Review

Biological responses towards cationic peptides and drug carriers

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In drug development, major resources are invested into the development of cellular delivery systems to increase the effectiveness of a large array of potential therapeutics, such as proteins and oligonucleotides. These carriers comprise cell-penetrating peptides (CPPs), cationic lipids and cationic polymers. In recent years, evidence has been accumulating that these carriers not only act as mere pharmacokinetic modifiers but also interfere with cellular processes in various ways. In this review, we present an overview of the biological side effects associated with carrier systems. The focus will be on CPPs, which have been explored for a diverse set of cargos. Reported activities range from an induction of receptor internalization to the generation of reactive oxygen species. Ultimately, cell-penetrating molecules with such biological side effects might evolve into new bioactive agents that combine delivery capacity and pharmacophore in a single molecular entity. First examples for such molecules will be presented.

Introduction

As research provides an ever greater understanding of disease at a molecular level, the number of potential therapeutic targets is increasing steadily. The challenge is to translate these targets into therapies [1]. Progress with classical, small-molecule based drug development has been slow. Underlying reasons include the need to target protein–protein interactions, reach targets inside cells and to achieve specificity [2,3].

Therefore, attention is shifting towards alternative approaches that enable a rapid rational design of active and specific molecules. Oligonucleotides, including siRNA, proteins and peptidomimetics are prominent examples [4–6]. However, for these molecules, a rapid and rational design is hampered by poor bioavailability, which prevents them from reaching their intended targets *in vivo*. The successful implementation of these molecules into new therapies therefore crucially depends on delivery and targeting strategies [7], which generally involve the use of peptides, proteins, lipids or polymers. Although the specific molecular design underlying these delivery strategies varies in detail, the incorporation of positive charge is a common denominator [8–10]. The positive charge mediates the interaction with negatively charged sugar moieties and lipids on the outer leaflet of the plasma membrane, which promotes cellular uptake.

Ideally, these carriers act exclusively as pharmacokinetic modifiers with no biological activity of their own. However, an increasing body of evidence indicates that these carriers can influence cellular activity in multiple ways [11–19]. Hence, testing of carriers should extend beyond mere cytotoxicity and delivery efficiency. In this review, we will therefore summarize recent results on biological responses towards cationic carriers. The focus will be on cationic cell-penetrating peptides (CPPs) and related transporters.

Research into the biological side effects of cationic and amphiphilic membrane-active peptides has a long tradition in the area of peptide toxins, with melittin and mastoparan being paradigmatic examples. Extensive studies on structure–activity relationships have been conducted for induction of mast cell degranulation [20] and hemolysis [21]. Mast cell degranulation has been related to the interaction of the positive charges of the cationic peptides with sialic acids present on the plasma membrane and a G-protein-coupled receptor (GPCR)-independent G protein activation [20].

Many membrane-active peptides also have cell-penetrating activity. Therefore, it is difficult to make a clear distinction between both types of peptides [22]. CPPs

Glossary

Amphipathic peptide: peptide that contains both polar and nonpolar regions.
Acid sphingomyelinase (ASMase): an enzyme that converts sphingomyelin to ceramide and phosphorylcholine.
Cationic peptide: peptide with a high positive net charge and few acidic amino acid residues.
Caveolae-dependent endocytosis: endocytosis emerging from membrane microdomains (lipid rafts) rich in the protein caveolin; sensitive to cholesterol depletion.
Cell-penetrating peptide (CPP): a short peptide that facilitates cellular uptake of membrane-impermeable macromolecules.
Ceramide: cleavage product of sphingomyelin. Lipid composed of sphingosine and fatty acid with both structural and signaling properties.
Clathrin-dependent endocytosis: endosomal uptake that originates from clathrin-coated pits, leading to formation of endosomes encapsulated by a clathrin cage.
Glycosaminoglycans (GAGs): class of linear, negatively charged carbohydrate polymers composed of repeating disaccharide units that are part of the extracellular matrix.
Hemolysis: destruction of red blood cells, commonly measured for determination of the membrane-disruptive properties of potential drug delivery agents.
Macropinocytosis: formation of larger endocytic vesicles by actin-driven ruffling of the plasma membrane.
Metabolomic profiling: quantitative detection of the entirety/a large part of cellular metabolites in parallel; typically done with mass spectrometry.
Phosphatidylserine (PS): membrane glycerophospholipid typically enriched in the cytoplasmic leaflet of the plasma membrane.
Transcriptome profiling: genome-wide analysis of changes in gene expression.

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 0165-6147/$ – see front matter doi:10.1016/j.tips.2010.11.005 Trends in Pharmacological Sciences, February 2011, Vol. 32, No. 2

might be considered membrane-active peptides with little toxicity. As a consequence, CPP research has focused on mechanisms of uptake and biomedical applications rather than potential side effects. However, because the biological side effects were reported to occur in the absence of acute toxicity, they could be very relevant in future clinical applications. In this review, we will present an overview of the *in vitro* and *in vivo* biological responses reported for the interaction of cells and organisms with cationic carrier systems, especially cationic CPPs. Frequently, these responses directly relate to uptake. Finally, we will provide examples of CPPs that have an intended intrinsic biological activity.

