disruption. In the first case, peptides accumulate on the outside of plasma membrane and form pores by associating into "toroidal" or "barrel-stave" multimers, whose formation is mainly driven by the binding occurring between guanidinium of arginine residuals and fatty acids groups at high pH [6]. The other mechanism of direct translocation is associated to membrane destabilization and can be ascribed to one of the following models, the "carpet-like" and the "inverted-micelle". In the first case, the interaction of CPPs leads to a transient increase in membrane fluidity and subsequent translocation, while, in the second, an invagination of the bilayer allows the encapsulation of CPPs in micelles followed by their release into the cytosol by inversion of micelles: this is the mechanism described for penetratin, which is dependent on the presence of GAG and specific lipid domain in the membrane. The entity of membrane perturbation activates a membrane repair response (MRR) that prevents a more severe damage to the cell [112]: if these defense mechanisms fail, peptides can be cytotoxic and this is the desired mechanism of action for antimicrobial peptides. The preference for passive uptake by more cationic peptides is also confirmed by their rapid cytosolic accumulation that is otherwise incompatible with times associated to endocytosis [113]. Some highly cationic CPPs show a dependence from anionic lipids in order to be uptaken by passive translocation, while more amphipathic CPPs are generally less dependent on lipid composition. In a recent paper by Hirose *et al.* arginine-rich peptides were observed while forming "particle-like" structures composed of multiple vesicles on the plasma membrane, shedding light on a new model of translocation to be further explored [114].

The energy-dependent mechanism of uptake is endocytosis, that is generally accepted as the prevailing mechanism of CPP translocation and can be classified into four pathways: macropynocytosis, clathrin-mediated endocytosis, caveolae/lipid-raft mediated endocytosis and clathrin/caveolae-independent endocytosis. Endocytosis by macropynocytosis is, for example, the primary mechanism of entry of TAT, but clathrin-mediated and caveolar endocytosis are involved as well. Differently from passive uptake, in which, being the CPP and its cargo directly delivered into the cytoplasm, they are promptly available to act at cytosol level, endocytosis has the disadvantage of the peptides entrapment into endosomal vesicles. The desirability of passive translocation with

respect to endocytic way will of course depend on the intended target for the drug associated with the CPP: if the target of the drug is one of organelles or the nucleus, an endocytic pathway could be preferable, provided that an adequate mechanism of targeting and escape from lysosomal degradation is considered. In addition, also the subtype of endocytic pathway contributes to determine the final delivery of the drug: clathrin-coated endosomes are subjected to transportation on microtubules towards Golgi complex and preferentially degraded, while caveolin-mediated endocytosis leads to cytosolic release of cargo bypassing the degradative route: this last is the route described for the internalization of amphipathic Pro-rich CPPs.

Even for endosomal escape, mechanisms remain elusive, but it is supposed that the same features of CPPs that aid initial uptake could be useful to promote vesicle disruption: for example the stiffening of the membrane due to interaction with positively charged peptides, the formation of pH gradients and the increases in concentration of vesicles content [6, 110], all could contribute to the rupture of endosomes and the release of their content. Selected strategies to obtain endosomal escape derive from modification of CPPs with the addition of fusion proteins or polymers with buffering capacity that elicit a "proton sponge effect" that induce osmotic swelling and rupture of endosomes.

As underlined before, these mechanisms of uptake should not be considered exclusive but rather woven together and complementary. For example, three endocytic pathways can be used simultaneously by penetratin, Arg_9 and TAT. For most of CPPs studied, it seems that size and **physico-chemical** nature of the cargo and the concentration of the peptide could act as a discriminant in the activation of one pathway or another. The term concentration is here generally referred as the peptide-to-cell ratio, which in turn has consequences on the binding stoichiometry between peptides and membrane components. In particular, when the concentration of CPPs is low, endocytosis seems to be the preferential way, while at high concentration of CPPs direct penetration occurs, probably in association with the formation of peptide multimers, as is suggested for TAT and Arg_9. It is not possible to define what is meant for "high" or "low" concentration of a CPP, since this value is extremely dependent on the characteristics of both the peptide and its cargo, as well as the model cell line: for example non-adhering cell lines usually appears to be more sensitive to CPPs [113]. As a reference, Arg_9 is reported to be translocated by endocytosis up to a concentration of 5 μM, while above 10 μM direct uptake is favored. A compromise between useful and toxic concentration for a CPPs must be found, since mechanisms involved in membrane damage can be functional to help the escape from endosomal vesicles, thus contributing to the correct targeting of the cargo.

In view of such variability, a clear knowledge of the target is necessary, in order to carry out all strategies to obtain the most efficient delivery of the drug.

6. CPPs in ocular drug delivery

The eye can be divided into an anterior segment (*i.e.* conjunctiva, cornea, anterior and posterior chambers, filled with the aqueous humor, iris and lens) and a posterior segment, consisting of posterior sclera, choroid, retina and vitreous cavity, enclosing a gel-like fluid called vitreous humor [115]. This distinction is important since, as a general rule, anterior segment diseases are treated by topical application, while posterior segment diseases are treated by local injections such as periocular, intravitreal, suprachoroidal and subretinal. Even if the eye is apparently an easily accessible organ, and this represents an advantageous condition for diagnostic and surgical purposes, ocular drug delivery is challenging, due to the presence of several static, dynamic and metabolic barriers [116]. A topically applied drug that has to be delivered into the anterior eye chamber has to cross the cornea, a tri-layer tissue composed by an outer epithelium, with tight junctions connecting the most superficial cells, a central connective tissue, made of highly organized collagen, and an endothelium, mainly involved in the maintenance of the correct corneal hydration [115] **(Figure 1).**

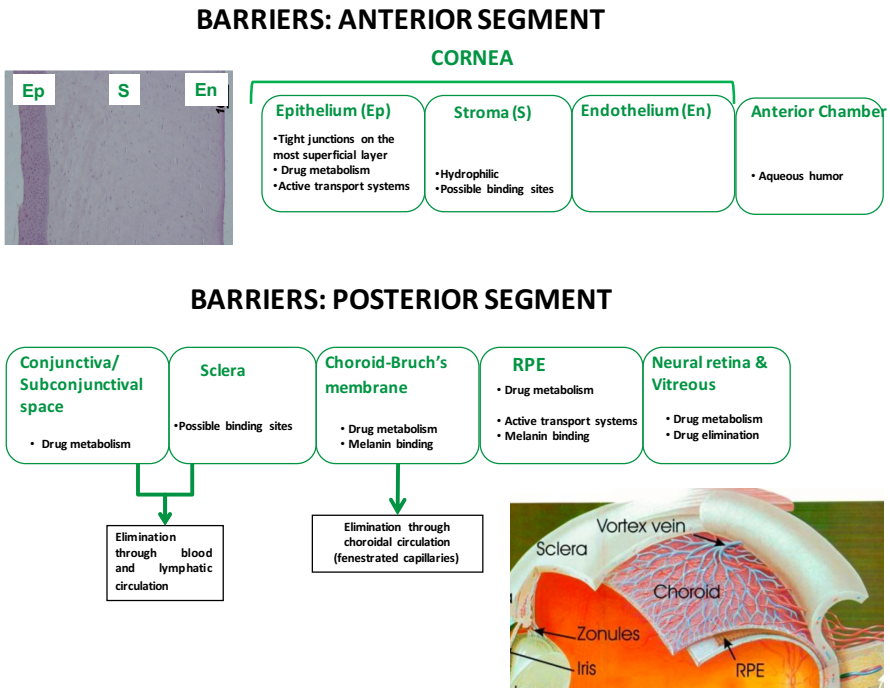


Figure 1: Schematic representation of the barriers involved in drug delivery to the anterior and posterior segment of the eye. Adapted from [117, 118] with permission

