The Effects of Lipidation on a TAT-Containing Peptide-Based Inhibitor of PSD-95

Eduardo F. A. Fernandes, Linda M. Haugaard-Kedström, and Kristian Strømgaard

Abstract

Stability and cell permeability are critical parameters in the development of peptide therapeutics. Conjugation to fatty acids and cell-penetrating peptides, such as TAT (YGRKKRRQRRR), are established strategies to increase peptide stability and permeation, respectively. Here, we prepared lipidated analogues of a potent TAT-containing dimeric peptide-based inhibitor of the intracellular scaffolding protein PSD-95, an emerging drug target in ischaemic stroke. Lipidation increased peptide stability in vitro and in vivo. Combining both lipidation and conjugation to TAT improved brain/plasma ratios, but caused acute toxic effects due to the potent haemolytic activity of the TAT-lipid moiety.

Introduction

The human proteome is composed of more than 20000 proteins that are predicted to engage in ~650 000 protein–protein interactions (PPI). These interactions are fundamental to control physiological cell homeostasis, and offer novel and exciting drug targets to modulate pathological signalling cascades. The design of PPI inhibitors frequently starts with a peptide template based on a fragment of one of the interacting protein partners. However, such native peptide sequences do not often display drug-like properties, that is, they do not efficiently cross biological membranes and are also prone to proteolytic degradation and renal elimination. Such limitations hinder their access to intracellular PPI targets and result in shot half-lives in vivo.

Conjugation of peptides to cell-penetrating peptide (CPP) moieties is an established strategy to increase cellular uptake. One of the most frequently used CPPs derives from the HIV-1 transactivator of transcription (TAT, YGRKKRRQRRR). The TAT-mediated internalisation mechanism is suggested to take place through either endocytosis or by a direct penetration pathway. Also, TAT CPP not only induces cell permeation of the cargo peptide in cells but also can increase the blood–brain barrier (BBB) penetration of conjugates in vivo. TAT peptides have been examined in human clinical trials seemingly without significant signs of toxicity, although no drug containing the TAT sequence has been approved yet.

Conjugation with long-chain fatty acids can improve peptide stability in vivo. The lipid binds to plasma albumin preventing both proteolytic cleavage and renal filtration. Lipidation was successfully employed to enhance the half-life of insulin and glucagon-like peptide-1 (GLP-1) derivatives, and these products are now commercially available drugs with improved pharmacokinetic properties. Lipid conjugation may also lead to increased brain uptake, as demonstrated by increased efficacies of lipidated peptides targeting brain proteins, compared with the native sequence. For example, the administration of palmitoylated oxytocins sustained improved parental social behaviour in a mice model of autism spectrum disorder (ASD) for 24 h, while oxytocin was active for only for 30 min. In another study, mice treated with myristoylated prolactin-releasing peptides lost more weight and had increased neuronal activity in the hypothalamus compared with the original peptide.

Lipid conjugation to CPPs can increase the peptide association to the cell membranes, promote endocytosis, and increase their resistance to serum proteases. For this reason, CPP–lipid conjugates have been applied as efficient transfection agents. A variant of the TAT sequence (GRKKRRQRRRPPQ) was lipidated with stearic acid and this conjugate was 100-fold more effective as a transfection agent in COS cells. However, applications of CPP–TAT conjugates for systemic delivery are still scarce; one example is the stearylization of the CPP TAT (48–60) that increased the efficacy of a siRNA knockout in mice.

The inhibition of the ternary PPI complex between the N-methyl-d-aspartate receptor (NMDAR), the postsynaptic density protein 95 (PSD-95), and the neuronal nitric oxide synthase (nNOS) has shown great promise in the treatment of ischaemic stroke. PSD-95 occupies a central position in this ternary complex interacting with both the NMDAR and nNOS through tandem postsynaptic density-95/disks-large/zonula occludens (PDZ) domains. The C-terminus of the NMDAR binds to the first PDZ domain (PDZ1) of PSD-95, while the second PDZ domain (PDZ2) binds to nNOS, both interactions at low micromolar affinities.

The first developed inhibitor of PSD-95 is a 20-mer peptide, NA-1, consisting of the nine last C-terminal amino acids of the NMDAR combined to the CPP TAT. NA-1 is the most advanced peptide inhibitor of PSD-95 and is currently in Phase III clinical
In Vitro Stability of the TAT–Lipid Conjugates