Cell-penetrating peptides: promising molecules for the intracellular delivery of therapeutics

CPPs are characterized by their ability to promote the receptor-independent cellular uptake of membrane-impermeable macromolecules, such as peptides, proteins, nucleic acids and nanoparticles [23]. CPPs contain fewer than 30 amino acids and are mostly cationic or cationic and amphipathic. Cationic and amphipathic CPPs show some differences in their internalization mechanisms [24], which might be attributed to the ability of amphipathic CPPs to more directly interact with membrane lipids [25]. Early CPPs include the *Drosophila melanogaster*-derived penetratin [26], the protein transduction domain from the Tat protein [27] and the more hydrophobic CPP transportan, which is a chimeric molecule designed from parts of the neuropeptide galanin and the wasp venom mastoparan [28].

Cellular internalization of CPPs is observed for virtually all cells, although with different efficiencies that depend on the CPP, the cargo and the cell type [29,30]. Especially for large molecular weight complexes, internalization occurs mainly through endocytosis. Therefore, even though the ultimate target compartments typically are cytosol and nucleus, in many (if not most) cases the major part of the imported cargo is trafficked to the endolysosomal compartment [24]. Endocytic pathways that have been reported to contribute to CPP uptake include macropinocytosis and the clathrin-dependent and caveolae-dependent pathways [31]. It has been shown that a given CPP is not necessarily restricted to only one import pathway. Instead, CPPs might exploit multiple entry routes at the same time [32].

Biological interactions involved in the internalization of cationic CPPs

A CPP approaching a cell first encounters glycosaminoglycans (GAGs), negatively charged carbohydrate polymers on the surface of the plasma membrane. It is therefore no surprise that GAGs have been implicated in the cellular uptake of CPPs [33]. Affinity measurements typically show K_d values between 100 nM and 1 μM for the interaction of cationic peptides with GAGs, which provides evidence for their role as a primary cellular interaction partner [34].

Conflicting evidence has been obtained on whether interaction with GAGs also triggers import or not. The role of GAGs as a direct trigger for uptake has been supported by a report from Letoha *et al.*, who found that the proteoglycan syndecan-4 bound the cationic CPP Tat, penetratin and octaarginine and facilitated their internalization in a protein kinase C (PKC) α-dependent manner [35]. Conversely, a role of GAGs in CPP import has been questioned entirely by research showing only minor differences in cellular delivery of a Tat–Cre recombinase into GAG-deficient or wild-type cells [36].

In addition to studies addressing interactions with GAGs, research on lipid bilayer model systems has given ample indications for the potential of CPPs to interact with

Table 1. Sequences of cationic peptides

Peptide	Sequence	Class	Origin	Reference
R9	RRRRRRRRR-NH_2	Cationic CPP	N/A	[48]
Tat	C-(acetamidomethyl) GRKKRRQRRRPPQQ	Cationic CPP	HIV Tat protein (48–60)	[27]
hLF	KCFQWQRNMRKVRGPPVSCIKR-NH_2	Cationic CPP	Human lactoferrin	[49]
(RXR)4	(R-Ahx[a]-R)4-PMO	Cationic CPP	N/A	[50]
Penetratin	RQIKIWFQNRRMKWKK	Cationic/amphipathic CPP	Antennapedia homeodomain	[26]
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-NH_2	Amphipathic CPP	Galanin and mastoparan	[28]
TP10	AGYLLGKINLKALAALAKKIL-NH_2	Amphipathic CPP	Galanin and mastoparan	[51]
Maurocalcine	GDCLPHLKLCKENKDCCSKKCKRRGTNIEKRCR-COOH	Bioactive CPP	Scorpion venom	[52]
Vasostatin 1	MRSAAVLALLLCAGQVTALPVNSPMNK GDTEVMKCIVEVISDTLSKP SPMPVSQECFETLRGDERILSILRHQNLL-COOH	Bioactive CPP	Vasostatin 1 (1–76)	[53]
p14ARF	MVRRFLVTLRIRRACGPPRVRV-NH_2	Bioactive CPP	p14ARF protein (1–22)	[54]
M511	FLGKKFKKYFLQLLK-NH_2	Bioactive CPP	Rodent angiotensin receptor	[55]
TBX2	GKMADWFRQTLLKKPKKRPNSPESTLQLRD ATPGGAIVS-NH_2	Bioactive CPP	Toll-receptor 4 adaptor protein (19–43)	[56]
Cyt *c*	GTKMIFVGIKKKEERADLIAYLKKA-NH_2	Bioactive CPP	Human cytochrome *c* (77–101)	[57]
mPrP	MANLGYWLLALFVTMWTDVGLCKKRPKP-NH_2	Bioactive CPP	Mouse prion protein (1–28)	[58]
$SAHB_A$	EDIIRNIARHLAX[b]VGDX[b]N_L[c]DRSIW-NH_2	Chemically modified bioactive CPP	BID BH3	[59]
Lactoferricin B	FKCRRWQWRMKKLGAPSITCVRRAF[d]	Host defense peptide	Bovine lactoferrin (17–41)	[60]
Buforin IIb	TRSSRAGLQWPVGRVHRLLRK[d]	Host defense peptide	Histone H2A (toad stomach)	[61]

[a]Ahx denotes 6-aminohexanoic acid.
[b]X refers to the position of α,α-disubstituted non-natural amino acids containing olefin-bearing tethers. These amino acids were used for the formation of a hydrocarbon staple (cyclic alkene).
[c]N_L corresponds to the non-natural amino acid norleucine.
[d]No information was available regarding the amidation status.

lipids, although affinities are substantially lower [34]. It has been shown that cationic CPPs directly affect lipid bilayer organization in model systems. Disturbance of bilayer organization is a major requirement for uptake-related membrane processes such as fusion, fission or pore formation [37,38]. In these processes, changes in membrane curvature are involved [38]. As an example, the CPP Tat was found to induce negative curvature in artificial vesicles [39], which is a necessary condition for pore formation, dimple formation in caveolae-based endocytosis and for membrane protrusions in macropinocytosis [38]. Induction of vesicle fusion has also been observed for cationic CPPs [40].