As a result of its structure, the permeability of the cornea is low and the diffusion of drugs, particularly hydrophilic and of high MW, is very difficult. Additionally, after instillation, drugs can also diffuse across the conjunctiva, a thin and transparent membrane, with a possible "non-productive" absorption into the blood and lymphatic capillaries and systemic distribution. Finally, a third penetration route

is possible, since the drug after crossing the conjunctiva can diffuse across the sclera, a collagenous highly hydrated tissue, to reach the choroidal space. From here, the drug can be either systemically absorbed into the highly fenestrated capillaries or diffuse across the Bruch's membrane to reach the retinal pigment epithelium (RPE) and the neural retina **(Figure 1)**. This penetration pathway, called conjunctival-scleral, is in principle the one followed by topically applied drugs to reach the retina, even if the possibility of achieving therapeutic concentration using this route is still object of investigation and debate. Given that the posterior segment of the eye is not easy accessible even through the systemic administration, because of the presence of the blood-retinal barrier, the intravitreal injection (*i.e.* direct injection of the drug in the vitreous body), is, at present, the most efficient option for drug administration to the retina. Alternative and less invasive delivery strategies to enhance and sustain drug release to the posterior segment, such as trans-scleral iontophoresis, microneedles, sonophoresis, have been proposed [119]. Cell penetrating peptides as well appear as a potentially useful tool to improve ocular drug bioavailability, by enhancing drug transport across the different ocular barriers **(Figure 1).**

In this section, we have summarized the published papers where CPPs have been used for ocular delivery, differentiating between anterior and posterior segment targeting, since the problems related to these two approaches are very different. We have then focussed our attention to the ocular models used for the study of CPPs in ocular drug delivery and finally commented on toxicity issues.

6.1. Anterior segment targeting (cornea, anterior chamber, lens)

POD, peptide for ocular delivery ($GGG[ARKKAAKA]_4$; 3.5 kDa) has been the first CPP expressly designed and developed for ocular administration from Johnson *et al.* [120] with the aim to treat posterior segment diseases, particularly affecting retina. Two years later, the same research group [121] published an interesting work on the *in vivo* topical administration of POD, in which the peptide was fused with green fluorescent protein (27 kDa), allowing for easy detection and, at the same time, mimicking therapeutic protein conjugation. A dose of 40 µg of POD-GFP was topically applied *in vivo* in male C57BL6/J mice and compared to GFP alone. POD-GFP had the best performance with a preferential localization in corneal epithelium, suggesting the potential use of this CPP for surface corneal diseases such as keratitis. On the contrary, the fluorescence detected inside stroma was weak and indicate a poor diffusion across the tissue [121]. However, this result could be due to the early analysis of distribution (45 minutes after the administration) that could have failed to spot a deeper diffusion. More recently, POD was conjugated with PLGA-PEG (poly(lactic-co-glycolic acid) and polyethylene glycol, respectively) nanoparticles (CPP-NPs), with the aim to increase corneal

epithelium penetration of flurbiprofen [122]. TAT conjugated nanoparticles were used for comparision. CPP-NPs were well tolerated since no irritation was detected neither *in vitro* (hen's egg test), nor *in vivo* (Draize test). The presence of CPPs increased flurbiprofen efficacy in preventing ocular inflammation in an *in vivo* inflammatory model induced by topical administration of sodium arachidonate in New Zealand white rabbits (NPs administration before arachidonate application). POD was more effective than TAT; being size and zeta potential of conjugated NPs comparable, a possible explanation of the different behaviour could be ascribed to a higher affinity of POD for corneal epithelial cells [121] as well as to the intrinsic difficulty for TAT to cross intact corneal epithelium [123]. This hypothesis could find a confirmation in the result obtained in the anti-inflammatory treatment (NPs administration after arachidonate application), when the final effect of TAT-NPs and POD-NPs was comparable [122], maybe due to an increased corneal permeability induced by the inflammatory process.

Table 3. List of CPPs proposed for topical ophthalmic administration to target either the anterior or the posterior eye segment.

CPP	**Cargo**	***In vivo* intraocular distribution and/or preclinical studies**	**Year**	**Ref**
Anterior segment				
TAT-C, penetratin, EB	-	-	2006	[124]
POD	GFP	C57BL6/J	2010	[121]
TAT and NPB (nucleolin binding peptide)	lissamine and green fluorescent protein	C57BL6/J	2011	[125]
TAT-Cd^0	-	BALB/C mice murine (HSV-1 keratitis model)	2013	[126]
PEP-1	FK506BP	C57BL/6 mice (dry-eye model)	2013	[127]
TAT, gC	αβ-cystallin fluorescently labelled	-	2013	[128]
TAT_2, $mTAT_2$	natamycin	-	2015	[129]
PEP-1	FK506BP	male Sprague-Dawley rats (inflammation model)	2015	[130]
PEP-1	FK506BP	male Sprague-Dawley rats (dry-eye model)	2015	[131]
POD, TAT	PLGA-PEG nanoparticles loaded with flurbiprofen	New Zealand white rabbit (inflammation model)	2015	[122]
TAT, SynB1	ELP fluorescently labelled	female New Zealand white rabbit (penetration) and female Dutch-belted rabbits (clearance)	2016	[132]
TAT	endostatin RGD	female and male C57BL/6 mice	2016	[133]
Penetratin, PEP-1 and PEP-1 derivatives	FAM	-	2016	[134]

Posterior segment				
POD	lissamine	C57BL6/J mice	2008	[120]
TAT	acidic FGF	male Sprague-Dawley rats (retinal ischemia model)	2010	[135]
TAT, polyarginine (R8), penetratin, protamine, polyserine (S8, negative control)	FAM	male Sprague–Dawley and albino rats	2014	[136]
TAT, FITC-TAT	endostatin	Kunming mice, laser-induced CNV rC57BL6/J mice	2015	[137]
penetratin (alone or with PAMAM dendrimer (3G))	pRFP	Sprague–Dawley rats	2016	[138]
CC12, penetratin	KV11	Rats, oxygen-induced retinopathy (OIR) in C57BL/6J mice	2017	[139]
TAT	PLGA nanoparticles loaded with fluorescent probes (coumarin, Nile red)	male Brown Norway rats (laser induced CNV model)	2017	[140]
FAM-polyarginine R6	bavacizumab, ranibizumab	male Sprague-Dawley rats; laser-induced CNV in wild-type (WT) C57BL/6J mice	2017	[141]
penetratin, penetratin derivatives	FAM	mice	2017	[142]
TAT, penetratin, polyarginine (R8)	HDL loaded with coumarin or pazopanib	laser-induced CNV rC57BL6/J mice	2017	[143]
penetratin (alone or with PAMAM (5G))	antisense oligonucleotide targeting luciferase gene	Bioluminescent U87 glioblastoma cells (U87-Luc) intravitreal injection in BALB/c Nu/Nu mice	2017	[144]
penetratin (alone or with PAMAM and HA (5G))	antisense oligonucleotide	ICR mice	2017	[145]

Abbreviations: EB, entry blocker peptide; ELP, elastin like polypeptide; FAM, 5-carboxyfluorescein; GFP, green fluorescent protein; HDL, high density lipoproteins; NBP, nucleolin binding peptide; PLGA, poly(lactic-co-glycolic acid); POD, peptide for ocular delivery; pRFP, red fluorescent protein plasmid; RGD, arginine-glycine-aspartic acid; ICR, Institute of Cancer Research; PAMAM, polyamidoamine dendrimer; HA hyaluronic acid; FITC, fluorescein isothiocyanate

Elastin-like polypeptide (ELP) is an artificial biopolymer intended as drug carrier thanks to its ability to diffuse across membrane. In the study presented by George and co-workers, ELP (*approx*. 60 kDa, neutral), fluorescently labelled with rhodamine or Alexa Fluor 633, was fused to TAT (TAT-ELP) or to SynB1 (SynB1-ELP) [132] to further increase its penetration enhancing properties. SynB1 is a 18 **aa** (RGGRLSYSRRRFSTSTGR), naturally derived CPP, able to deliver doxorubicin through the blood brain barrier in tumor targeting [146]. Even if ELP-CPPs fusion complexes showed a significant increase in human corneal epithelial cells uptake (ranging from 2 to 3-fold higher with respect to ELP alone), they did not enhance corneal uptake when topically applied *in vivo* on rabbit ocular surface. Particularly, ELP and SynB1-ELP were found in both epithelium and stroma in comparable amount after two hours after the last administration; fluorescence inside cornea lasted for

at least 3 hours after the application. Another important finding was a wide distribution of ELP inside different ocular tissues, such as sclera and retina, suggesting a lack of specificity.

