



Photochemical internalisation of a macromolecular protein toxin using a cell penetrating peptide-photosensitiser conjugate

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ABSTRACT

Photochemical internalisation (PCI) is a site-specific technique for improving cellular delivery of macromolecular drugs. In this study, a cell penetrating peptide, containing the core HIV-1 Tat 48–57 sequence, conjugated with a porphyrin photosensitiser has been shown to be effective for PCI. Herein we report an investigation of the photophysical and photobiological properties of a water soluble bioconjugate of the cationic Tat peptide with a hydrophobic tetraphenylporphyrin derivative. The cellular uptake and localisation of the amphiphilic bioconjugate was examined in the HN5 human head and neck squamous cell carcinoma cell line. Efficient cellular uptake and localisation in endo/lysosomal vesicles was found using fluorescence detection, and light-induced, rupture of the vesicles resulting in a more diffuse intracellular fluorescence distribution was observed. Conjugation of the Tat sequence with a hydrophobic porphyrin thus enables cellular delivery of an amphiphilic photosensitiser which can then localise in endo/lysosomal membranes, as required for effective PCI treatment. PCI efficacy was tested in combination with a protein toxin, saporin, and a significant reduction in cell viability was measured versus saporin or photosensitiser treatment alone. This study demonstrates that the cell penetrating peptide-photosensitiser bioconjugation strategy is a promising and versatile approach for enhancing the therapeutic potential of bioactive agents through photochemical internalisation.

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1. Introduction

Photochemical Internalisation (PCI) is a technology developed by Berg and co-workers [1–3], which can enhance drug delivery using light in combination with photosensitisers localised in endo/lysosomal membranes. The technique is aimed at improving the delivery of bioactive agents which become sequestered within endo/lysosomal vesicles and are therefore unable to exert their action upon the target site. With PCI, the light-induced rupture of the photosensitised vesicle membranes enables the agents to reach their intracellular target. The aim of this study was to investigate whether a cell penetrating peptide bioconjugate can be used to enhance drug delivery.

Pharmaceutical agents can suffer from low bioavailability due either to poor cell membrane penetration and/or the lack of water solubility. In tackling this key problem in drug delivery, an active area of research

to improving bioavailability is the conjugation of drugs with cell penetrating peptides (CPP), which can enhance the water solubility and cellular uptake of drugs [4]. Two studies independently reported the cell membrane translocation ability of the trans-activating transcriptional activator (Tat), a protein from human immunodeficiency virus type I (HIV-1) [5,6] using the 86-mer Tat or the purified Tat protein. Both Tat proteins were able to enter cultured cells and induce the trans-activation of the viral promoter. The full-length protein was soon characterised as a protein with multiple functional domains [7,8] and the highly basic region which contains six arginine and two lysine in nine residues is assigned to the protein translocation activity [9]. Since then, other natural or designed proteins or peptides have also demonstrated the property of translocation [10]. These proteins and peptides all contain the highly positive charged protein transduction domains (PTDs) or cell penetrating peptides (CPPs) that are responsible for protein transduction, *i.e.* the translocation process across the cell membrane.

Several approaches to linking pharmaceutical cargos to Tat peptides or other CPPs to overcome the cell membrane barrier have been carried out. To date, Tat peptides have been proven to facilitate the cell entry of various molecules ranging from peptides [11] with molecular weights of hundreds of Dalton to larger structures such as liposomes [12]. The remarkable success of Tat peptides in drug delivery has drawn considerable attention to the mechanisms involved

Abbreviations: CPP, cell penetrating peptide; DMF, dimethylformamide; DMPC, dimyristoyl phosphatidylcholine; PCI, photochemical internalisation; PDT, photodynamic therapy; SPPS, solid phase peptide synthesis; Tat, trans-activating transcriptional activator; TFA, trifluoroacetic acid; TPP, TP-porphine.

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in the internalisation and intracellular delivery. A reduction of cellular uptake was found when Tat peptides were modified with a single deletion or substitution of the core sequence 'GRKKRRQRRR' in the basic domain resulting in a change of charges [13,14]. Diminished cellular uptake indicated the unique highly cationic nature of Tat peptides certainly plays a key role in the cell uptake process, and the direct electrostatic interaction between the positively charged Tat peptides and the negatively charged proteoglycans or glycosaminoglycans on the cell surface is believed to be required in the internalisation mechanism [15,16].

Despite earlier misinterpretations of the uptake mechanism resulting from artefacts caused during the cell fixation procedures, endocytic uptake is the generally recognised mechanism for Tat peptide and most other CPP mediated drug delivery [17,18]. Both lipid raft-mediated caveolar endocytosis and the clathrin-mediated endocytosis have been identified as endocytic routes of Tat-fusion proteins [19,20], in addition to macropinocytosis, a rapid non-specific lipid raft cell uptake pathway [21]. To date, more than 20 Tat-derived short peptides with various modifications at either the N-terminal or the C-terminal ends have been used for delivering drugs into cells [22]. The advantages and versatility of the Tat peptide-mediated drug delivery have also been applied *in vivo*. For example, a study conducted by Schwarze et al. used a Tat fusion protein (120 kD) in which β -galactosidase (116 kD) was ligated to the protein transduction domain [23]. After intraperitoneal injection in mice, the conjugate was found to distribute into virtually every organ and even cross the blood brain barrier after 4 h. These promising results have led to the possibility of Tat peptides being used for *in vivo* drug delivery.

