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ORIGINAL RESEARCH

The Neuroprotective Efficacy of Cell-Penetrating Peptides TAT, Penetratin, Arg-9, and Pep-1 in Glutamic Acid, Kainic Acid, and In Vitro Ischemia Injury Models Using Primary Cortical Neuronal Cultures

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Abstract Cell-penetrating peptides (CPPs) are small peptides (typically 5–25 amino acids), which are used to facilitate the delivery of normally non-permeable cargos such as other peptides, proteins, nucleic acids, or drugs into cells. However, several recent studies have demonstrated that the TAT CPP has neuroprotective properties. Therefore, in this study, we assessed the TAT and three other CPPs (penetratin, Arg-9, Pep-1) for their neuroprotective properties in cortical neuronal cultures following exposure to glutamic acid, kainic acid, or in vitro ischemia (oxygen–glucose deprivation). Arg-9, penetratin, and TAT-D displayed consistent and high level neuroprotective activity in both the glutamic acid (IC50: 0.78, 3.4, 13.9 μM) and kainic acid (IC50: 0.81, 2.0, 6.2 μM) injury models, while Pep-1 was ineffective. The TAT-D isoform displayed similar efficacy to the TAT-L isoform in the glutamic acid model. Interestingly, Arg-9 was the only CPP that displayed efficacy when washed-out prior to glutamic acid exposure. Neuroprotection following in vitro ischemia was more variable with all peptides providing some level of neuroprotection (IC50; Arg-9: 6.0 μM, TAT-D: 7.1 μM, penetratin/Pep-1: >10 μM). The positive control peptides JNKI-1D-TAT (JNK inhibitory peptide) and/or PYC36L-TAT (AP-1 inhibitory peptide) were neuroprotective in all models. Finally, in a post-glutamic acid treatment experiment, Arg-9 was highly effective when added immediately after, and mildly effective when added 15 min post-insult, while the JNKI-1D-TAT control peptide was ineffective when added post-insult. These findings demonstrate that different CPPs have the ability to inhibit neurodamaging events/pathways associated with excitotoxic and ischemic injuries. More importantly, they highlight the need to interpret neuroprotection studies when using CPPs as delivery agents with caution. On a positive note, the cytoprotective properties of CPPs suggests they are ideal carrier molecules to deliver neuroprotective drugs to the CNS following injury and/or potential neuroprotectants in their own right.

Keywords Cell-penetrating peptides · TAT · Arg-9 · Penetratin · Pep-1 · Excitotoxicity · In vitro ischemia

Introduction

The development of cell-penetrating peptides (CPPs), also referred to as peptide transduction domains (PTDs) as facilitators of therapeutic drug delivery has progressed significantly since the initial discovery of the PTD within the human immunodeficiency virus-type 1 trans-activating transcriptional activator (Frankel and Pabo 1988; Green and Loewenstein 1988). Since then, the active transporting portion of this sequence has been isolated ($TAT_{48–58}$: referred to as the TAT peptide or TAT), as well as the discovery and synthesis of over 100 novel CPPs (Milletti 2012).

Potential therapeutics fused to CPPs have been assessed in neuronal culture systems and animal models that mimic

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neural injury mechanisms in a variety of disorders, including cerebral ischemia, epilepsy, Parkinson's disease, and Alzheimer's disease (Lai et al. 2005; Liu et al. 2006; Arthur et al. 2007; Colombo et al. 2007; Nagel et al. 2008; Meade et al. 2009). The use of CPP for neurological disorders is especially attractive due to their ability to transport cargo across the blood–brain barrier and then enter into neural cells within the brain parenchyma (Aarts et al. 2002; Zhang et al. 2013). Two examples of CPP-fused neuroprotective peptides that have entered clinical trials are the JNK inhibitor peptide (JNKI-1D-TAT or XG-102; ARAMIS 2011) and the NMDA receptor/postsynaptic density-95 inhibitory peptide (NR2B9c or NA-1; Dolgin 2012); both peptides fused with TAT.

