Journal of Controlled Release 140 (2009) 237–244

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Cellular uptake, distribution and cytotoxicity of the hydrophobic cell penetrating peptide sequence PFVYLI linked to the proapoptotic domain peptide PAD

Catherine L. Watkins [a], Paul Brennan [b], Christopher Fegan [c], Kentaro Takayama [d], Ikuhiko Nakase [d], Shiroh Futaki [d], Arwyn T. Jones [a,*]

[a] Welsh School of Pharmacy, Cardiff University, Cardiff, CF10 3NB Wales, UK
[b] Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Heath Park, Cardiff, CF4 4XX, Wales, UK
[c] Department of Haematology, University Hospital of Wales, Cardiff, UK
[d] Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

ARTICLE INFO

Article history:
Received 23 March 2009
Accepted 25 April 2009
Available online 3 May 2009

Keywords:
Cell penetrating peptides
Octaarginine
Proapoptotic domain
Apoptosis
Endocytosis

ABSTRACT

The capacity of cell penetrating peptides (CPPs) to breach biological membranes offers hope for their utilisation as vectors for the delivery of small molecule drugs and macromolecular therapeutics. Using three different cell systems, including primary human cells, we have studied the uptake, subcellular localisation and effect on cell viability of the well characterised octaarginine and the more recently discovered hydrophobic PFVYLI peptide, either alone, or conjugated to the proapoptotic domain peptide PAD $(klaklak)_2$. Octaarginine and PFVYLI were efficiently endocytosed into cells at 37 °C but an ability to translocate directly across the plasma membrane at higher peptide concentrations or when uptake experiments were performed on ice was confined to the cationic variant. Octaarginine- and PFVYLI-PAD conjugates were cytotoxic, with KG1a leukaemia cells being more sensitive than HeLa cells and octaarginine-PAD being the most potent conjugate in both cell lines. The effects of the CPP-PAD conjugates on cell morphology and permeability was rapid suggesting that cytotoxicity is partially mediated at the plasma membrane rather than exclusively through induction of apoptosis at the mitochondria. Primary human leukaemia cells were more similar to KG1a cells than HeLa cells, suggesting the relative sensitivity of leukaemia cells to these peptides could be exploited *in vivo*.

1. Introduction

Cell penetrating peptides (CPPs) are amino acid sequences of usually <30 residues in length that have the capacity to breach biological membranes [1,2]. They have also been shown to efficiently deliver membrane impermeable molecules to cells, and these range from small fluorescent probes to much larger macromolecular structures including polyplexes, lipoplexes and microparticles [3–5]. Much interest in CPPs lies in their utilisation as delivery vehicles for therapeutic entities such as genes and proteins [6–8] and the best characterised are those with sequences enriched in cationic residues lysine and arginine. Notable examples include the HIV-TAT peptide and synthetic oligoarginines R6-20 [9]. The mechanism by which these cationic peptides enter cells as single or fluorescent entities is still largely unknown despite intense studies over the past two decades. For the cationic variants, the extent and mechanism of cell entry is heavily dependent on the incubation temperature and the extracellular peptide concentration [10–13]. Generally at concentrations ≤2 μM, endocytosis of R8 and TAT seems to be the prominent uptake mechanism but the plasma membrane has a concentration threshold that when breached leads to an increase in permeability. However at these concentrations the membrane is not permeable to probes such as propidium iodide but whether escape into the cytosol is mediated through a distinct location at the plasma membrane, remains to be determined [10,12,13].

Cationic residues are not absolutely required for cell penetration and efficient internalisation and vector capacity has been observed in peptides with a variety of net charges, isoelectric points, hydrophilicity and folding properties [1]. A number of hydrophobic CPP sequences have been described and examples include the signal sequences from integrin ß 3 (VTVLALGALAGVGVG) and Kaposi fibroblast growth factor (AAVALLPAVLLALLAP) [14,15]. Hydrophobic amino acids are also integral to amphipathic CPPs such as MAP [16] and to longer chimeric CPPs containing additional cationic residues for enhancing uptake and delivery capacity [17]. More recently a short

Abbreviations: CPP, cell penetrating peptide; CLL, chronic lymphocytic leukaemia; CLSM, confocal laser scanning microscopy; PAD, proapoptotic domain; PBS, phosphate buffered saline; R8, octaarginine.