Results and Discussion

In Vivo Stability and Pharmacokinetics

Next, we tested if the improved stability found in vitro is also observed in vivo. Peptides are degraded not only by plasma enzymes but also by tissue proteases.[28] For example, renal filtration with subsequent proteolytic degradation is a common elimination mechanism of peptides.[29] We examined if the enhanced plasma stability of lipid derivatives will lead to a higher brain uptake, measuring the compound levels using liquid chromatography–tandem mass spectrometry (LC-MS/MS). We obtained plasma and brain pharmacokinetic profiles in rats that were administered intravenously (i.v.) at 5 mg kg\(^{-1}\) or subcutaneously (s.c.) at 10 mg kg\(^{-1}\), and sampled for the treatment of ischaemic stroke. Another peptide, UCCB01-144, contains two pentameric binding sequences connected through a polyethylene linker and an N-terminal CPP TAT (Chart 1). Due to its dimeric design, UCCB01-144 binds simultaneously to PSD-95 PDZ1 and PDZ2 domains. UCCB01-144 displayed a 1000-fold affinity improvement relative to NA-1 and a superior reduction in brain infarct areas in a mouse model of ischaemic stroke.[6,22] Here we designed and prepared lipidated analogues of UCCB01-144 and evaluated their stability and affinity in vitro together with plasma and brain pharmacokinetics. The TAT–lipid analogues retained plasma stability and had higher brain/plasma ratios. However, we also observed increased haemolytic effects of these compounds caused by the combined effects of lipidation and CPPs.

Results and Discussion

In Vitro Stability of the TAT–Lipid Conjugates

Previously we prepared lipidated derivatives by acylations of the dimeric binding motif N-dimer peptide (Chart 1) with fatty acids of different lengths.[23] These peptides retained low nanomolar affinity to PSD-95 and improved stability both in vivo and in vitro compared with UCCB01-144. However, neither the lipidation of a TAT-containing peptide nor the direct measurement of brain permeation was previously studied. Thus, we have prepared four lipidated analogues, two of them acylated with stearic acid at the N-terminal of the N-dimer (1 and 2) and two (3 and 4) combining stearic acid acylation and TAT conjugation (Chart 1). Peptide 3 is linked directly at the N-terminal of TAT, while peptide 4 has an additional \(\gamma\)-aminobutyric acid (GABA) linker between the TAT sequence and the fatty acid. The presence of a linker between the fatty acid and the peptide moiety has previously been shown to influence their serum stability.[24,25]

First, we assessed the effects of fatty acid acylations on the binding affinity to the PSD-95 tandem PDZ12 domain using a fluorescence polarisation (FP) assay.[22,26] All analogues showed similar binding to the parent peptide UCCB01-144 (12.1 ± 0.6 nM), but were much more potent than the native, monomeric GluN2B-PSD-95 interaction (7100 ± 0.2 nM) (Fig. 1). Hence, fatty acid modification at the N-terminus of UCCB01-144 had no or little influence on binding to PSD-95, which encouraged us to proceed with a metabolic stability characterization of peptides 1–4.

To evaluate if the TAT-conjugated lipidated compounds 3 and 4 retain stability in biological tissues, we incubated them with both human plasma and mice liver microsomes. We found that UCCB01-144 lipidation prevented enzymatic degradation in human plasma (Fig. 2a). All lipidated analogues have half-lives of >24 h, suggesting that lipid conjugation is the primary structural determinant of the increased stability. Mice liver microsomes did not extensively metabolise the lipidated peptides after one hour of incubation (Fig. 2b), and their intrinsic clearance values were similar to the low metabolised control propranolol, indicating the hepatic clearance as a minor metabolism route of these compounds.[27]

In Vivo Stability and Pharmacokinetics

Next, we tested if the improved stability found in vitro is also observed in vivo. Peptides are degraded not only by plasma enzymes but also by tissue proteases.[28] For example, renal filtration with subsequent proteolytic degradation is a common elimination mechanism of peptides.[29] We examined if the enhanced plasma stability of lipid derivatives will lead to a higher brain uptake, measuring the compound levels using liquid chromatography–tandem mass spectrometry (LC-MS/MS). We obtained plasma and brain pharmacokinetic profiles in rats of peptides that were administered intravenously (i.v.) at 5 mg kg\(^{-1}\) or subcutaneously (s.c.) at 10 mg kg\(^{-1}\), and sampled...
after 0.5, 1, 4, and 8 h. Intravenous injection of peptide 2 resulted in plasma concentrations more than three times higher than the levels of peptides 3 and 4 after 30 min of administration (Fig. 3a). As their in vitro metabolism were similar, this suggests that either the TAT–lipid analogues distribute fast to the tissues, or their elimination route proceeds via a different mechanism than plasma proteolysis or liver metabolism. On the other hand, brain levels of 3 and 4 were ~2-fold the levels of 2 after 30 min, which indicates that TAT–lipid combination increases brain permeation (Fig. 3b). Unexpectedly, after i.v. administration of compounds 3 and 4 systemic toxic effects were observed. Two of nine animals died, and the remainder had signs of toxicity such as cyanosis, hypoxia, dyspnea, and loss of limb function.