An important feature of any CPP is limited toxicity at clinically relevant doses, while a CPP that displays endogenous neuroprotective activity is an added advantage. In fact, the TAT peptide, the most widely used CPP used in neuroprotection experiments appears to possess intrinsic neuroprotective properties. Recent studies (Xu et al. 2008; Vaslin et al. 2009; Meade et al. 2010a, b; Craig et al. 2011) have reported that the TAT peptide displays neuroprotective actions in vitro following excitotoxicity and oxygen–glucose deprivation, and in vivo following cerebral ischemia in P12 rats after intraventricular injection. While the exact mechanisms of TATs neuroprotective action are not fully understood, there is speculation that it interferes with NMDA receptor activation (Xu et al. 2008; Vaslin et al. 2009), although one study failed to detect a binding interaction (Li et al. 2008). Additionally, in a RNAi study using CPPs to deliver constructs, both the TAT and penetratin peptides alone were shown to down-regulate MAP kinase mRNA in the lung following intratracheal administration (Moschos et al. 2007). This data suggests that both membrane and intracellular mechanisms may be operating, and which requires further investigation, as does the assessment of other CPPs for neuroprotective properties.

In this study, we have examined the neuroprotective efficacy of four CPPs, namely TAT, penetratin, Arg-9, Pep-1 in a glutamic acid, kainic acid and in vitro ischemia injury model. The three different neuronal injury models are likely to activate different damaging cellular pathways, and thereby will provide further insight into the neuroprotective spectrum and possibly mode of action of the CPPs.

Methods

Primary Neuronal Cortical Cultures

Establishment of cortical cultures was as previously described (Meloni et al. 2001). Briefly, cortical tissue from E18–E19 Sprague–Dawley rats was dissociated in Dulbecco's-modified Eagle medium (DMEM; Invitrogen, Australia) supplemented with 1.3 mM L-cysteine, 0.9 mM $NaHCO_3$, 10 U/ml papain (Sigma, USA), and 50 U/ml DNase (Sigma) and washed in cold DMEM/10 % horse serum. Neurons were resuspended in Neurobasal (Invitrogen) containing 2 % B27 supplement (B27; invitrogen). Before seeding 96-well sized glass wells (7 mm diameter, ProTech, Australia) or 96-well plastic plates (Nunc, Australia) were coated with poly-D-lysine overnight (50 µl/well: 50 µg/ml; 70–150 K, Sigma). Excess poly-D-lysine solution was then removed and replaced with Neurobasal (60 µl: containing 2 % B27; 4 % fetal bovine serum; 1 % horse serum; 62.5 µM glutamic acid; 25 µM 2-mercaptoethanol; and 30 mg/ml streptomycin and 30 mg/ml penicillin). Neurons were plated (90 µl) to obtain ≈10,000 viable neurons for each well on day in vitro 11–12. Neuronal cultures were maintained in a CO_2 incubator (5 % CO_2, 95 % air balance, 98 % humidity) at 37 °C. On day in vitro 4, one-third of the culture medium was removed and replaced with fresh Neurobasal/2 % B27 containing the mitotic inhibitor, cytosine arabinofuranoside (1 µM final concentration; Sigma). On day in vitro 8, one half of the culture medium was replaced with Neurobasal/2 % B27. Cultures were used on day in vitro 11 or 12 after which time they routinely consist of >97 % neurons and 1–3 % astrocytes (Meloni et al. 2001).

Cell-Penetrating Peptides and Control Peptides

All peptides were synthesised by Mimotopes Pty Ltd (Australia), except TAT-L, which was synthesised by Pepscan Presto (The Netherlands). The peptides were HPLC purified to greater than 93–96 %. TAT-L, penetratin, Arg-9 and Pep-1 were synthesised in the L-isoform and TAT-D in the protease resistant D-retro-inverso form, synthesised from D-amino acids in reverse sequence (referred to as D-isoform hereafter) (Table 1). A TAT-fused JNK inhibitory peptide (JNKI-1D-TAT) in the D-isoform and a TAT-fused AP-1 inhibitory peptide (PYC36L-TAT) in the L-isoform were used as positive controls (Table 1; Borsello et al. 2003; Meade et al. 2010b). All peptides were prepared as 100 × stocks (500 µM) in normal saline and assessed in a concentration range from 0.1 to 15 µM, dependent upon injury model. The TAT-L peptide was only used in the glutamic acid excitotoxicity model.