* Corresponding author. Welsh School of Pharmacy, Redwood Building, Cardiff University, Cardiff, CF10 3NB, Wales, United Kingdom. Tel.: +44 2920876431; fax: +44 2920874536.
E-mail address: jonesat@cardiff.ac.uk (A.T. Jones).

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doi:10.1016/j.jconrel.2009.04.028

hydrophobic sequence PFVYLI was shown, as a fluorescent conjugate, to penetrate the plasma membrane of a number of different cell lines [18]. Uptake of this peptide was unaffected by lowering the incubation temperature to 4 °C or by treating cells with a number of commonly used endocytosis inhibitors such as cytochalasin D and methyl ß-cyclodextrin.

CPPs have been widely used to deliver bioactive peptides including sequences that accelerate or inhibit apoptosis. One example is the proapoptotic domain peptide PAD $(klaklak)_2$ that was originally investigated as an antimicrobial peptide [19], but was more recently shown to induce mitochondrial swelling and apoptosis in *in vitro* and *in vivo* models of cancer and obesity [20,21]. PAD peptide alone is non-toxic to eukaryotic cells at relatively high concentrations (>300 μM) but PAD conjugated to a number of CPPs inhibits cell proliferation in the low micromolar range and inhibited the growth of solid tumours in mice [20,22–25].

In this study we investigated whether the PFVYLI sequence has the capacity to deliver a proapoptotic peptide into cells, thus testing its potential as a vehicle for delivering therapeutics into the cytosol. We show that a PFVYLI-PAD conjugate is toxic to adherent and leukaemia cells lines, including primary leukaemia cells, in the low micromolar range. Though less effective at cell killing compared with an octaarginine-PAD conjugate, it suggests that this short hydrophobic sequence can be exploited to deliver other bioactive entities to cells.

2. Materials and methods

2.1. Reagents

Alexa Fluor® 488-C5-maleimide was purchased from Invitrogen (Paisley, U.K). Glass-bottomed culture dishes (35 mm) for microscopy were from MatTek Corporation, (Ashland, USA). Propidium Iodide (PI) was purchased from Sigma-Aldrich (Gillingham, UK). All tissue culture reagents were from Invitrogen (Paisley, U.K).

2.2. Peptide synthesis and conjugation

Sequences of the peptides used in this study are shown in Table 1; lower case letters denote D isoforms. R8 peptide was obtained from American Peptide Company (California, USA). All other peptides used in this study were generated by Fmoc (fluoren-9-ylmethoxycarbonyl) solid-phase synthesis. Peptides were extended at the C termini by addition of GC to allow fluorescent labelling using maleimide-C5-Alexa488 sodium salt as previously described [11]. Purification of peptides was by HPLC using a C18 Luna 100 Å 5 μm semi preparative column (Phenomenex, Macclesfield, UK) as previously described [11,13]. Peptide masses were confirmed using electrospray- and matrix-assisted laser-desorption ionization–time-of-flight spectroscopy.

Table 1
Peptides used in this study.

Unlabelled peptide	Sequence
PFV	PFVYLIGC-amide
PAD	klaklakklaklakGC-amide
PFV-PAD	PFVYLIGGklaklakklaklakGC-amide
r8-PAD	rrrrrrrrGGklaklakklaklakGC-amide
Alexa488-Peptides	**Sequence**
$(GS)_4$GC-Alexa488	GSGSGSGSGC-Alexa488
R8-Alexa488	RRRRRRRRGC-Alexa488
PFV-Alexa488	PFVYLIGC-Alexa488
PFV-PAD-Alexa488	PFVYLIGGklaklakklaklakGC-Alexa488
r8-PAD-Alexa488	rrrrrrrrGGklaklakklaklakGC-Alexa488

Lower case denotes D amino acids, C-Alexa488 denotes cysteine labelled with Alexa488.