To date, structure–activity relationships of CPPs have been explored in rather simplistic model systems. Even though these systems have succeeded in identifying lipid composition, transbilayer potential and vesicle size as important determinants for peptide translocation, even the most complex of these models has failed to account for cellular responses that dynamically remodel the lipid composition.

Biological activity of cationic CPPs

Cationic CPPs were originally considered as 'Trojan horse' delivery vehicles that enter cells without eliciting a cellular response [41]. However, CPPs can induce a wide range of side effects that might be more subtle than cell death. These side effects have been related to the uptake itself as well as to interactions inside the cell.

Effects of cationic CPPs on membrane integrity and cell viability

Cationic CPPs (for peptide sequences, see Table 1) have a sound track record with regard to toxicity, both *in vitro* and *in vivo*. *In vitro*, the low toxicity holds for both acute membrane toxicity (as determined by a variety of membrane integrity assays) and for cell viability (as determined mainly by assays probing for mitochondrial activity) [32,42,43]. Tat and penetratin (with or without cargo) at concentrations of up to 50 μM had only minor effects on cell proliferation and membrane integrity [42]. By comparison, the more amphipathic CPPs MAP and TP10 affect proliferation at lower concentrations and show considerable membrane destabilization. For example, MAP and TP10 induce lactate dehydrogenase leakage at concentrations of 10 μM [42,43].

At the high concentration of 50 μM, little hemolysis was observed for penetratin and none for Tat, further supporting the lack of acute membrane toxicity for cationic CPPs [43]. Although amphipathic peptides (see Glossary) were somewhat more hemolytic, none of the tested CPPs had a hemolytic activity comparable to the one of membrane-active peptides such a mastoparan, which clearly distinguishes CPPs from other membrane-active peptides [43].

Novel methods to detect subtle side effects of cationic CPPs

Now that cationic CPPs are moving steadily towards preclinical and clinical development, the traditional toxicity analysis methods are being complemented with '-omics' assays that globally profile cell activities. These assays are more sensitive in profiling potential side effects of CPP-based therapeutics than the traditional assays. Consistent with results on membrane integrity and viability, a toxicity analysis based on metabolomic profiling also demonstrated that amphipathic peptides such as TP affect cellular metabolism at concentrations of 5–10 μM, whereas the cationic CPP penetratin, Tat and nonaarginine had little effect at 10 μM [44]. No specific enzymes were identified as being directly affected by TP. Instead, reduced levels of reduced glutathione, glucose, purines, pyrimidines and related molecules led the authors to hypothesize that the cells countered oxidative stress caused by the peptide [44].

A transcriptome analysis for H1299 lung adenocarcinoma cells treated with a Tat-conjugated dsRNA-binding domain indicated very few changes both after 12 h and 24 h [45]. None of the upregulated or downregulated genes clustered into a specific genetic pathway. Similarly, a transcriptome analysis in HeLa cells found that several unrelated genes were affected by penetratin, whereas for Tat hardly any effects were observed [46].

To detect possible subtle side effects of CPPs on the immune system, Kuo *et al*. performed genome-wide profiling of U-937 macrophages treated with octaarginine [47]. Although these researchers found an increase in the expression of several immune-related genes, this response did not result in cytokine release or reduced viability for concentrations of up to 40 μM. They did, however, find elevated superoxide levels 30 min after peptide addition, which normalized after 8 h [47].

Biological mechanisms of side effects of cationic CPPs

Even though scientific understanding of the biological effects of CPPs is far from complete, individual molecular mechanisms along the route of uptake and inside cells have been identified (Figure 1). At the first step of cellular entry, an interaction between heparan sulfate proteoglycans and the CPP Tat has been found to affect the cell by reducing its responsiveness to exogenous polyamines via a competition for heparan sulfate binding sites [17].

Following their interaction with heparan sulfates, a principal effect of cationic CPPs is their ability to induce endocytosis of unconjugated constituents and transmembrane receptors, as detected by an increased uptake of fluorescently labeled dextran via fluid-phase endocytosis [15] and the internalization of tumor necrosis factor receptors (TNFRs) [14], respectively. Although the precise trigger has not yet been identified, this endocytosis induction can have profound effects on the capacity of a cell to respond to extracellular signals. Internalization of TNFRs occurred without receptor activation. As a consequence, downregulation resulted in a reduced capacity to respond to TNF-α by up to 50% [14]. Downregulation was also observed for epidermal growth factor receptor and was induced by penetratin, Tat and nonaarginine [14].