Glutamic Acid and Kainic Acid Excitotoxicity Models and Peptide Incubation

Peptides were added to culture wells (96-well plate format) 15 min prior to glutamic acid or kainic acid exposure by removing media and adding 50 µl of Neurobasal/2 %

Table 1 Amino acid sequences, molecular weights, and charge of peptides

Peptide	Sequence	Amino acids:molecular weight (Da)	Net charge at pH 7	Physical–chemical properties
Arg-9	H-RRRRRRRRR-NH_2	9:1,422	10	Cationic
TAT-D	H-GrrrqrrkkrG-NH_2	10:1,453	9	Cationic
TAT-L	Ac-GRKKRRQRRRG-NH_2	10:1,494	8	Cationic
Penetratin[a]	H-RQIKIWFQNRRMKWKK-NH_2	16:2,246	7	Cationic
Pep-1	H-KETWWETWWTEWSQPKKKRKV-NH_2	21:2,847	4	Amphiphilic
PYC36L-TAT	H-GRKKRRQRRRGGLQGRRRQGYQSIKP-NH_2	26:3,180	13	Cationic
JNKI-1D-TAT	H-tdqsrpvqpflnlttprkprpprrrqrrkkrG-NH_2	32:3,925	12	Cationic

At the N-terminus, H indicates free amine, and Ac indicates acetyl. At the C-terminus NH_2 represents amide. Sequence is in standard single letter code with L-isoform amino acid residues represented in uppercase and D-isoform amino acid residues represented in lowercase

[a] Penetratin is also known as antennapedia peptide

B27-containing CPPs, control peptides, or MK801/CNQX. To induce excitotoxicity 50 µl of Neurobasal/2 % B27-containing glutamic acid (200 µM) or kainic acid (400 µM) was added to the culture wells (100 µM glutamic acid and 200 µM kainic acid final concentrations). Cultures were incubated at 37 °C in the CO_2 incubator for 5 min for glutamic acid and 45 min for kainic acid exposure, after which time the media was replaced with 100 µl of 50 % Neurobasal/2 % N_2 supplement (Invitrogen) and 50 % balanced salt solution (BSS; see below). Cultures were incubated for a further 24 h at 37 °C in the CO_2 incubator. The untreated controls with or without glutamic acid or kainic acid treatment received the same wash steps and media additions.

In one experiment, following the 15 min CPP incubation (5 or 10 µM), the media in wells was removed and wells washed once in 300 µl of BSS before the addition of Neurobasal/2 % B27-containing glutamic acid (100 µM/100 µl). Following this step, cultures were treated as described above. Untreated controls with or without glutamic acid exposure received the same wash steps and media additions. In addition, a post-glutamic acid exposure CPP treatment (5 µM) experiment was performed for the Arg-9 peptide and the JNKI-1D-TAT control peptide. In this experiment, neurons were exposed to glutamic acid (100 µM) in 100 µl Neurobasal/2 % B27 for 5 min as described above, after which time the media was removed and replaced with 50 µl Neurobasal/2 % N_2 supplement, followed by peptide (10 µM/50 µl in BSS) addition at 0 and 15-min post-glutamic acid exposure.

In Vitro Ischemia Model and Peptide Incubation

The in vitro ischemia model has previously been described (Meloni et al. 2011). Briefly, culture media was removed from wells (glass 96-well plate format) and washed with 315 µl of glucose free balanced salt solution (BSS; mM: 116 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 1 NaH_2PO_4; pH 6.9) before the addition of 60 µl BSS containing cell-penetrating or control peptides (see Table 1). A non-peptide positive control consisting of the glutamate receptor blockers (5 µM MK801/5 µM 6-cyano-7-nitroquinoxaline: MK801/CNQX) was also included. In vitro ischemia was initiated by placing wells in an anaerobic incubator (Don Whitely Scientific, England; atmosphere of 5 % CO_2, 10 % H_2, and 85 % argon, 98 % humidity) at 37 °C for 55 min. Upon removal from the anaerobic incubator, 60 µl of Neurobasal/2 % N_2 supplement was added to the wells and cultures incubated for a further 24 h at 37 °C in the CO_2 incubator. Control cultures received the same BSS wash procedures and media additions as ischemic treated cultures before incubation at 37 °C in the CO_2 incubator.