Our group has recently investigated the capability of different CPPs to cross porcine cornea. We investigated penetratin (PNT), PEP-1, (a synthetic amphipathic CPP) and six mimotopic derivatives designed to cover the whole PEP-1 sequence (KETWWETWWTEWSQPKKKRKV) [134]. Newly synthesized CPPs were composed of 6 or 8 **aa** and differed for the charge, positive (pep-5, pep-6, pep-7) or negative (pep-2, pep-3, pep-4). All CPPs were fluorescently labelled with carboxyfluorescein (FAM) and their distribution within corneal epithelium as well as their capacity to diffuse across the entire corneal thickness were studied using an *ex vivo* porcine model. **Figure 2** reports the permeability coefficients obtained (Panel A) and the confocal sections (Panel B) of vital corneal epithelium after incubation with pep-7, PEP-1, and PNT. All the peptides showed a permeability comparable or even higher than the fluorescent probe alone. The best performing peptide was PNT, its permeability being at least 8-fold higher than other CPPs. Apparently, differences in charge, molecular weight and charge to mass ratio did not influence the diffusion of PEP-1 derivatives. Even if newly synthesized CPPs did not reach PNT performance, their diffusion was not negligible, thus suggesting their possible future use in ophthalmic field. The confocal images (Figure 2B) highlight a different permeation mechanisms: PNT appears as spot in the plasma membrane of wing cells and in cytoplasm and nucleus of basal cells while Pep-7 and PEP-1 are localized in the extracellular space suggesting a paracellular penetration pathway. This result is interesting, considering that, if the corneal epithelium is not the target, the avoidance of cellular uptake can represent an advantage. At the same time it was unexpected, since literature reports the ability of PEP-1 to enter HeLa cells [147] (a discussion on this specific point is reported in section 6.4). Finally, PEP-1 and pep-7 demonstrated to be safe when tested *in vitro* in conjunctival cells.

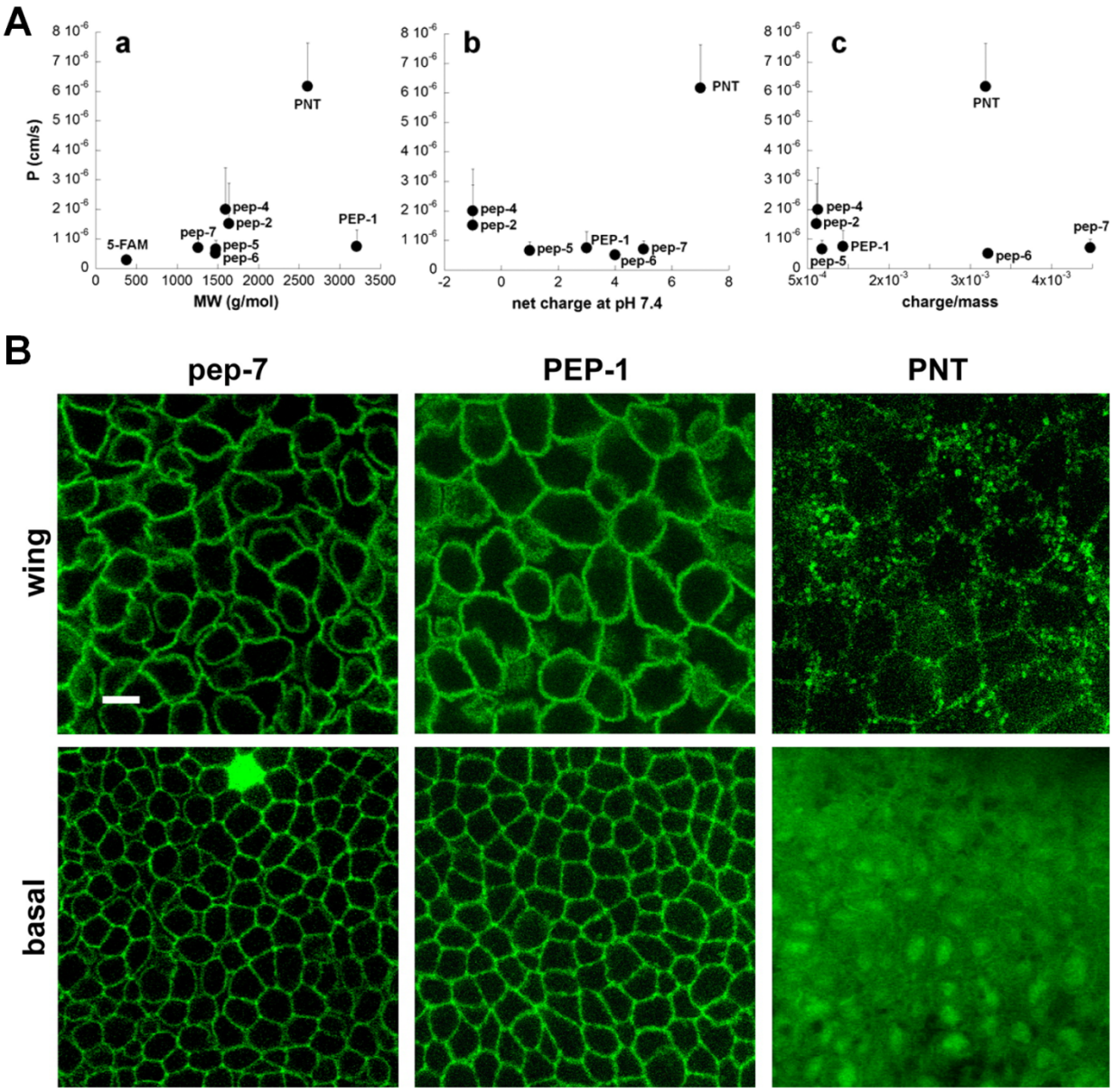


Figure 2. Panel A. Permeability coefficients of peptides as a function of MW (a), net charge (b), and charge/mass ratio (c) (mean values ± SD). Panel B. Confocal sections of *ex vivo* porcine corneal epithelium incubated with pep-7, PEP-1, and PNT. Images of the first line shows wing cells (*i.e.* intermediate layer) while second line represents basal layers (*i.e.* inner). In both cellular populations pep-7 is mainly localized in the extracellular space or in the plasma membrane and similar scenario is observable also for PEP-1. Contrarily, PNT shows a spot localization on plasma membrane in wing cells and appears within cytoplasm and nucleus in the inner layer (scale bar: 10 μm). Reprinted with permission from [134] (Copyright 2016 American Chemical Society).

6.1.1. CPPs for the treatment of corneal affections

Cornea represents not only the outer barrier of the eye, but also a therapeutic target, being itself potentially affected by pathologic conditions, such as keratitis, keratoconus or limbal stem cells deficiency (LSCD). Particularly, keratitis is a serious inflammatory condition affecting cornea, having an infective (bacterial, fungal, viral) or non-infective etiology and leading to the impairment

of corneal integrity and functionality. The most common cause of infective keratitis is *Herpes simplex* virus type 1 (HSV-1): approved therapeutics can control the acute symptom of the primary infection, but cannot successfully prevent the latent infection [124]. For this reason, attempts to find new strategies are ongoing and also the use of CPPs has been proposed. [148]. Indeed, penetratin and TAT-C (HIV-1 TAT with C-terminal cysteine: GRKKRRQRRRC) are CPPs with intrinsic antiviral activity, thanks to the presence of a protein transduction domain [149]. Brandt and collaborators described another antiviral CPP: the Entry Blocker peptide (EB peptide, RRKKAAVALLPAVLLALLAP) a 20 **aa** FGF4 modified sequence, and compared its toxicity with penetratin and TAT-C, both *in vitro* and *in vivo* [124]. All three peptides were non-toxic for primary and immortalized human keratocytes, while, considering non-ocular cell-lines, penetratin demonstrated some toxicity. After *in vivo* topical application (4 times a day for a week) to BALB/C mice, only TAT-C caused a temporary blepharitis and a slight keratopathy in one animal, but overall, no other toxic effect in both anterior and posterior ocular segments were observed, suggesting *in vivo* safety of all the three CPPs [124].