Cationic CPPs also evoke side effects by remodeling the plasma membrane. Tat–GFP fusion proteins were found to induce phosphatidylserine (PS) exposure, although without initiating apoptosis or necrosis [12]. Recently, we confirmed and extended this observation by showing that cationic CPPs have the ability to not only alter the distribution of

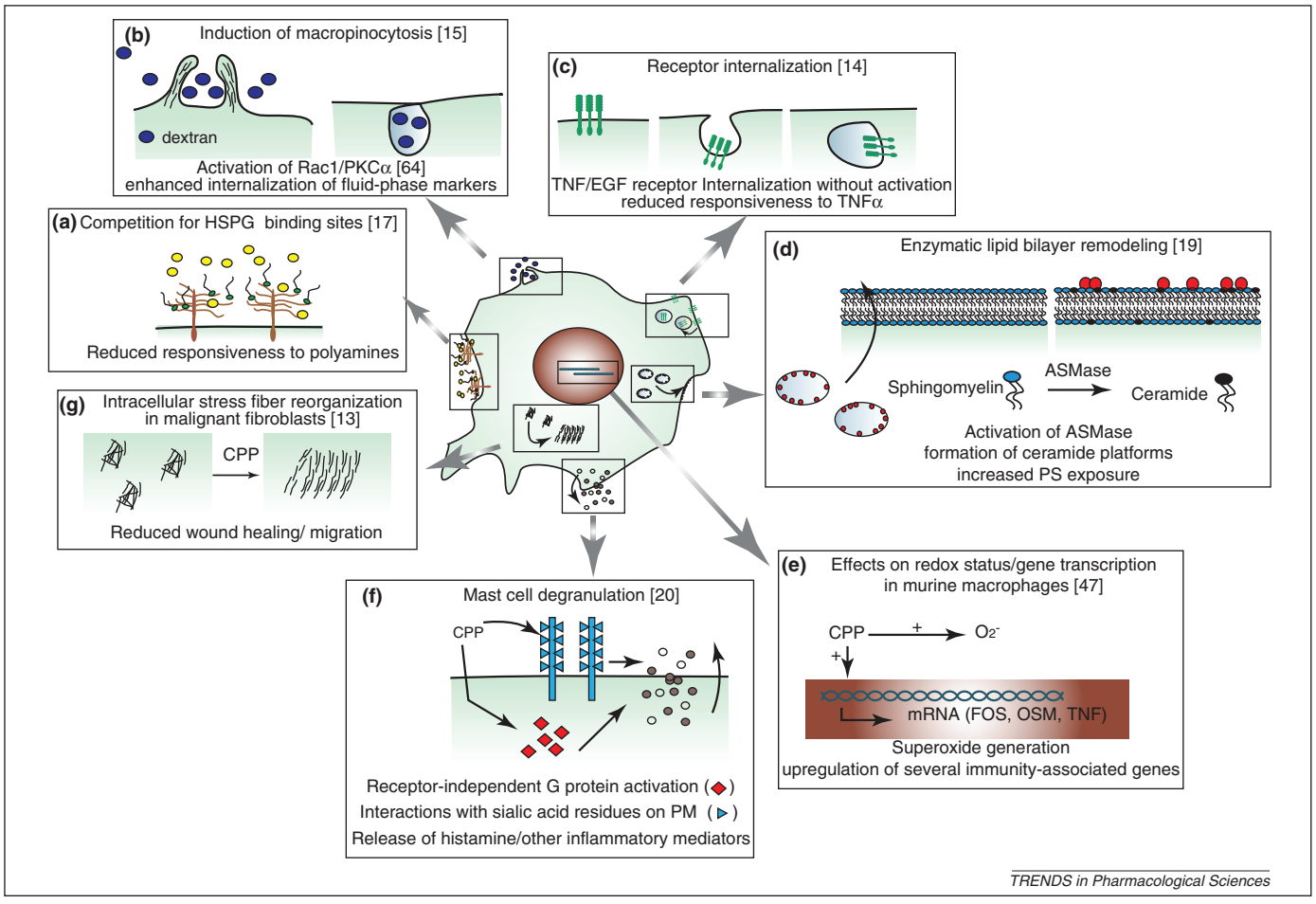

Figure 1. Common side effects elicited by cationic CPPs. In recent years, various side effects of cationic CPPs have been described that can, in some instances, be directly related to their internalization mechanisms. The first step of internalization, the high-affinity interaction with heparan sulfates, is the primary reason for the reduced responsiveness to polyamines **(a)**. The induction of internalization of fluid-phase markers and receptors are related to the ability of cationic CPPs to activate the endocytic machinery **(b,c)**. The molecular triggers that lead to plasma membrane remodeling **(d)** or affect the redox status and/or gene transcription **(e)** have not yet been identified, whereas interactions with sialic acid residues and G proteins are thought to be responsible for mast cell degranulation **(f)**. A direct interaction with intracellular actin has been proposed to underlie the ability to mediate stress fiber reorganization in malignant fibroblasts **(g)**. HSPG, heparan sulfate proteoglycan; PM, plasma membrane.

lipids in the bilayer but also to induce changes in the lipid composition of the plasma membrane that depend on enzymatic activity [19]. The mechanism involves the induction of acid sphingomyelinase (ASMase), which converts sphingomyelin to ceramide at the outer leaflet of the plasma membrane. Because ceramide is a lipid with important second-messenger functions, this finding could have important implications for the interpretation and design of studies using CPP-based therapeutic strategies (Box 1). ASMase was identified as a key player in the plasma membrane repair response [62], which is known to be induced by several CPPs including TP10 and penetratin [63].