Neuronal Viability Assessment and Statistical Analysis

Twenty-four hours after insult neuronal cultures were examined by light microscopy for qualitative assessment of neuronal cell viability. Neuronal viability was quantitatively measured by 3-(4,5,dimethyliazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt (MTS) assay (Promega, Australia). The MTS assay measures the cellular conversion of the tetrazolium salt to a water-soluble brown formazan salt, which is detected spectrophotometrically at 490 nm. MTS absorbance data were converted to reflect proportional cell viability relative to both the untreated and treated controls, with the untreated control taken as 100 % viability, and presented as mean ± SEM. Viability data was analyzed by ANOVA, followed by post hoc Fischer's PLSD test, with $P < 0.05$ values considered statistically significant. Four to six wells were used in all assays with sister neuronal cultures and repeated a minimum of two to three times independently.

Results

Neuroprotection Following Glutamic Acid Exposure

The CPPs TAT-D, Arg-9, and penetratin provided significant neuroprotection in a dose–response manner (Fig. 1a; Table 2). Visual assessment of cultures post-injury also confirmed the neuroprotective effect, which ranged from ≈5 % for untreated glutamic acid exposed cultures to 100 % survival for Arg-9 treated cultures. Arg-9 was the most potent peptide with an IC50 value of 0.78 μM, followed by penetratin (IC50: 3.4 μM) and TAT-D (IC50: 13.9 μM). The Pep-1 peptide was ineffective. The glutamate receptors blockers and control peptides (JNKI-1D-TAT, PYC36L-TAT) were also highly effective in this model (Fig. 1a; Table 2). In addition, the TAT-D peptide displayed a similar level of neuroprotection as the TAT-L peptide (Fig. 1b). When CPPs were washed-out prior to glutamic acid exposure only Arg-9 displayed high level neuroprotection (Fig. 1c). Finally, the Arg-9 peptide was highly effective when added immediately after glutamic acid exposure, and mildly effective when added at 15-min post-insult. In contrast, the JNKI-1D-TAT peptide did not significantly increase neuronal survival when added immediately after, or at 15-min post-glutamic acid exposure (Fig. 1d).

Neuroprotection Following Kainic Acid Exposure

Following kainic acid exposure TAT-D, Arg-9 and penetratin were neuroprotective, but less effective than in the glutamic acid model, and did not always display a typical dose–response pattern (Fig. 2; Table 2). Pep-1 was ineffective. Arg-9 was the most potent peptide, increasing neuronal survival from ≈20 % to a maximum of ≈80 %. The respective IC50 values for Arg-9, penetratin, and TAT-D were 0.81, 2.0, and 6.2 μM. The glutamate receptors blockers, JNKI-1D-TAT, and PYC36L-TAT were also effective in this model (Fig. 2).

Table 2 IC50 values of cell-penetrating and control peptides for the three injury models

Peptide	IC50: glutamic acid model (μM)	IC50: kainic acid model (μM)	IC50: in vitro ischemia model (μM)
Arg-9	0.78	0.81	6.0
TAT-D	13.9	6.2	7.1
Penetratin	3.4	2.0	N/A
Pep-1	N/A	N/A	>15
PYC36L-TAT[a]	1.5	–	–
JNKI-1D-TAT[a]	2.1	6.5	–

N/A not applicable because peptides were either ineffective or increased cell death at higher doses. – data not available

[a] IC50 values for JNKI-1D-TAT and PYC36L-TAT peptides from Meade et al. (2010a, b)