TAT-C was later modified, using D-amino acids and with the C terminus holding an amide instead of a carboxylic group. The obtained CPP, named TAT-Cd^0, a 11 **aa** cationic and hydrophilic peptide, demonstrated an higher ability in blocking HSV-1 [150]. TAT-Cd^0 was then formulated in four different vehicles, *i.e.* artificial tears, PBS solution, 2% methylcellulose gel (in PBS) and a cream (70% healing ointment, 15% mineral oil, 15% water), and evaluated *in vivo* in a murine herpetic keratitis model [126]. For this purpose, BALB/C mice were inoculated with HSV-1 and the treatment with peptide (1 mg/ml) either started within 4 hours or was delayed until 24 hours. Every day, for 7 days, 3 µl of artificial tears or PBS, 5 µl of gel or a small volume of cream, covering the entire cornea, were administered for five times. Over the observation period (15 days after infection), blepharitis, stromal keratitis and corneal neovascularisation were observed, however TAT-Cd^0 was able to partially reduce ocular herpetic effects. The best result against blepharitis was obtained using solutions, while gel and mostly cream reduced the CPP effectiveness. Signs of stromal keratitis and neovascularisation appeared always delayed when TAT-Cd^0 was used, regardless the formulation applied. Furthermore, the reduction of TAT-Cd^0 concentration or the treatment delay to 24 hours post-inoculation, prevented the effective control of the viral infection.

As previously mentioned, also filamentous fungi (*Fusarium* and *Aspergillus* species) can cause infective keratitis. Infections are often a consequence of ocular trauma or contact lens wearing and may lead to blindness. The first line treatment is represented by natamycin, effective but responsible for systemic toxicity. Its topical delivery is prevented by its high molecular weight (*approx.* 666 g/mol) with consequent low corneal permeability, and by concomitant low water solubility. In fact,

natamycin suspension is not effective when fungal infection affects the deep stroma. With the aim to increase corneal bioavailability, Jain and collaborators [129] successfully conjugated natamycin with two different CPPs, using amide linkage. CPPs chosen were TAT_2, that is TAT dimer (RKKRRQRRRRKKRRQRRR) and $MTAT_2$ (AKKRRQRRRAKKRRQRRR), both fluorescently labelled with FITC. The first positive consequence of natamycin conjugation with CPP was the increase of its water solubility: authors considered this improvement by itself could be a possible explanation for internalization observed in both HeLa and HCE cells. By treating HCE cells with endocytic inhibitors, the authors found that endocytosis is the principal mechanism of uptake. These promising results were further corroborated by the increased antifungal activity obtained when TAT_2-natamycin was used with respect to natamycin alone. This result finds an explanation in the ability of the CPP complex to reach fungal hyphae and spores as well as in a possible intrinsic antifungal activity of TAT [129]. Finally, toxicity studies demonstrated TAT_2-natamycin safety towards HCE cells, being over 90% the cell survival after incubation.

CPPs could be used also in the treatment of non-infective keratitis and several examples are reported in the literature. Non-infective keratitis could be induced, among others, by the dry eye syndrome with a not completely clarified pathogenesis. A condition that typically characterize dry eye disease is the increased expression of several pro-inflammatory cytokines, that can be controlled by the use of immunosuppressant drugs such as cyclosporine and corticosteroids [127]. Kim and collaborators used FK506BP-12, a 12 kDa binding protein belonging to immunophillins having anti-inflammatory and immunomodulatory properties, to treat corneal injury in a dry eye model. FK506BP-12 was fluorescently labelled with Alexa Fluor 488 and conjugated to PEP-1 (KETWWETWWTEWSQPKKKRKV) to enhance cell uptake. Indeed, after incubation up to 1 hour at concentrations ranging from 0.5 to 5 μM, PEP-1-FK506BP was detected inside HCE-2 cells cytoplasm and demonstrated its stability within the cells at least for 48 hours. PEP-1-FK506BP had previously demonstrated its efficacy in treating atopic dermatitis in a mouse model [151]. The conjugate was also tested *in vivo* using different animal models, in which corneal injuries, simulating dry eye disease, were produced either using *Botulinum* toxin A in male C57BL/6 mice [127] or low humidity air flow in male Sprague-Dawley rats [131]. In both cases, topical administration of PEP-1-FK506BP induced an improvement of ocular lesions if compared to FK506BP alone. In other words, FK506BP efficacy, which was mainly explained with a reduction in the expression of pro-inflammatory, apoptotic and angiogenic factors, was strictly dependent on cell internalization, possible only when the protein is conjugated to a CPP. In general, the use of a pathological model represents a strong evidence of efficacy, allowing for the collection of solid data both in term of

capability of the CPP complex to entry inside target cells and efficacy in producing a therapeutic effect. PEP-1-FK506BP appears a powerful tool to cure corneal damages, particularly those having a non-infective etiology: in fact, besides the efficacy in dry eye disease, promising results have been obtained also in an *in vivo* rat model of an alkali burn-induced corneal inflammation [130]. From the safety point of view, it could be of relevant interest to follow PEP-1-FK506BP *in vivo* distribution in all ocular tissues, to evaluate its specificity, as well as to perform toxicity test, even if the positive effect on corneal injuries could be seen as an indirect demonstration of complex safety.

6.1.2. CPPs for the treatment of lens diseases

Another interesting target in the anterior segment of the eye is represented by the lens, a transparent biconvex structure, located immediately behind the iris and responsible for the focus of the images. The most abundant protein within the lens, α-crystallin, belongs to the molecular chaperone family and prevents the aggregation of other proteins. Therefore, when deficiency of α-crystallin occurs, proteins tend to aggregate and this leads to lenticular opacification, a pathologic condition known as cataract. In order to prevent cataract onset, a possible approach consist in the delivery of α-crystallin proteins to the lens epithelial cells. Unfortunately, reaching the target is not simple, being the lens enveloped in a capsule. In 2013 Mueller *et al.* worked on the CPP mediated delivery of α-crystallin proteins to the lens [128]. In particular, they prepared a recombinant human αB-crystallin fused with TAT or gC (glycoprotein C from HSV-1) CPPs in amino-terminus. Particularly, gC was chosen since interacts with lenticular glycosaminoglycans and cell surface heparin sulphate. The obtained αβ-crystallin-CPPSs complexes were soluble; only in gC complex chaperone-like activity of α-crystallin was retained, while TAT-αB-crystallin failed in preventing protein aggregation. Cellular uptake was studied on Human Lens Epithelial B3 cells (HLE-B3) incubating fluorescently labelled (Alexa Fluor 488) TAT-αB-crystallin, gC-αB-crystallin or αB-crystallin for 1 hour at 37°C. Cellular internalization was followed using confocal microscopy and after 24 hours revealed a poor penetration of αB-crystallin, while TAT-αB-crystallin and gC-αB-crystallin were clearly observable inside each cell. Furthermore, no α-crystallin degradation occurred within HLE-B3 cells at least up to 48 hours after the uptake. Among the two CPP complexes, gC-αB-crystallin appeared as the best candidate. However, authors underlined that primary human lens epithelial cells may differ from native lens epithelial cell in term of protein expression pattern and therefore the uptake data should be considered with caution. Additionally, the present data were collected on isolated cells, and do not consider the relevant barriers hindering *in vivo* the penetration, such as the cornea and the lens capsule. These aspects should be taken into account to evaluate the feasibility of a topical administration.

6.2. Posterior segment targeting (choroid, retina, vitreous)

6.2.1. Intravitreal and subretinal injection

Different peptides have demonstrated the capability to enhance cargo cell uptake *in vivo* following either subretinal or intravitreal injection in mice and rat models. CPPs evaluated were VP22 [152], TAT [153-155], POD [120, 121, 156, 157] and nucleolin binding peptide (NBP) [125]. Overall, the published data highlight that, the CPPs-cargo complex (fusion protein, CPP-protein physical complex, DNA-containing nanoparticles or the simple CPP linked to a fluorescent probe) when localized in proximity of the target cells is able to enter retinal cells and, if the case, transcript the encoded proteins. Despite these relevant results, subretinal and intravitreal administration routes present the downside of being highly invasive; this issue become particularly relevant considering that many ocular diseases affecting the posterior segment are chronic and request regular lifelong injections. A possible approach to prolong the dosing interval has been recently evaluated by Bhattacharya [158], who hypothesized the use of RPE cells as an intraocular drug reservoir after intravitreal injection. Controlled drug release from the intracellular depots was obtained by the use of cleavable peptide linkers between cargo peptides and CPPs. The studied cargo peptides were both hydrophobic and hydrophilic and their intracellular release was controlled by the specific sequence of the peptide linker.