Porphyrins and their analogues constitute the major class of photosensitising agents in photodynamic therapy (PDT), which is a local treatment for both malignant and no-malignant pathologies. Its therapeutic effects arise from the photo-oxidative damage initiated by the photosensitised generation of reactive oxygen species [24]. As with other pharmaceutical agents, many photosensitising agents exhibit poor bioavailability being either too hydrophilic or conversely too hydrophobic and with limited water solubility. Conjugation strategies that modify photosensitisers by linking to certain peptides or proteins with targeting function have been used to improve the selectivity of photosensitiser delivery [25,26]. For example, penetratin coupled to 5-aminolevulinic acid was shown to be taken up by cells and could be successfully converted to photoporphyrin IX *via* the haem biosynthetic pathway [27]. CPP-porphyrin conjugates have been recently synthesised as photoactive compounds. Sibrian-Vazquez et al. synthesised different porphyrin-peptide conjugates bearing PEG linkers to several targeting sequences including the Tat peptide (48–60) to compare their cellular uptake, subcellular localisation and the phototoxicity *in vitro* [28]. Sehgal et al., from the same group, examined the cellular uptake and phototoxicity of five porphyrin-peptide conjugates using a prostate cancer cell line [29]. Recently we have also shown that a Tat-porphyrin conjugate is effective for antimicrobial PDT [30].

In photochemical internalisation (PCI), the light-induced rupture of the endo/lysosomal membranes enables therapeutic agents sequestered within endo/lysosomes to reach their intracellular target. In contrast to photodynamic therapy, PCI is a site-specific drug delivery technique where the therapeutic action is exerted by the drug itself but not the by photosensitiser or the reactive oxygen species. For example, previous studies in our laboratory have demonstrated successful reversal of doxorubicin drug resistance *in vitro* by PCI [31,32]. Other agents successfully delivered using PCI include siRNA [2] and a peptide nucleic acid (PNA), where the PNA was conjugated to a cell penetrating peptide but not the photosensitiser [33].

Using an *in vivo* rat liver model, we have also demonstrated that PCI-mediated release of low doses of a protein toxin can give enhanced tissue damage [1]. Type I ribosome inactivating proteins (RIPs) such as gelonin and saporin exert a lethal effect on protein synthesis only if

delivered into the cytosol, however due to their size they can become sequestered within endo/lysosomes [34]. Enhanced drug delivery of saporin and other RIPs (gelonin) to the cytosol has previously been demonstrated using PCI technology and amphiphilic disulfonated photosensitisers *in vitro* [35–37]. These amphiphilic disulfonated photosensitisers were selected since they exhibit preferential localisation in endo/lysosomal membranes in order to promote the PCI effect. It is important to note that the criteria for selection of a photosensitiser for PCI are distinct from PDT where localisation in several subcellular compartments may confer an advantage (e.g. in mitochondria) rather than the specific endo/lysosomal localisation required for PCI. Identification and optimisation of new photosensitising compounds for PCI therefore presents a different experimental challenge.

In the present study, we investigated the hypothesis that a Tat-TPP conjugate could be particularly suitable for PCI because of the resulting amphiphilicity conferred by cationic Tat peptide sequence and the hydrophobic porphyrin. Such an amphiphilic structure should favour membrane binding essential for PCI, but retain good water solubility. The presence of the CPP moiety should in principle favour endo/lysosomal uptake but, unlike CPP-mediated drug delivery where segregation within these vesicles is a drawback, with PCI the CPP-photosensitiser conjugate is used to induce rupture of these vesicles for co-delivery of the bioactive agent. Photophysical and biological studies of a peptide-porphyrin conjugate were carried out to assess its efficacy for PCI in enhancing intracellular delivery and cytotoxicity of the macromolecular toxin, saporin.

2. Materials and methods

2.1. Chemicals

Chemical reagents were purchased from Sigma-Aldrich (Gillingham, UK), Fluka (Gillingham, UK), Acros (Loughborough, UK), Lancaster (Lancashire, UK) and Novabiochem (Nottingham, UK). All other solvents were purchased from Fisher Scientific (Loughborough, UK) and used as received. Peptide grade dimethylformamide was purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). Preparative RP-HPLC was performed on a Dionex HPLC system (Leeds, UK) consisting of an Ultimate 3000 WPS autosampler, a P680 preparative pump (HPG), a UVD 170U multiwavelength detector, and a Foxy Jr fraction collector. The purifications were performed on a Phenomenex Gemini 5 μ m C-18 (250 \times 30 mm) column with a flow rate of 22.5 ml/min. Mobile phase A was 0.1% TFA in water, mobile phase B was 0.1% TFA in acetonitrile. High resolution mass spectrometry was performed using a Bruker MicroTOF autospec ESI mass spectrometer (Coventry, UK). TPPS₄ (tetrasulfonated tetraphenylporphine) was supplied by Frontier Scientific (Frontier Scientific Europe, Lancashire, UK). TPPS_{2a} (disulfonated tetraphenylporphine) was a gift from PCI Biotech (Oslo, Norway).

2.2. Synthesis of Tat-TPP conjugate

The Tat-TPP conjugate **2** was prepared by the ligation of the maleoylporphyrin derivative **1** (5 eq) to a HIV-1 Tat 48–57 analogue (1 eq), wherein the peptide was extended at the C-terminus by the tetrapeptide amide GYKC-NH₂ (Fig. 1), as used elsewhere [38]. The peptide was synthesised by 9-fluorenylmethoxycarbonyl (Fmoc)-SPPS, and the ligation reaction was performed in solution in DMSO containing pyridine, shielded from light, as recently described by us [30]. The crude product was isolated on a Discovery DSC-18 solid phase extraction cartridge (Supelco, Gillingham, UK). The desired compound was eluted with (5% MeCN-0.1% aq TFA) mobile phase: and lyophilised. **2** was subsequently characterised by high resolution mass spectrometry, and its purity confirmed as >95% by analytical RP-HPLC. The molecular weight of the conjugate is 3869 Da.

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