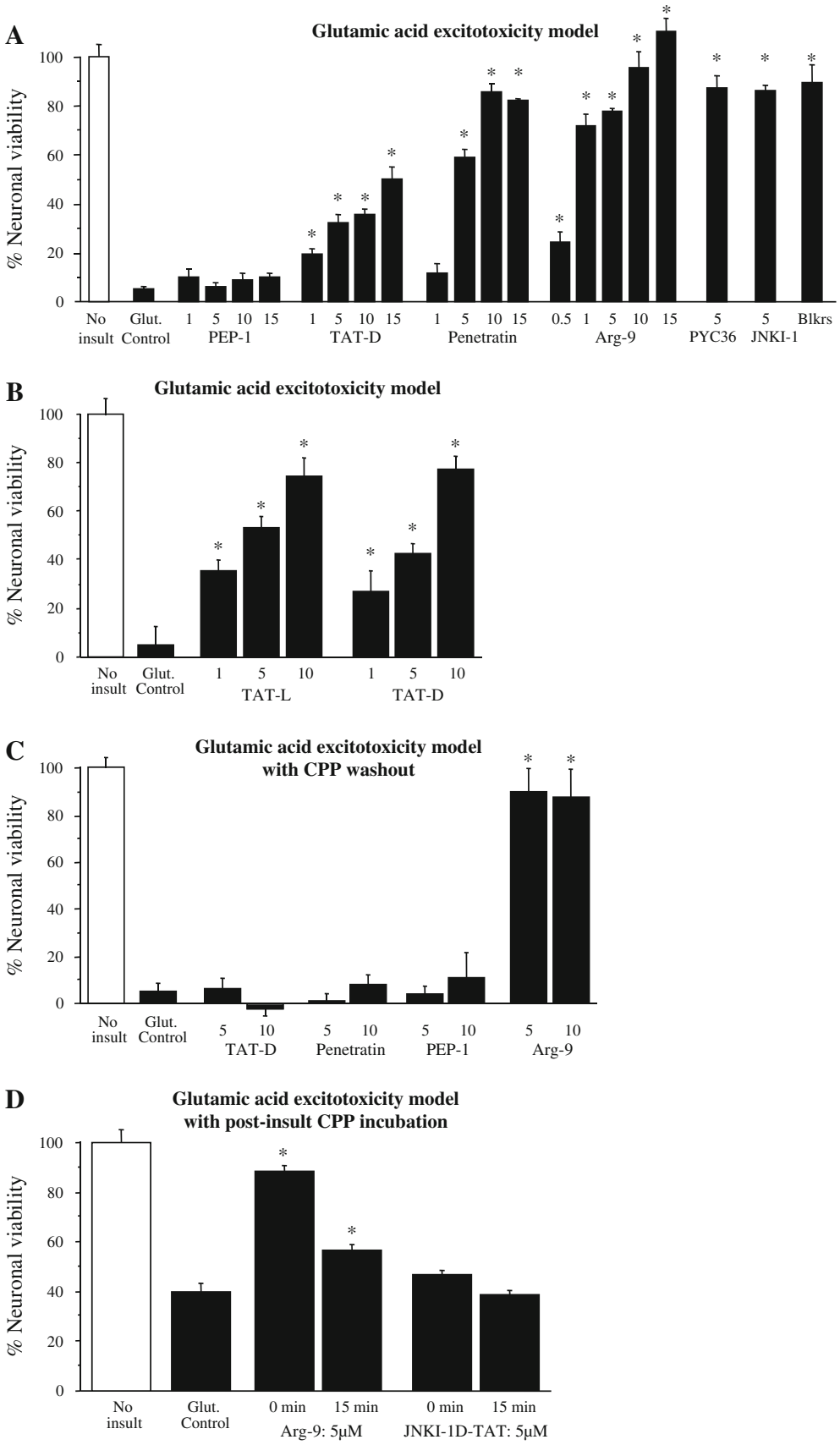
Fig. 1 Glutamic acid excitotoxicity model; concentration of peptide in μM. **a** Neuronal viability 24 h following glutamic acid exposure and treatment with CPPs, positive control peptides (JNKI-1D-TAT/PYC36L-TAT) and glutamate receptor blockers (Blkrs; 5 μM: MK801/5 μM: CNQX). **b** Neuronal viability 24 h following glutamic acid exposure and treatment with TAT-L and TAT-D peptides. **c** Neuronal viability 24 h following glutamic acid exposure when CPPs were washed-out prior to insult. **d** Neuronal viability 24 h following glutamic acid exposure when Arg-9 peptide and control peptide JNKI-1D-TAT were added 0 or 15-min post-insult; note in this experiment glutamic acid exposure resulted in less cell death in controls than in other experiments (60 vs. 95 %). MTS data were expressed as percentage neuronal viability with no insult control taken as 100 % viability (mean ± SEM; $n = 4$–6; $^*P < 0.05$) ▶

Neuroprotection Following In Vitro Ischemia

Following in vitro ischemia all four CPPs displayed neuroprotective effects (Fig. 3; Table 2). Neuroprotection with Arg-9 (IC50: 6.0 μM) and TAT-D (IC50: 7.1 μM) was similar, efficacy followed a dose–response pattern and increased neuronal survival from ≈10 to 40–50 %. Neuroprotective efficacy was lost with increasing concentrations of penetratin (≥5 μM), while Pep-1 was only neuroprotective at lower concentrations (1–5 μM). Glutamate receptors blockers and PYC36L-TAT were also effective in this model (Fig. 3).