6.2.2. Non-invasive or minimally-invasive administration

In the last 10 years the research has explored the possibility of a non-invasive or minimally-invasive administration modality to target the posterior segment of the eye. A preclinical study in 2015 has confirmed that brimapitide (XG-102), a compound obtained by the fusion of a peptide that selectively inhibits c-Jun N-terminal Kinase activity to TAT, (DQSRPVQPFLNLTTPRKPRPPRRRQRRKKRG) has potential for treating intraocular inflammation via the subconjunctival injection, a good compromise to get a therapeutic effect while limiting side effects [159]. More recently, a phase III clinical study showed that a single subconjunctival injection of XG-102 (90 μg or 900 μg) administered at the end of a surgical procedure is non-inferior to the administration of dexamethasone eye drops, 4 times a day for 21 days [4].

Many recent investigations are performed to evaluate the possibility of attaining the posterior segment by using a completely non-invasive application, in the form of eye drops (Table 3). Thus, in this case, the research focus has moved from CPPs capability to obtain intracellular delivery to their capability to cross biological barriers.

6.2.2.1. Topical instillation of CPPs-conjugates

One of the first paper evaluating topical application of CPPs for retinal targeting was published by Wang and coworkers, willing to deliver a potent protector against retinal ischemia-reperfusion (IR)

injury [135]. The molecule was the human acidic fibroblast growth factor (aFGF) topically applied in male Sprague-Dawley rats as such (FGF-His) or TAT-conjugated (TAT-FGF-His). Only TAT conjugated FGF was found in the retina. The retinal highest levels were found between 30 minutes and 1 hour after topical application, then the concentrations declined, but the conjugate was still detectable after 8 hours. Preclinical data on ischemia-reperfusion injury model showed less severe pathological changes in the retina and accelerated recovery measured by electroretinogram (ERG) upon topical instillation (administration at days 0, 2 and 4 with respect to IR injury) of TAT-FGF-His for comparison to FGF-His, demonstrating that the presence of TAT improved the permeation across ocular tissue while preserving the biological functionality of FGF. Concerning the penetration pathway, the authors excluded a transcorneal pathway, since after application on the ocular surface TAT-FGF-His was found in the corneal epithelium, but not in the stroma and endothelium, in agreement with *ex vivo* data on TAT-β-galactosidase [123]. Indeed, a conjunctival-scleral penetration route is consistent with the much higher permeability of these tissues for comparison to the cornea [160].

De Cogan recently reported the possibility of delivering high molecular weight proteins (bevacizumab and ranibizumab) to the posterior eye segment upon topical application using a fluorescently labelled polyarginine (5(6)-carboxyfluorescein-RRRRRR-COOH) [141]. In this case, a simple charge-based interaction complex was prepared, demonstrating that protein activity was not compromised. Pharmacokinetic studies were performed on a rat model after topical application of one drop of either CPP-bevacizumab complex or appropriate controls. The results highlight a significant CPP enhancing property and a fast diffusion to the posterior segment (vitreous + retina) with a maximum tissue concentration obtained after 45 minutes. Pre-clinical data obtained on a laser-induced choroidal neovascularization (CNV) mouse model demonstrated the efficacy of the administration of the bevacizumab-CPP complex twice a day for 10 days: this treatment schedule resulted comparable to either daily systemic administration of dexamethasone or one single intravitreal injection of bevacizumab (performed at day 0). Interestingly, the authors also took into consideration the issue related to the model used, very different from the human eye in terms of volumes and delivery path-length. They addressed this problem by evaluating bevacizumab uptake in an *ex vivo* model of porcine eye and again demonstrated the capability of the complex to deliver the drug to the vitreous and the retina. In this paper, the authors underpin a corneal penetration pathway. Indeed, by using optical coherent tomography (OCT), they detected the presence of the fluorescent complex in the aqueous humor of rats already 6 minutes after the topical application. However, the penetration path could not be established with precision since the quantification of the drug and/or the CPP was not performed in the anterior segment, cornea, sclera choroid or conjunctiva.

Chen used a Phage display technology to select the CPP sequence to be linked to a therapeutic peptide (KV11) that had previously demonstrated to reduce pathological neovascularization [139]. The selected peptide, called CC12 (EMFTPPSMIERLK-FITC), demonstrated to significantly increase KV11 penetration across both the cornea and the sclera-choroid-retina trilayer *ex vivo* on rabbit tissues. *In vivo* studies showed the capability of CC12-KV11 (but not of the cargo alone) to reach the neural retina after only 30 minutes from topical instillation. The fluorescence retina levels increased with time and peaked after 3-6 hours. Preclinical studies demonstrated that development of pathologic retinal changes was inhibited by topical instillation of the peptide CC12-KV11, but not by KV11 alone. Concerning the penetration path, the authors hypothesized a conjunctival/sclera penetration mainly by intercellular/paracellular translocation. This hypothesis was supported by the relatively low cell uptake measured with ARPE-19 cell lines and the lack of obvious fluorescence in the entire cornea, where the peptide seems mainly localized in the epithelium (at 0.5 and 3 h after instillation).
Another promising anti-angiogenic compound is represented by endostatin, a 20kDa fragment of collagen. Zhang *et al.* prepared a FITC-labelled fusion protein with TAT and demonstrated that anti-proliferative and anti-angiogenic properties of endostatin were maintained (in some cases even increased) and cellular uptake by human EA.hy926 cells was greatly enhanced for comparison to the non-conjugated endostatin [137]. TAT-endostatin cellular uptake was both concentration and time dependent. The studies performed on uptake mechanisms highlighted the simultaneous presence of direct translocation, both clathrin- and caveolar-mediated endocytosis and micropinocytosis. The *in vivo* studies demonstrated the capability of TAT-endostatin (but not endostatin alone) to reach the retina after topical instillation for 3 days (6 times a day). A similar result was obtained in preclinical studies where a significant reduction of the neovascularization area was found after topical instillation of the fusion protein. The same authors evaluated the further association of RGD to the TAT-endostatin complex [133] to further enhance its binding affinity to endothelial cells.
The penetration pathway was not investigated and, given the relatively long application and dosage (6 times a day for 3 days) it is not possible to highlight if the enhanced permeation was obtained across the cornea, the RPE or the conjunctiva and if a systemic absorption took place.

6.2.2.2. Topical instillation of CPPs-based nanoparticles

Penetratin was used to condense a model gene (red fluorescent protein plasmid, pRFP) and promote its topical absorption and delivery to the posterior segment of the eye [138]. Indeed, the 20:1 penetratin to plasmid complex (420 nm, +24 mV) dramatically increased pRFP transfection on both

corneal and conjunctival cell lines. The authors also evaluated a double condensed penetratin-PAMAM pRFR (155 nm, +30 mV) that demonstrated *in vivo* on Sprague–Dawley rats a significantly higher distribution into the posterior segment and transfection with respect to the penetratin-pRFR complex, after topical instillation of the complexes for three time every 10 minutes. The better performance of the polyamidoamine dendrimer (PAMAM) containing complex was attributed to its smaller size and to a different internalization process. Interestingly, the levels found in the corneal tissues were very low. This result suggests a conjunctival-scleral penetration route and indicate that the data collected on isolated cells do not necessarily translate to tissues, where a specific cell organization is present. Similar results were found when studying the association between PAMAM (5G), hyaluronic acid and penetratin to deliver antisense oligonucleotides to the posterior segment of the eye [145]. In fact, while the presence of penetratin in the complex gave only a limited advantage on *in vitro* cell uptake, a much higher retinal distribution was found after *in vivo* administration on ICR mice, demonstrating the ability of penetratin to promote the absorption across complex epithelia and tissues, such as the ocular barriers. The topical application of a physical complex between PAMAM (5G), penetratin and an oligonucleotide gave also encouraging results for the treatment of an orthotopic intraocular tumor model on Balb/c nude mice after topical instillation: a significant reduction of tumor cell proliferation was found, with respect to both the nude oligonucleotide and the PAMAM-oligonucleotide complex [144].