Arginine-rich peptides also induce actin rearrangement in a proteoglycan-dependent manner [64]. Given the central role of the cytoskeleton in signaling processes, it remains to be determined to what extent this activity affects signaling cascades [65]. Very interestingly, differential effects on healthy and malignant fibroblasts were

Box 1. The many faces of acid sphingomyelinase and ceramide

ASMase and ceramide are subjects of intense research due to their recently discovered roles in the pathophysiology of common diseases [101] and the multifaceted roles of ceramide as a second messenger in several antiproliferative and/or apoptotic responses, in the arrangement of the cellular cytoskeleton and in motility. Currently, there is increasing interest in ceramide metabolism as a potential target for cancer therapy [102].

In lipid membranes, ceramide segregates into rigid domains and induces negative monolayer curvature [103], which has been associated with the internalization of several pathogens [104] and cationic CPPs [19]. The initial trigger for ASMase translocation and ceramide formation is poorly understood, although reactive oxygen species have been implicated independently by various groups [105]. Although octaarginine also induced superoxide in U-937 macrophages [47], a direct link to ASMase activation has not been established.

The observation that cationic CPPs have the potential to influence ceramide metabolism could have important implications for cancer therapy. CPPs could serve to enhance the sensitivity of tumor cells to chemotherapeutic drugs. In fact, ceramide generation induced by cationic CPPs might be part of the mechanism of enhanced toxicity of CPP-based chemotherapeutic approaches [106]. CPP–drug conjugates showed enhanced cytotoxicity and induced ceramide formation through acid sphingomyelinase, whereas the drugs by themselves did not. Although the mode of drug delivery was seen as the probable reason, the bioactivity of the cationic CPP could very well have contributed to the increased cytotoxicity.

observed for several arginine (R9) and arginine/tryptophan-based (R/W)9 peptides [13]. Whereas these peptides restored a disrupted actin cytoskeleton in malignant fibroblasts, the peptides did not affect actin organization in untransformed fibroblasts. Furthermore, the CPPs reduced cell motility and wound healing. These activities were only observed with CPPs that had the ability to bind actin *in vitro* as determined by isothermal titration calorimetry [13]. The effects were not observed upon treatment with non-actin binding peptides, such as an (R/W)9 peptide consisting of D-amino acids or penetratin, which both had a comparable capacity to enter the fibroblasts.

In addition to these activities, oligoarginines also act as protease inhibitors. Octaarginine reduced proteasome activity, leading to the accumulation of high-molecular-weight ubiquitin-conjugated proteins inside the cells as determined by analysis of whole cell lysates [16]. Oligoarginines more generally acted as furin inhibitors [11]. As again another mechanism-of-action, HIV replication was inhibited by Tat, which is achieved through competition with the full HIV-1 Tat protein for binding to the TAR element in the nucleus [18].

Given all these biological activities, cationic CPPs are thus far from true Trojan horses. Because arginine and tryptophan are residues enriched on protein interaction surfaces and because of the high energy contribution of these residues to protein–protein interactions [66], it should come as no surprise if arginine- and tryptophan-rich peptides had further proteinaceous interaction partners inside the cell. As we will detail more extensively below, many other specific intracellular interaction partners have been identified for particular bioactive CPPs.

Toxicity and bioactivities of cationic drug delivery systems

The scientific focus for other cationic carriers is also shifting from the application of standard toxicity assays towards a more complete understanding of the side effects through the analysis of specific biological interactions and global gene expression profiling [67,68]. Selected examples that are pertinent to the discussion of cellular responses towards cationic carriers are presented. For the cationic polymer polyethyleneimine (PEI), two distinct phases in cytotoxicity were detected [69]. First, PEI induced lactate dehydrogenase leakage and PS externalization in a caspase-3-independent manner, which was then followed by the induction of caspase-3-dependent mitochondrial apoptosis, most probably caused by a direct interaction of PEI with the negatively charged mitochondrial membrane. A role for lysosomal destabilization could not be ruled out because PEI has been shown to disrupt lysosomes [70]. A similar caspase-3-independent PS externalization was observed for poly(L-lysine)s (PLLs), which was also followed by a caspase-3-dependent apoptosis [71]. Another bioactivity that could be implicated in PLL-mediated apoptosis, but that has also been observed for poly-L-arginine, was the induction of phospholipase D through PKC [72]. Similar to PEI and PLLs, chitosan and chitosan-derivates also induce apoptosis through caspase-3 [73].

Cholesterol-based cationic lipids, which are being investigated for gene delivery, have the potential to inhibit PKC activity [74]. Factors that affect the inhibition are charge, the presence of steroid versus linear hydrocarbons and the presence of a quaternary ammonium cation versus a tertiary amine headgroup [74]. Reminiscent of the induction of receptor internalization by cationic CPPs, the transfection agent lipofectamin, which has similarities with cationic lipids that are being developed into drug delivery constructs, activates the insulin receptor at higher concentrations. This leads to a subsequent downregulation of insulin receptors and a lack of responsiveness to insulin in transfected cells [75].