Discussion

This study extends findings from our own and other laboratories reporting neuroprotective activity for the TAT peptide in excitotoxic and ischemic injury models (Xu et al. 2008; Vaslin et al. 2009; Meade et al. 2010a, b, Craig et al. 2011). In addition, for the first time we show that other CPPs exhibit neuroprotective properties in different in vitro injury models. The demonstration that the Arg-9 peptide was neuroprotective in the excitotoxic and ischemic injury models was not surprising due to its relatedness to TAT in terms of size (9 vs. 10 mer), arginine residues (9 vs. 6), structure, and transduction efficiency (Ho et al. 2001). However, what was surprising was the superior neuroprotective action of Arg-9, at least in the excitotoxic models; based on IC50 values Arg-9 was 17- and 7-fold more potent than TAT-D in glutamic acid and kainic acid models, respectively, and the only effective peptide when washed-out prior to glutamic acid exposure. This finding suggests the increased arginine residues and/or the slightly higher net charge (10 vs. 9 at pH 7) of Arg-9 are important factors for neuroprotection following excitotoxicity.


A
Glutamic acid excitotoxicity model
% Neuronal viability
100
80
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No insult
Glut. Control
1 5 10 15
PEP-1
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TAT-D
1 5 10 15
Penetratin
0.5 1 5 10 15
Arg-9
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PYC36
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Glutamic acid excitotoxicity model
% Neuronal viability
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Glut. Control
1 5 10
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Glutamic acid excitotoxicity model
with CPP washout
% Neuronal viability
No insult
Glut. Control
5 10
TAT-D
5 10
Penetratin
5 10
PEP-1
5 10
Arg-9
D
Glutamic acid excitotoxicity model
with post-insult CPP incubation
% Neuronal viability
No insult
Glut. Control
0 min
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Arg-9: 5μM
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15 min
JNKI-1D-TAT: 5μM

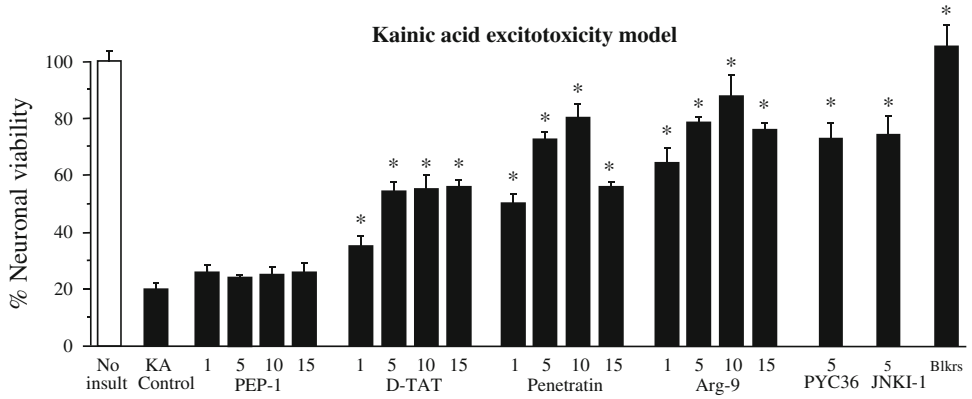

Fig. 2 Kainic acid excitotoxicity model; concentration of peptide in μM. Neuronal viability 24 h following kainic acid exposure and treatment with CPPs, positive control peptides (JNKI-1D-TAT/PYC36L-TAT) and glutamate receptor blockers (Blkrs; 5 μM: MK801/5 μM: CNQX). MTS data were expressed as percentage neuronal viability with no insult control taken as 100 % viability (mean ± SEM; $n = 4$; $^*P < 0.05$)