Suda *et al.* explored the possibility to target the retina by topical instillation of CPPs-fused high-density lipoproteins (HDL) nanoparticles, designed as a drug delivery system [143]. They prepared human apolipoprotein (apoA-I) mutants with or without a C-terminal cationic CPP (TAT, penetratin or polyarginine) and then HDL using different kind of phospholipids. HDL were loaded either with a fluorescent probe (coumarin-6) or with a tyrosine kinase inhibitor (pazopanib). Distribution studies on mice (C57BL6/J) highlighted the capability of CPPs-modified HDL to increase coumarin deposition in the inner layer of the retina for comparison to non-modified HDL, after 30 minutes from the instillation of one drop of formulation. The best performing CPP was penetratin, and the reason was linked to neither NP size nor charge, since nanoparticles size (15-20 nm) and zeta potential (-4 - 8 mV) were comparable for the three CPPs. Within penetratin fused NP, the lipid used and the size played a role, with the best performance found for DSPC (1,2-Distearoyl-sn-glycero-3-phosphorylcholine) and 15 nm penetratin-derivatized HDL. Preclinical studies on a laser-induced CNV murine model confirmed that, after one week of treatment (twice a day instillation) pazopanib-loaded penetratin-fused HDL significantly reduced the neovascularization area for comparison to a cyclodextrin-based pazopanib formulation (Captisol®) (**Figure 3**). The data collected did not allow to determine the nanoparticles penetration pathway. In fact, after 30 minutes application, high

fluorescence levels were detected in both the cornea and the conjunctiva. Additionally, after instillation in the right eye, fluorescence was detected also in the contralateral eye, suggesting an absorption through conjunctival capillaries and/or lymphatic vessels.

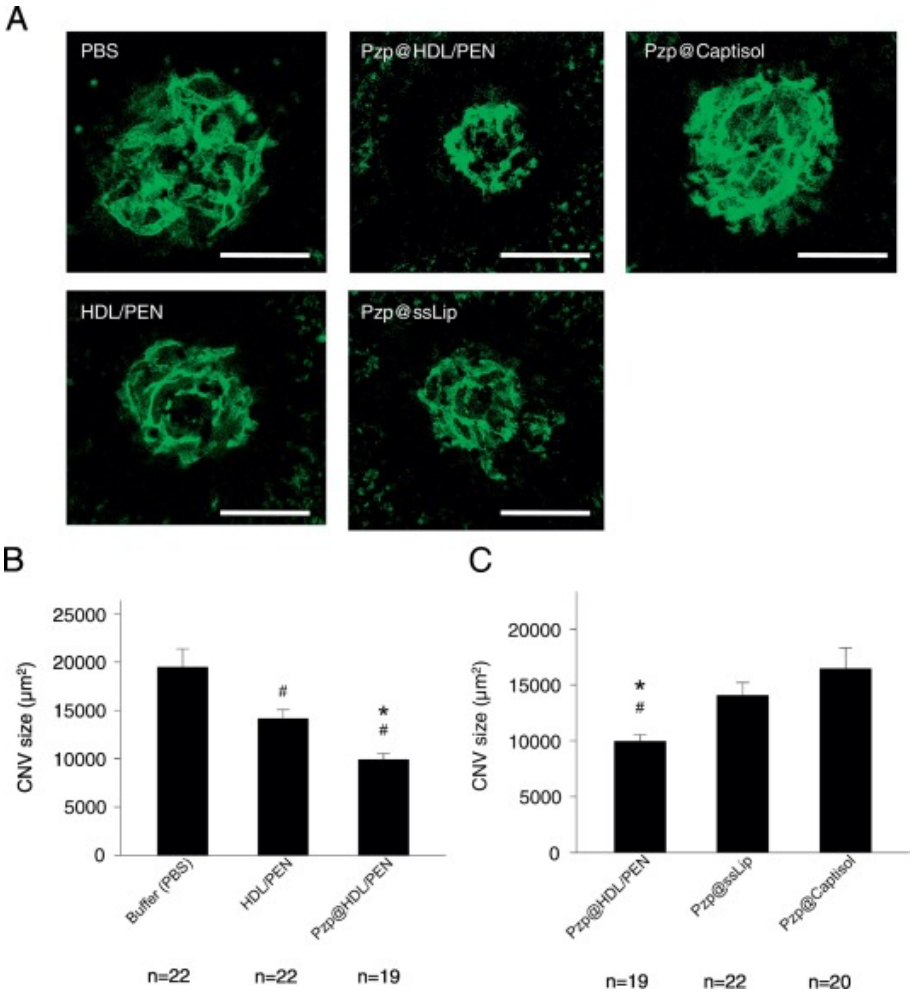


Figure 3. Preclinical evaluation of pazopanib (Pzp) loaded HDL in a murine laser-induced CNV model. (Panel A) Confocal images of the CNV area stained with anti-ICAM2-antibody after 1 week of topical treatment with PBS, HDL mutant with penetratin (HDL/PEN), or Pzp formulations containing HDL/PEN (Pzp@HDL/PEN), Captisol® (β-cyclodextrin) (Pzp@Captisol), or a small-size liposome (Pzp@ssLip). Scale bars, 100 µm. (Panel B) A comparison of CNV areas 1 week after treatment (Panel C) A comparison of CNV areas on confocal images 1 week after Pzp loaded eye drop administration using three different drug carriers (reproduced from reference [143] with permission).

Chu *et al.* derivatized PEG-PLGA nanoparticles either with RGD (arginine-glycine-aspartic acid) or with TAT or with both peptides [140]. They evaluated nanoparticles uptake using umbilical vein endothelial cells (UVECs) with overespressing integrin αvβ3 (probe: nile red), nanoparticles penetration across a monolayer of HCE (probe: coumarin 6) and *in vivo* nanoparticles distribution in the retina and choroid after 30 minutes of *in vivo* instillation in a rat model of laser-induced CNV

(Brown Norway male rats). The cellular uptake results permitted to select the right RGD/TAT density. The particles selected for further studies had an average size of 60-70 nm, polydispersity index lower than 0.2 and a slightly negative zeta potential. Uptake studies on UVECs overespressing integrin αvβ3 showed fluorescent intensity levels in the order RGD-TAT-NP>TAT-NP≈RGD-NP>NP, suggesting that both TAT and RGD promote NP cellular uptake even if with different mechanisms. Transport studies using HCE model, show, on the contrary, that only TAT was able to promote the transport, probably due to the absence of integrin αvβ3 on these cells. Finally, *in vivo* results show that fluorescence of the choroid-retina was in the order RGD-TAT-NP>TAT-NP>RGD-NP>NP. Contrarily to the transport experiments across HCE where NP and RGD-NP had the same low performance, the results obtained *in vivo* highlighted a better performance of RGD modified nanoparticles compared to unmodified ones.