2.3. Cell culture

Human acute myeloid leukaemia KG1a cells were cultured and maintained at a confluency of $0.5–2\times10^6$ cells/mL in RPMI 1640 medium, supplemented with 10% (v/v) foetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin. Human cervical carcinoma, HeLa cells were maintained as a subconfluent monolayer in D-MEM supplemented with 10% (v/v) heat inactivated foetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin. Both cell lines were maintained in a humidified 5% CO_2 incubator at 37 °C.

2.4. Cellular localisation of R8-Alexa488, PFV-Alexa488, r8-PAD-Alexa488 and PFV-PAD-Alexa488 in suspension KG1a and adherent HeLa cells

KG1a cells (0.5×10^6) were washed in complete media and equilibrated at 4 or 37 °C for 15 min. The medium was replaced with fresh temperature equilibrated medium containing 2–20 μM Alexa488-labelled peptides and the cells were then incubated with the peptides at these temperatures for 1 h. Cells were then washed twice in serum-free RPMI 1640 medium (SFM), once in imaging medium (SFM without Phenol red) and finally resuspended in 500 μL of imaging medium. 100 μL of the cell suspension was transferred to a glass-bottomed, 35 mm culture dish and allowed to settle for ~1 min prior to analysis on a Leica SP5 confocal laser scanning microscope equipped with an Ar and HeNe laser and a 63x oil immersion objective. Cells were imaged through a single section and images acquired using the Leica LAS-AF software were finally arranged using Adobe Photoshop.

HeLa cells (1.8×10^5) were seeded into glass-bottomed, 35 mm culture dishes and allowed to adhere for 24 h. The cells were equilibrated at 4 or 37 °C for 15 min prior to replacing the medium with fresh temperature equilibrated complete medium containing 2–20 μM Alexa488-labelled peptides and the cells were then incubated with the peptides at these temperatures for 1 h. The cells were washed twice in SFM, once in imaging medium and then imaged by confocal microscopy as described above.

2.5. Flow cytometry

HeLa cells (0.6×10^5) were seeded into 12 well tissue culture plates and grown to 90% confluency under tissue culture conditions. On the day of the experiment HeLa and KG1a cells (0.5×10^6) were washed once on complete media and were equilibrated at 4 or 37 °C for 15 min, prior to incubation for 1 h with 2 μM R8-Alexa488, PFV-Alexa488 or $(GS)_4$GC-Alexa488 at either 4 or 37 °C. Cells were washed once with ice cold phosphate buffered saline (PBS), incubated with 0.25 mg/mL trypsin/EDTA solution at 37 °C for 5 min and then placed as a suspension to 1.5 mL centrifuge tubes. The cells were washed once in ice cold PBS, twice with PBS containing 14 μg/mL heparin and finally resuspended in 200 μL ice cold PBS for flow cytometry. Cellular fluorescence was then immediately quantified using a Becton Dickinson FACSCalibur analyser. Live cells were gated on a forward and side scatter basis, and 10,000 viable cells were assayed.

2.6. Effect of PAD peptides on plasma membrane permeability

KG1a cells (0.5×10^6) were washed once in complete media equilibrated at 4 or 37 °C for 15 min and co-incubated with 10 μM unlabelled PAD, PFV-PAD or r8-PAD peptide and 5 μg/mL PI for 10 min or 1 h at 37 or 4 °C. Cells were then washed twice in SFM, once in imaging medium and finally resuspended in 500 μL of imaging medium. 100 μL of the cell suspension was transferred to a glass-bottomed, 35 mm culture dish and these were analysed by confocal microscopy as described.

HeLa cells (1.8×10^5) were seeded into sterile glass-bottomed, 35 mm culture dishes and allowed to adhere for 24 h under tissue