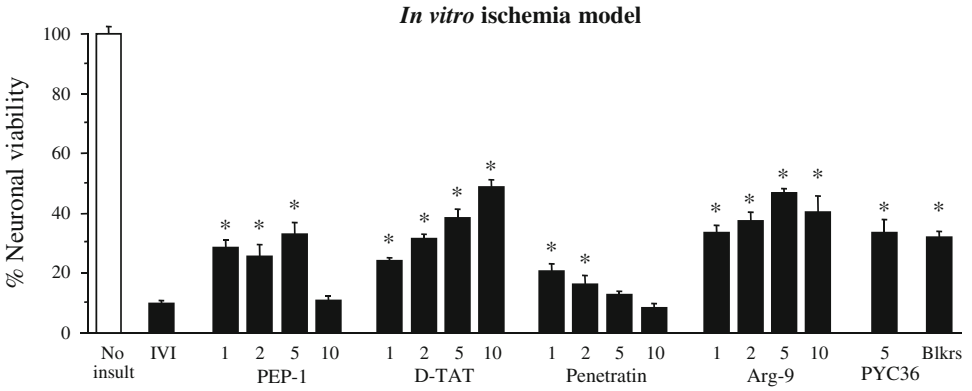

Fig. 3 In vitro ischemia model; concentration of peptide in μM. Neuronal viability 24 h following in vitro ischemia and treatment with CPPs, positive control peptide (PYC36L-TAT) and glutamate receptor blockers (Blkrs; 5 μM: MK801/5 μM: CNQX). MTS data were expressed as percentage neuronal viability with no insult control taken as 100 % viability (mean ± SEM; $n = 4$; $^*P < 0.05$)

Furthermore, while the exact reason for the loss of efficacy of TAT and penetratin, but not Arg-9 following wash-out prior to glutamic acid exposure is unknown, it may relate to the speed of Arg-9 intracellular up-take, rather than some extracellular mechanism. This is supported by the finding that Arg-9 was effective when added after glutamic acid exposure, while the JNKI-1D-TAT peptide was ineffective; the JNKI-1D-TAT result is in line with our pervious study (Meade et al. 2010b).

As eluded above, a novel result in the study was the demonstration that penetratin and Pep-1 also exhibited neuroprotective properties. The penetratin and Pep-1 peptides bear no amino-acid sequence relatedness to each other, or the TAT/Arg-9 peptides. Interestingly penetratin was highly neuroprotective in the excitotoxic models (IC50s: 3.4 and 2 μM), but less effective in the in vitro ischemia model, with increasing concentrations reducing efficacy. The Pep-1 peptide was generally ineffective in the excitotoxic models and in some experiments appeared to increase neuronal death (data not shown), but was neuroprotective following in vitro ischemia at lower concentrations. Interestingly, when penetratin was washed-out from neuronal cultures prior to glutamic acid exposure, visual observations revealed that the peptide did display some early neuroprotective effects (data not shown). Hence, both penetratin and Pep-1 behaved differently to each other and the TAT/Arg-9 peptides in the injury models.

At present the reason for the differential neuroprotective responses for the four CPPs in the excitotoxic and ischemic injury models is unknown, but is likely to be related to the peptides physical–chemical properties. Furthermore, it is unknown if the neuroprotective action of the CPPs is mediated at the cell membrane (e.g., receptors, ion channels) or intracellular level (e.g., proteins, mitochondria) or both. Xu et al. (2008) have suggested that TAT may alter the cell membrane and thereby affect the function of cell surface receptors, such as the NMDA receptor, resulting in reduced calcium influx. However, data from our (Meade

et al. 2010b) and another laboratory (Aarts et al. 2002) using TAT fused peptides (e.g., PYC36D-TAT, NR2B-AA-TAT), show that these peptides do not reduce calcium influx following glutamic acid or NMDA exposure, respectively, suggesting other mechanisms may also be operating. This is supported by another study, which showed that the larger $TAT_{31–61}$ peptide increased neuronal intracellular calcium when added to cultures (Nath et al. 1996). An alternative hypothesis is that the CPPs interact and stabilize the outer mitochondrial membrane and thereby help to preserve mitochondrial function. Potential benefits are maintenance of ATP synthesis, reduced reactive oxygen species production, and improved calcium handling. To this end, we have observed that Arg-9 can increase MTS absorbance levels above baseline levels in normal neurons and following injury (e.g., Fig. 1a, 15 μM). Since reduction of MTS to its formazan product primarily occurs in mitochondria, the ability of Arg-9 to increase formazan levels is supportive that the peptide is improving mitochondrial function. Another potential mechanism especially in relation to Arg-9, is that this poly-arginine peptide is inhibiting the calcium-dependent pro-protein convertase enzyme furin (Kacprzak et al. 2004), and thereby blocking activation of potentially damaging proteins. It is also possible that Arg-9 is acting as a NMDA receptor antagonist, as it has been reported that arginine rich hexapeptides selectively block this receptor channel (Ferrer-Montiel et al. 1998).