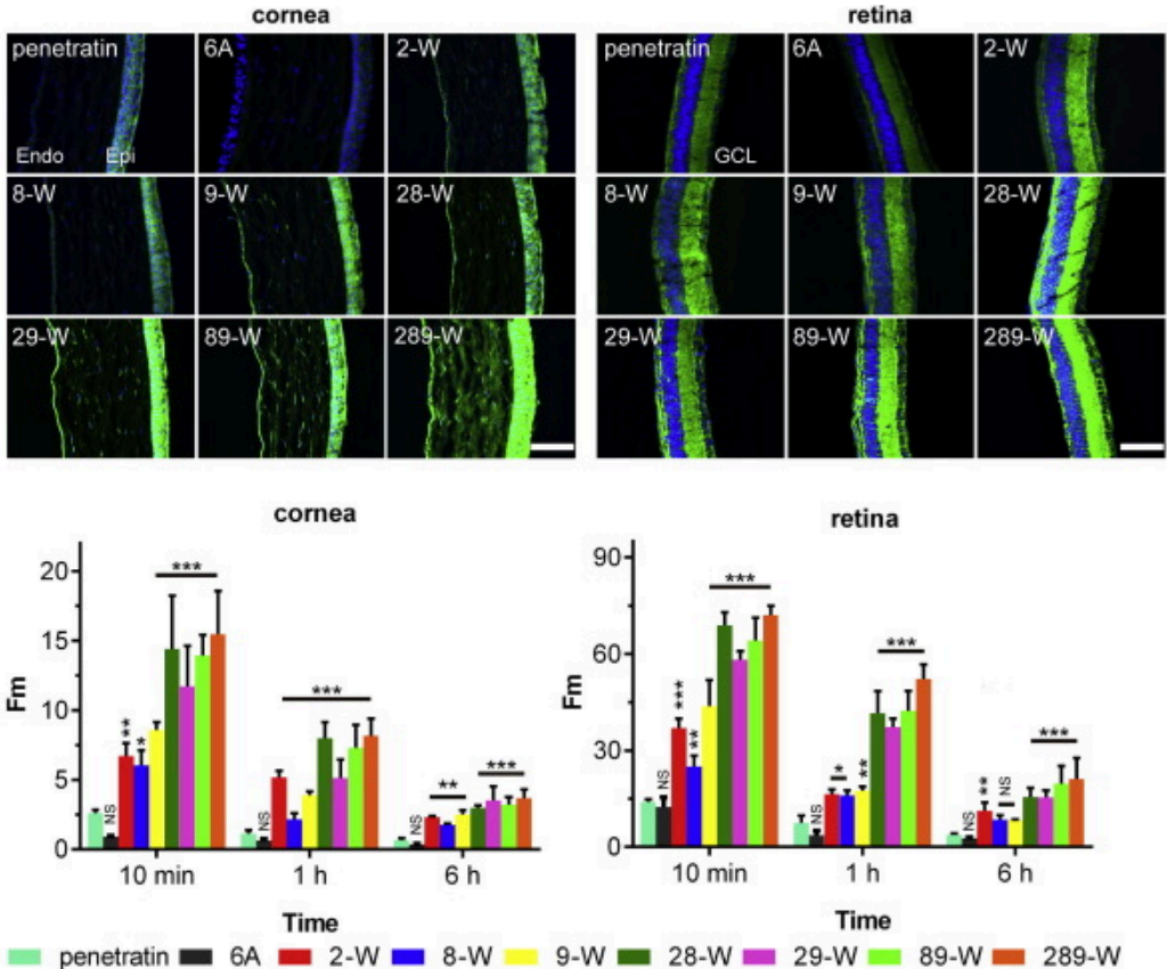
6.3. The influence of CPPs structure on ocular distribution following topical administration

Liu *et al*. screened different cationic CPPs (polyarginine R8, protamine, TAT, polyserine S8 and penetratin), to find out the more suitable to promote drug delivery to eye tissues [136]. Penetratin demonstrated the best performance in terms of cell uptake and *ex vivo* permeation across isolated cornea. *In vivo* studies highlighted penetratin distribution in both cornea (mainly in the epithelium) and in the retinal layer after only 10 minutes from the instillation. The fluorescence peaked after 30 minutes and was still detectable at 6 hours. The best performance of penetratin for comparison to the other peptides was explained by the authors on the basis of its structure and, in particular, of its lipophilicity, that was the highest among the tested CPPs. The better performance of penetratin for comparison to TAT and polyarginine was also reported by Suda *et al.* [143] (see also section 6.2.2.2.). Even if authors did not investigate the reason, their data permit to exclude the role of the number of positive charges.

In a further work, a change in penetratin aminoacidic sequence was done, by partially substituting hydrophilic **aa** with tryptophan. Eight different peptides were prepared (**Figure 4**) and again the results showed a dramatic enhancement of both uptake in conjunctival and corneal cells and *in vivo* drug distribution to cornea and retina for the most lipophilic ones (**Figure 4**) [142]. The results are in agreement with the impact of the presence of tryptophan on cell uptake in case of cationic CPPs, as discussed in section 3.1..

Abbreviation	Sequence [a]	Mw (Da)	Positive charge	Number of Trp	miLogP [b]
Penetratin	RQIKIWFQNRRMKWKK	2246.77	7	2	-6.01
6A	RQΔKΔΔΔQNRRΔKΔKK	1796.12	7	0	-6.72
2-W	RWIKIWFQNRRMKWKK	2304.86	7	3	-5.57
8-W	RQIKIWFWNRRMKWKK	2304.86	7	3	-5.57
9-W	RQIKIWFQWRRMKWKK	2318.88	7	4	-5.75
28-W	RWIKIWFWNRRMKWKK	2362.94	7	4	-4.76
29-W	RWIKIWFQWRRMKWKK	2376.97	7	4	-5.13
89-W	RQIKIWFWWRRMKWKK	2376.97	7	4	-5.13
289-W	RWIKIWFWWRRMKWKK	2435.05	7	5	-3.40

A



B

Figure 4. Structure and physico-chemical characteristics (Panel A) and *in vivo* ocular distribution of penetratin and its derivatives (Panel B). Peptides were instilled every 10 min for three times and eye bulbs were harvested 10 min, 30 min, 1 h, 2 h, 4 h and 6 h after the last administration. Panel B reports the fluorescence-merged images of the cornea and retina 10 min after the last topical instillation. Peptides are labelled with 5-carboxyfluorescein (green) while cells with DAPI (blue). The histograms represent the semi-quantitative analysis of mean fluorescent intensity in cornea and retina at different time points after topical instillation (modified from reference [142] with permission).

Not all CPPs are suitable for posterior segment targeting: contrarily to TAT, penetratin and penetratin derivatives, data on POD do not support its use for topical delivery if the retina is the target tissue. In fact, after 45 minutes of topical application, the fluorescently labelled POD was found to be localized in the corneal epithelium, in the *dura madre* of the optic nerve and in the sclera, but no fluorescence was found in the underlying tissues [120]. In analogy, the topical application of nucleolin binding peptide (NBP), a CPP designed to evade endosomal loss and enhance crossing of nuclear membrane, only results in retention in the optic nerve sheath and in the superficial epithelial cells of the cornea [125].

6.4. Models for the study of CPPs in ocular drug delivery

Different *in vitro*, *ex vivo* and *in vivo* models have been used to evaluate CPPs activity in ocular drug delivery. Table 4 summarizes the different models described in scientific literature. Toxicity and uptake studies are generally performed using isolated human conjunctival/corneal/RPE cell lines. Although the results obtained *in vitro* using cell-lines are in principle more reproducible and avoid species-dependent differences, at least two possible drawbacks can be identified. The first inconvenience concerns the difficulty in extrapolating the CPP's behavior observed in a specific cell-line to different cell types. For instance it is known that non-adherent cell lines display an higher permeability towards CPPs [113]. Another clear example is provided by TAT-C and penetratin [124]. TAT-C showed lack of toxicity in various ocular (primary and immortalized human keratocytes) and non-ocular (HeLa, Vero, TM-1, DP-9) cell-lines, while penetratin demonstrated no clear cytotoxic effect on primary and immortalized human keratocytes, but produced a toxic response in non-ocular cell-lines, with different specificity (TM-1>Vero>HeLa>DP-9) [124]. The second problem concern the predictivity of results obtained on isolated cell. Indeed, the difference between *in vitro* cell uptake experiments and *in vivo* set-up can be due not only to pharmacokinetic reasons (presence of tear fluid, clearance, blood and lymphatic absorption) but also to a different CPP-cell interaction when the cells are part of a structured and organized tissue. The studies performed by Johnson [120] on isolated cells in the presence/absence of chondroitin sulfate and heparan sulfate found a relevant reduction of cell uptake after pre-incubation of POD with the two proteoglycans. This experiment has been used to support the use of cell-surface proteoglycans for binding and cell entry, but it is worth reminding that similar molecules are also present in the intercellular spaces and could be responsible of a different results between *in vitro* and *ex/in vivo* experiments. PEP-1 is reported as capable of entering the cell nucleus of different cells in less than 10 minutes [42], but *ex vivo* data on isolated viable porcine cornea showed a paracellular localization and no cell uptake (see also **Figure 2B)** [134]. Additionally, significant changes take place in corneal epithelial cells during their differentiation and migration toward the surface, thus the behavior of CPPs can be different in basal, wing or superficial cells, due to the different cell features and glycoconjugate composition [161]. Indeed, PNT behaves differently depending on the degree of cell maturation, with an intercellular spotty localization in wing cells layer, and an intracellular (both cytoplasm and nucleus) localization in case of basal cells (see again **Figure 2B**) [134].