Toxicity and side effects of cationic CPPs in vivo

As very recently reviewed by Järver *et al.*, there is currently little information regarding the *in vivo* toxicology of cationic CPPs [76]. Despite numerous preclinical studies that indicate activity of CPP-based therapeutics (for examples, see below), few dedicated toxicity assessments of therapeutic CPP constructs in preclinical or clinical development have been published. Nevertheless, the absence of obvious toxic effects in most of the studies assessing therapeutic effects is promising. Several notable constructs that are or have been in clinical development include: PsorBan, which is cyclosporin A coupled to heptaarginine via a pH-dependent linker [77]; KAI-9803, a Tat-coupled peptide inhibitor of PKCδ [78]; (RXR)4 peptides coupled to anti-c-myc phosphorodiamidate morpholino oligomers (PMOs) [79]; and XG-102, a Tat-coupled c-Jun N-terminal kinase inhibitor [80–83]. In multiple studies with these compounds in rat, mouse and porcine models, but also in an exploratory study in humans (KAI-9803) [84], no apparent signs of acute or long-term toxicity were reported at therapeutic doses. Administration through various routes was well-tolerated, including: the topical administration of PsorBan (Psorban; http://goliath.ecnext.com/coms2/gi_0199-2736134/CellGate-Announces-Phase-IIa-Study.html); the intracoronary administration of KAI-9803 [78,84]; the intravenous administration of (RXR)4–PMO [79] and XG-102 [81]; and the intraperitoneal (IP) administration of XG-102 [80,82]. Similarly, direct stereotactic delivery into the brain of Tat coupled to a dsRNA-binding domain, a platform which is also being developed for clinical trials, seems to be well-tolerated at therapeutic levels in a glioblastoma mouse model [85].

In line with the aforementioned findings, 14 subsequent days of IP injection of 20 nmol of a Tat–PKC peptide conjugate in rats did not result in organ toxicity, despite widespread biodistribution [86]. Frequent IP injections of Tat coupled to the enzyme purine nucleoside phosphorylase at a dose of 80 mg/kg body weight/week in mice also did not result in any detectable toxicity, as determined by blood-based parameters of liver, kidney and hematological function [87]. By contrast, Tat and penetratin were found to induce by themselves a reduction in the expression of the kinase p38 in a mouse lung delivery study after intratracheal administration at 10 nmol [88]; however, it was not investigated in more detail if the expression of other genes was also affected. When coupled to siRNA, penetratin also activated innate immunity [88]. The mechanisms are not understood.