With respect to CPP intracellular entry, the predominant mechanism is considered to be by endocytosis (macropinocytosis) (Palm-Apergi et al. 2012). Although less relevant to the present study, a recent report has demonstrated that cargo properties may also promote a direct cell entry mechanism by certain CPPs (Hirose et al. 2012). However, what is potentially highly relevant is how specific cargos, peptide, or otherwise, may affect CPPs by enhancing their neuroprotective action, improving translocation efficiency and/or as demonstrated by Cardozo et al. (2007) increasing their toxicity. This is especially important when the cargo itself is neuroprotective, because this makes discerning the neuroprotective effect between the CPP and the cargo very difficult. For example, in one of our previous studies (Meade et al. 2010a), the addition of three amino acid residues (Pro, Lys, Ile) from the PYC36 peptid to the TAT-D peptide (AM8D-TAT) resulted in IC50 values decreasing from >15 μM for TAT-D to 1.1 μM for AM8D-TAT in the glutamic acid model. At present, we do not know if the improved efficacy of the AM8D-TAT peptide was due to enhanced neuroprotection of TAT and/or the inhibitory action of the three PYC36 amino acids on its target protein, AP-1.

It also needs to be asked in the studies that have included TAT as a control in neuroprotection experiments, why a positive effect with the TAT peptide control has not always been observed. We believe there are a number of possible explanations, and that in order to address this question, it is first necessary to differentiate between studies using the TAT peptide only (i.e., GRKKRRQRRRG), versus studies using TAT fused to a reporter protein (e.g., GFP, β-gal) or peptide (e.g., HA and/or 6X HIS tag, scrambled peptide) as a control. With respect to the studies that have used the TAT only peptide as a control, it is possible TAT was ineffective at the dose used and/or the injury model was too severe to uncover a neuroprotective effect. For example, Borsello et al. (2003) did not detect a neuroprotective effect with the TAT peptide following a 12, 24, or 48 h exposure of cortical neuronal cultures to 100 μM NMDA. In contrast the L-JNKI-1 peptide was effective at 12 and 24 h, while the protease resistant D-JNKI-1 peptide was effective at all time-points. Given, the superior efficacy of the JNKI-1 peptides compared to the TAT peptide, it is possible that at the concentration tested, TAT was not neuroprotective or that any neuroprotective effects were overridden due to NMDA insult severity. Interestingly, in a study by Ashpole and Hudmon (2011) a modest protective effect with the TAT peptide was observed in cortical neuronal cultures following glutamic acid exposure. Furthermore, the authors concluded that since the TAT peptide provided little protection, the neuroprotection observed for their CAMKII inhibitory peptide was not due to the "import sequence" (i.e., TAT). However, it cannot be ruled out that the CAMKII inhibitory peptide increased the potency of the TAT peptide. Lastly, it is possible that the TAT peptide is only neuroprotective in specific injury models and cell types.

In studies using TAT fused to a reporter protein or control peptide, in addition to the points raised above, it is also likely that the control protein/peptide may act to dampen or nullify the TAT peptide's neuroprotective properties. Based on the many studies that have used TAT-fused proteins/peptides as controls and showed no neuroprotective effects, this would appear to be the case (e.g., Kilic et al. 2003; Doeppner et al. 2009).

In conclusion, our findings show that TAT-like peptides and other non-related CPPs possess intrinsic neuroprotective properties. The extent to how widespread other known CPPs also have neuroprotective qualities is currently unknown, however we have recently isolated a diverse set of CPPs that also displayed neuroprotective properties (unpublished data). Together, our findings raise a number of important issues: (i) they highlight the potential advantages of using CPPs to deliver neuroprotective cargos to the CNS; (ii) raise the possibility that CPPs themselves could be developed into neuroprotective drugs; (iii) pose the question of the contribution of the CPP versus cargo in the neuroprotective effect

observed in previous studies; and (iv) do CPPs possess the same range of neuroprotective properties when used in vivo. With respect to the last point, there is evidence that this may be the case (Vaslin et al. 2009). Finally, it will be essential for future studies using CPPs in neuroprotection experiments to adequately address the issues raised above.

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Conflict of interest Paul Watt is the Chief Executive Officer and Richard Hopkins is the Chief Operating Officer for Phylogica Ltd Pty. Nadia Milech is a Senior Scientist working for Phylogica. Bruno Meloni is a Phylogica shareholder. The other authors declare no conflict of interest.

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