The lack of *in vitro-in vivo* agreement was also reported by George *et al.* [132]. They found a significant increase in elastin-like polypeptide (ELP) uptake in HCE cells, when fused to two different CPPs, but this result was not observed *in vivo*, when ELP amount inside corneal layers was

independent from the presence of CPPs. Other papers, as discussed in the previous sections, report a discrepancy between *in vitro* and *in vivo* results [138, 140, 145].

In vivo studies are generally performed using rats or mice (Table 4). This is often a necessary choice, but is however very important to take into account the differences between animal models and human eye concerning ocular volumes and delivery path lengths. Studies on larger species can be relevant to confirm the obtained data and also to investigate the penetration pathway, possibly using live imaging techniques.

Permeability studies have been mainly performed across excised tissues (Table 4). Despite the absence of dynamic clearance mechanisms, this kind of experiments has often the advantage to be performed using porcine or rabbit ocular tissues, *i.e.* animal models having ocular size and permeability relatively similar to the human eye [118]. An alternative is represented by immortalized human corneal epithelial cell line (HCE-T) [162], the human-derived cell line most extensively used in transcorneal permeability studies, but its permeability is considerably higher with respect to excised tissues and is also very variable as a function of the culture conditions [163].

Table 4. *In vitro*, *ex vivo* and *in vivo* models used for the study of CPPs cell uptake, transport and distribution in topical ocular drug delivery

Cell uptake	human conjunctival epithelial cells (NHC)	[136, 138, 142, 145]
	human embrionic retinal cells (HER)	[120, 125, 157]
	human corneal epithelial cells (HCE)	[123, 127, 129, 138, 142, 145]
	human adult RPE (ARPE-19)	[139]
	human lens epithelial cells (HLE-B3)	[128]
	excised porcine cornea	[134]
	excised rat cornea	[123]
Toxicity	human conjunctival epithelial cells (NHC)	[136, 138, 142, 144, 145]
	human corneal epithelial cells (HCE)	[129, 132, 138, 142, 144, 145]
	human corneal endothelial cells	[129]
	human adult RPE (ARPE-19)	[141]
	primary adult rat retinal cells	[141]
	primary adult human corneal fibroblasts	[141]
	immortalized human corneal epithelial cell line (HCE-T)	[143]
***In vitro* or *ex vivo* transport studies**	excised porcine ocular tissues	[134, 141]
	excised rabbit ocular tissues	[136, 139, 142]
	immortalized human corneal epithelial cell line (HCE-T)	[140]
	rabbit corneal endothelial cells (RCEC)	[133]
	primary rat retina microvascular endothelial cells (RRPC) and primary rat endothelial cells (RCEC) co-cultured	[133]
***In vivo* intraocular distribution**	male Sprague-Dawley rats	[130, 131, 135, 136, 138, 139, 141]
	albino rats	[136]
	C57BL6/J mice	[120, 121, 125, 143]
	C57BL/6 mice	[127, 133]
	Kunming mice	[137]
	BALB/C mice	[125, 126, 157]
	mice/ICR mice	[142, 145]
	male Brown Norway rats (laser induced CNV model)	[140]
	New Zealand white rabbit	[122]
	female New Zealand white rabbit (penetration) and female Dutch-belted rabbits (clearance)	[132]

6.5. Toxicity issues

A drawback of using CPPs is their general lack of cell and tissue specificity. This could be considered a limited problem for a topical application route, but the issue, in case of ocular delivery, still remains. In fact, the CPPs lack of specificity could cause an unwanted wider drug distribution in the different ocular structures, with potential accumulation in the RPE and retina cells of drugs that, for instance, should be delivered only to the corneal epithelium. However, different CPPs appears to be characterized by somewhat specific distributions: while penetratin, penetratin derivatives and TAT quickly accumulate in the retina after topical application (see sections 6.2 and 6.3), POD [120] and nucleolin binding peptide (NBP) [125] remain localized to the superficial epithelial cells of the cornea and in the optic nerve sheath. Additionally, POD seems to be more effective than TAT in the prevention of corneal inflammation *in vivo*, when flurbiprofen is administered topically in PLGA-PEG nanoparticles [122]. However, this point, *i.e.* the possibility of targeting different eye structure by using different CPPs, deserves further investigation, since this information was extrapolated by various reports where experiments were performed in different conditions and using different *in vivo* models.

An important advance to improve CPP specificity could be represented by activatable CPPs (ACPPs), penetrating peptides whose cellular uptake is hindered by a covalently attached inhibitory domain. Cleavage of the linker by tissue-specific proteases dissociates the inhibitory domain, enabling the CPP to enter cells [6]. This strategy, mainly applied to enhance drug delivery to tumor tissue, could be in principle exploited also for ocular delivery. Another approach to obtain selectivity could be the use of targeted delivery systems, for instance directed towards the melanin contained in the choroid and RPE cells [164].

Concerning the cell toxicity, the papers analyzed in the present review have mainly evaluated the cytotoxicity on conjunctival and corneal cells (Table 4) and found a good tolerability of these compounds. However, the toxicity is a function of the CPP considered. For instance Liu *et al*. [136] found a cytotoxicity on NHC in the order polyarginine (R8) = protamine>TAT ≈ polyserine (S8)> penetratin, this last did not demonstrated any effect on NHC viability even at the highest concentration tested (30 mM). Together with CPP nature and concentration, also the presence of the cargo can impact on the toxicity. For instance, one study reported a concentration-dependent toxic effect on HCE cells exerted by the fusion complex TAT-ELP, while, at the same concentrations (*i.e.* up to 40 μM), ELP alone or fused to SynB1 CPP, did not affect cell survival [132]. As previously mentioned, also the choice of cells type used is of outermost importance. Penetratin demonstrated lack of toxicity on keratocytes (CC_{50} 200 μM), but reduced the survival in Vero (CC_{50} 70 μM), HeLa (CC_{50} 93 μM) and TM-1 cells (CC_{50} >50 μM) [124]. Given these results, it appears necessary a careful

investigation of toxicity not only towards conjunctival and corneal but also on endothelial and retinal cell lines.

CPPs toxicity is however limited by their extensive degradation by proteases [165]. Extracellular proteases may degrade CPPs before they attain their target, reducing the efficacy of the cargo drug that may cumulate in extracellular space. Even CPP–cargo complexes that are effectively internalized by endocytosis can be degraded within endosomes or lysosomes. An improved stability can be obtained using peptides containing non-natural aa or D enantiomers of natural aa, that exhibit a reduced sensitivity to enzyme degradation (see section 4).

7. Concluding remarks

There are some general considerations and open questions regarding CPPs in general, such as how to increase CPPs stability and specificity, what are the uptake mechanisms involved, what is the impact of the cargo characteristics on the CCP-cargo behavior and if this impact can be in some way predicted. These issues have been widely treated in previous reviews, duly cited in the present paper. There are, however, also questions and remarks specifically related to the ocular administration route. The specificity, for instance, is of outermost importance, even in the case of a local application like the administration to the eye. Indeed, when the target is represented by the cornea or the anterior segment, the retinal uptake of the cargo after formulation administration could really represent an important drawback worth of further investigation. Connected with this, the understanding of the penetration pathways is of outermost importance and should be better clarified possibly using relevant *in vivo* models. The ocular administration also suffers from a rapid clearance that often impose frequent administrations. This is true for both topical eye drops application and intravitreal solution injection, even if with different timing and administration site-related problems. Thus, CPPs should be integrated in a formulation capable of sustaining dug release, protect them from degradation and/or increase overall residence time. Indeed, the problem of peptide drugs formulation becomes relevant also when considering their stability during the storage.

In summary, even if the amount of knowledge and data accumulated on CPPs mediated ocular delivery is still limited, this field appears very promising to tackle an important number of drowbacks of traditional ocular delivery and could significantly contribute to improve ocular therapies for several difficult to treat eye disease.

Conflicts of interest

The authors report no conflicts of interest.

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