Subcutaneous administration resulted in higher absorption of lipid-only peptides 1 and 2 than TAT–lipid peptides 3 and 4 after 4 h (Fig. 4). All peptides reached their maximum concentration in plasma after 8 h of administration indicating a slow release of these peptides from the injection site. Peptides 1 and 2 are negatively charged and have molecular weights of ~2 kDa, while peptides 3 and 4 are positively charged and are roughly twice as large. The extracellular matrix of the subcutaneous tissue comprises charged proteins such as collagen and glycosaminoglycans, with net negative charge. The lower absorption of peptides 3 and 4, therefore, may derive from their higher molecular weights and enhanced electrostatic interactions with components of the skin extracellular matrix. In accordance with the reduced plasma levels, the toxic effects of 3 and 4 were less severe after s.c. administration and no animal died. However, all animals showed signs of depression after 15 min of injection, and after 8 h all animals displayed leg oedema.

Toxicity Mechanism of Peptides Containing TAT–Lipid Combinations

The pharmacokinetic data suggested that the toxic effects observed after administration of TAT–lipid peptides correlated with their plasma concentration. We wondered if the acylation...
of the cationic CPP TAT resulted in a peptide amphiphile with increased membrane-disruption effects in human erythrocytes. To examine this, we incubated peptides 1–4 at increasing concentrations with diluted human red blood cells. As controls, we tested the hemotoxic bee venom peptide melittin and the TAT peptide conjugated with stearic acid, but without the dimeric PDZ binding motif (5, Chart 1).\(^4\)

Peptides 3 and 4 sharing TAT–lipid combinations resulted in 50% of hemolysis (HC\(_{50}\)) at 50 μM, explaining the toxic effects found in vitro. In the in vivo studies, the administered formulation contained 3 and 4 at concentrations of \(\sim 1.3\) mM, a value that may induce extensive cell lysis, and match the clinical signs of hypoxia and cyanoysis. The lipid-only peptides 1 and 2, the CPP TAT, UCCB01-144, and the N-dimer peptide did not display haemolytic effects up to 50 μM (Table 1). In turn, peptide 5, the TAT–lipid conjugate without the PDZ binding motif, was almost 20-fold more haemolytic than peptides 3 and 4 (HC\(_{50}\) of 2.6 μM) demonstrating that lipidation on the N-terminus of the CPP TAT can lead to haemolytic effects in vitro and is likely the cause for the toxic effects of peptides 3 and 4.

Even though the conjugation of lipids to CPPs have been studied, in particular in the context of transfection agents, few reports indicate their toxicity to cells and, as far as we know, none reported acute toxic effects. Polyarginines, for example, have been acylated with myristic acid and tested both in vitro and in vivo with no described hemolysis.\(^{32,33}\) However, polyarginines with more than 15 residues, and acylated with either lauric (C\(_{12}\)) or palmitic acid (C\(_{16}\)) were toxic to Jurkat cells.\(^{34}\) A similar reduction in cell viability was also reported for tetraarginines conjugated with stearic acid, but only for the HepG2 cell line, while no cell death was found in the Caco-2 line.\(^{15}\)

### Conclusion

CPP and lipid conjugation improved ADME properties and advanced the development of bioactive peptides towards approved medicines. However, to the best of our knowledge, this is the first description of the haemolytic effects of lipidated TAT-conjugated peptides. Our result points out that even though it may be tempting to simultaneously increase peptide stability and cell penetration, there is a balance between membrane permeability and haemolytic properties of lipidated CPPs. Structurally, this effect may be related to both the hydrophobic chain length and the number of charged species in the hydrophilic moiety. A structure–activity study of 5 could result in the design of less toxic and plasma-stable CPPs. In another way, if the membrane effects of 5 could be directed towards bacterial membranes, it could serve as a starting point in the design of a new class of antimicrobial peptides.\(^{16}\)

### Experimental

See the Supplementary Material.

### Supplementary Material

Experimental procedures and peptide characterization results are available on the Journal’s website.

### Conflicts of Interest

The authors declare no conflicts of interest.

### Acknowledgements

E.F.A.F was funded by the Brazilian CNPq program Science without Borders (Ciências sem Fronteiras, grant 205513/2014-0).

### References


### Table 1. Haemolytic activity of peptides 1–5 and experimental controls to human red blood cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>HC(_{50}) [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melittin</td>
<td>0.6 ± 0.02</td>
</tr>
<tr>
<td>TAT</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>N-dimer</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>UCCB01-144</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>1</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>2</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>3</td>
<td>46 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>46 ± 0.7</td>
</tr>
<tr>
<td>5</td>
<td>2.6 ± 0.02</td>
</tr>
</tbody>
</table>

\(^A\)Concentration that induces 50% of maximum haemolytic activity.

Values shown are mean ± s.e.m., \(n \geq 3\).
Dimeric Inhibitor of PSD-95