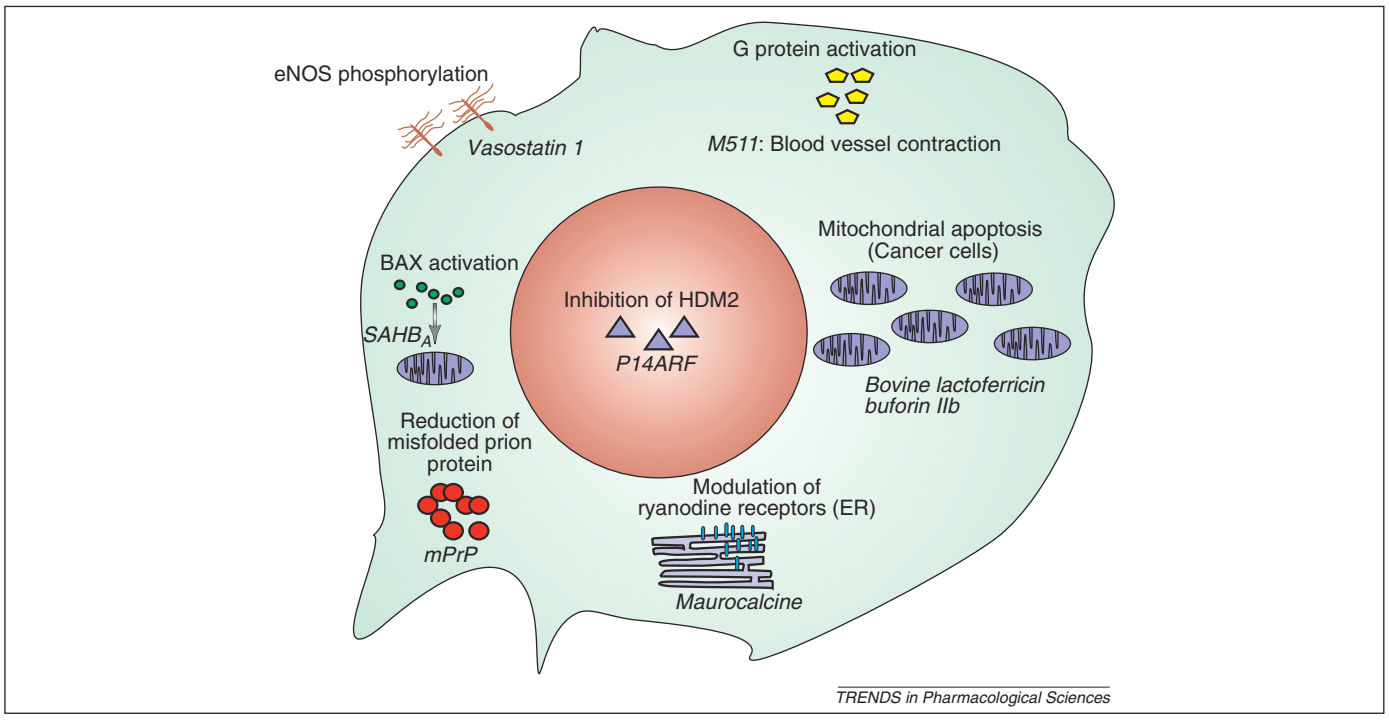

Figure 2. Examples of CPPs with intrinsic bioactivity. Cationic CPPs can have diverse effects on cellular physiology. At the plasma membrane, binding of vasostatin 1 to heparan sulfate proteoglycans is involved in endothelial nitric oxide synthase phosphorylation [53]. In the cytosol, diverse activities have been described that include a reduction in the pathogenic isoform of the prion protein by prion-derived peptides [58] and blood vessel contraction through G proteins [55]. A cell-penetrating BH3 domain with a stabilized α-helical conformation acts by recruiting BAX to the mitochondria, where it is activated [59]. Several host defense peptides have been shown to cause mitochondrial apoptosis in cancer cells after having traversed the plasma membrane without major disturbances [60,61]. Ryanodine receptors, located in the membrane of the ER, are modulated by a bioactive CPP derived from scorpion venom [52]. In the nucleus, a p14ARF-derived peptide is proposed to sequester HDM2 to the nucleolus, which leads to p53 activation and apoptosis [54]. ER, endoplasmic reticulum.

A recent report from Aguilera *et al*. described the *in vivo* distribution and toxicity of C-terminally amidated r9 (all D-amino acids) following intravenous administration in mice. The peptide was found to bind rapidly to the local vasculature and to redistribute within 30 min, with over 90% localized to the liver [89]. These researchers observed that mice went into shock with dilated blood vessels and died of respiratory collapse within 1 min after an injection of 100 nmol r9 (5 μmol/kg, which is ~7 mg/kg), although four out of five mice survived an injection of 50 nmol. The toxicity of r9 could be reduced by masking the arginine moieties in the bloodstream via a glutamic acid-based polyanion stretch coupled to the CPP via a protease-cleavable linker. A suspected reason for this adverse effect is massive mast cell degranulation, leading to fatal vasodilation. These strong adverse effects of r9 in mice were in contrast to the results observed with peptide–PMO constructs in rats, where no adverse effects occurred at 15 mg/kg, and the LD_{50} lay between 210 and 250 mg/kg. Of note is that the total amount of positive charge at 15 mg/kg is approximately half of that for r9 at 7 mg/kg. The conjugate (RXR)4–PMO has one positive charge less than r9 and its molecular weight is approximately 4-fold higher. Moreover, the charge density is substantially lower due to the insertion of four 6-aminohexanoic acid moieties in the octaarginine sequence [79]. In the exploratory study in human patients with Tat–PKCδ, corrected-for-weight quantities of the peptides were around 100-fold lower (~7 mg/kg in the mice study [89] vs max. 70 μg/kg in the human study [84]). Other reasons that could contribute to the diverse biological responses include the different route of administration (intravenous vs intracoronary), the difference in CPPs and the conjugation to a peptide inhibitor (PKCδ). Typically, the studies at therapeutic doses use far lower concentrations which make toxicity comparisons difficult, if not impossible.

Another consideration regarding the toxicity of CPPs *in vivo* is their lack of specificity. The implementation of targeting strategies to direct the CPP to the site of action could therefore help to avoid side effects in multiple tissues [76,90]. So far, targeting strategies to improve specificity have focused on targeting CPP conjugates to tumors. These strategies either exploited the tumor microenvironment [91,92] or tumor-specific antigens [93,94]. For instance, Whitney *et al*. increased specificity through a phage display-based selection of tumor-homing activatable CPPs, which incorporate a tumor protease-cleavable linker attached to an arginine-masking polyanion chain [92]. As indicated above, this strategy also decreases toxicity. The Torchilin group made use of hydrazone-linked polyethylene glycol shells on liposomes, which shield the Tat moiety at physiological pH, but expose Tat at lower pH values [91], which are normally encountered in the tumor microenvironment [95]. Miyamoto *et al*. and Myrberg *et al*. achieved enhanced specificity of CPPs through coupling to tumor-targeting antibodies or peptides, respectively [93,94].

In the near future, cell-targeting peptides with an intrinsic cell-penetrating activity might expand the repertoire of strategies available to increase specificity [96].

CPPs with intrinsic bioactivity

In addition to the traditional cationic CPPs that are applied as vectors for cargo delivery, CPPs with intrinsic activities

are rapidly gaining interest (Figure 2). This class of peptides demonstrates that cell entry and intracellular function can be combined effectively (for examples, see Table 1).

One example of a bioactive CPP is a seven arginine residue-containing CPP derived from the p14 ARF protein [54]. Internalization of this peptide is as efficient as uptake of the CPP TP10 and is associated with little membrane disturbance. Inside the cells, this peptide activates the apoptotic machinery by mimicking the function of the intact p14ARF protein, most probably by increasing p53 activity through inhibition of HMD2. In a similar vein, bioactive CPPs derived from cytochrome *c* were developed, which also combine efficient cellular internalization with the induction of tumor cell apoptosis. Another example is maurocalcine, which is a CPP derived from a scorpion venom that induces intracellular Ca^{2+} release through activation of ryanodine receptors [52]. The peptide vasostatin 1 also shares multiple characteristics with cationic CPPs, because it also binds to heparan sulfate proteoglycans and is internalized via endocytosis. In addition, vasostatin 1 activates endothelial nitric oxide synthase phosphorylation in a PI3K- and proteoglycan-dependent manner, which stimulates caveolae-dependent endocytosis [53]. The increase in caveolae-dependent endocytosis is somewhat surprising, because PI3K is commonly associated with macropinocytosis [97]. A highly interesting finding has been presented for prion-derived peptides, which successfully entered cells and also caused a reduction of intracellular levels of the pathogenic prion isoform [58].

Furthermore, the G protein-binding parts of GPCRs have been employed as a source for CPPs with an intrinsic bioactivity [55]. A CPP prediction algorithm was used to identify CPPs from various GPCRs. One of the peptides (M511) induced blood vessel contraction. This indicates that the peptide successfully mimicked the respective domain of its parent protein [55]. By contrast, a peptide derived from the Toll-receptor 4 (TLR-4) adaptor protein blocked parent protein activity [56]. This peptide, TBX2, penetrated the plasma membrane and eliminated lipopolysaccharide-induced production of proinflammatory cytokines. Presumably, it prevents TLR-4-mediated activation of downstream signaling events by competition for TLR-4-binding sites [56].

Walensky *et al.* followed a different route to confer cell-penetrating capacity to a cell-impermeable BID BH3 peptide, which is the conserved BH3 α-helical domain of the pro-apoptotic protein BID (a member of the Bcl-2 family) [59]. Unlike the aforementioned examples, cell permeability of this peptide was not a function of its primary sequence, but instead the result of the inclusion of a cyclic alkene using α,α-disubstituted non-natural amino acids that constrains the molecule to assume an α-helical conformation. This chemical modification is referred to as stapling. The stapled BID BH3 peptide enters cells through macropinocytosis, localizes to mitochondria and binds to and activates Bcl-2-associated protein X (BAX), thereby initiating the apoptotic machinery [59]. *In vivo*, this peptide analog slows the growth of human leukemia xenografts.

Host defense peptides

A separate class of bioactive peptides that also comprises CPPs is the class of host defense peptides (Table 1). Although some of these peptides act by being preferentially membranolytic for cancer cells or bacteria, others have the ability to translocate into cells without major membrane disturbance. An example is the 7 arginine residues-containing buforin IIb, a 22 amino acid peptide derived from histone H2A [61]. In studies, this peptide required cell surface gangliosides or sialic acids for efficient entry into cancer cells. Remarkably, buforin IIb did not enter healthy cells even at high concentrations, despite being very arginine-rich. After cell entry, buforin IIb causes mitochondria-dependent apoptosis through the activation of procaspase-9 and procaspase-3. It has not been established whether a direct interaction is involved. Mitochondria-dependent apoptosis has also been described for bovine lactoferricin [60], a host defense peptide that also has the capacity to enter cells [98]. For bovine lactoferricin B, generation of reactive oxygen species has been implicated in the mechanism of apoptosis induction. Notably, the cyclic structure, although being essential for internalization of the human lactoferrin (hLF)-derived CPP (which is a part of human lactoferricin), was not required for the cancer cell-selective induction of apoptosis of bovine lactoferricin, emphasizing the distinct structure–activity relationship of these two peptides [49,60].

Concluding remarks

The data reviewed in this article indicate that side effects of cationic delivery agents can be diverse and are not necessarily directly related to cellular uptake. Therefore, when screening for side effects, a comprehensive analysis of the interactions between the delivery agent and the biological system is required. Global gene expression profiling is the most straightforward screen. Ideally, such a screen should be complemented with a more directed search for side effects based on available precedents. Testing procedures could encompass diverse assays for the detection of superoxide levels, receptor internalization, mast cell degranulation or the effect on lipid composition.

At present, it is difficult to foresee which side effects that were observed *in vitro* will be relevant *in vivo*. There are still very limited data to translate *in vitro* peptide concentrations into relevant *in vivo* concentrations. Nevertheless, we predict the start of an exciting new era in which it will be possible to use knowledge regarding molecular interactions of CPPs to design delivery agents that have themselves desired pharmacodynamic activity. Alternatively, the fundamental knowledge could be used to rationally engineer delivery agents without undesired side effects. A notable example is the (R/W)9 synthesized with either D- or L-amino acids, which have equal internalization efficiencies, but remarkably different molecular interactions and activities inside the cell. A further example is the recent design of a pharmacologically inert D-maurocalcine, which, unlike its L-counterpart, does not affect calcium signaling inside the cell [99]. Another emerging trend is to identify cell-permeant proteomimetic peptides using quantitative structure–activity relationship-based algorithms [100], which have already provided several interesting new bioactive agents in which the delivery capacity and pharmacophore are present in the primary sequence in overlapping, but not identical, regions [55–57].

In conclusion, cationic peptides and cationic drug carriers show a large variety of side effects that might be related or unrelated to their internalization mechanisms. If bioactivities are associated with the internalization mechanisms of this type of carrier, then the choice of the class of delivery system should be compatible with or contribute to the therapeutic goal, such as for example the import of anticancer drugs with apoptosis-inducing peptides. Ultimately, the understanding of the structural determinants underlying these effects should allow us to rationally engineer new bioactive agents that only possess the properties that are deliberately introduced.

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