

# Membrane translocation of penetratin and its derivatives in different cell lines

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The third helix of the homeodomain of the Antennapedia homeoprotein can translocate through the cell membrane into the nucleus and can be used as an intracellular vehicle for the delivery of oligopeptides and oligonucleotides. A 16-amino acid-long peptide fragment, called penetratin, is internalized by the cells in a specific, non-receptor-mediated manner. For a better understanding of the mechanism of the transfer, penetratin and two analogs were synthesized:

- |                            |                  |              |
|----------------------------|------------------|--------------|
| (1) penetratin             | RQIKIWFQNRRMKWKK | (peptide 1); |
| (2) (6,14-Phe)-penetratin, | RQIKIFFQNRRMKFKK | (peptide 2); |
| (3) dodecapeptide,         | RQIKIWF-R-KWKK   | (peptide 3); |

The conformation of penetratin peptides 1–3 was examined in both extracellular matrix-mimetic and membrane-mimetic environments. <sup>1</sup>H-NMR and CD spectroscopic measurements were performed in mixtures of TFE/water with different ratios. Peptides 1–3 were labeled by reacting their N-terminal free amino group with fluorescein isothiocyanate (FITC). Membrane translocation of the labelled peptides was studied with cell cultures [WEHI 164 murine fibrosarcoma cells (WC/1); chicken fibroblast cells (CEC-32); chicken monocytic cells (HD-11); human fibroblast (SV 80) and human monocytic cells (MonoMac-6)]. Confocal laser scanning microscopy and flow cytometry assay were used to study membrane translocation. Amphiphilicity was calculated for each peptide. In our experiments all the penetratin peptides penetrated into the cells. Helical conformation and membrane translocation ability showed little correlation: substitution of the two Trp with Phe increased the stability of helical conformation but decreased membrane translocation activity. The results of fluorescence microscopy and flow cytometry show that penetratin can be translocated into the cells by two mechanisms: endocytosis and direct transport through the cell membrane. Copyright © 2003 John Wiley & Sons, Ltd.

**Keywords:** cell penetrating peptides; penetratin; NMR; CD; conformation; FITC-labeling; translocation mechanism; amphiphilicity

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## INTRODUCTION

Many therapeutic agents (drugs, oligonucleotides, polypeptides) act at intracellular sites. Their biological efficacy depends on an efficient delivery into the target cell. Charged and large compounds cannot penetrate through lipid membranes and require active transport mechanisms for uptake into cells. A series of delivery systems (viral vectors, cationic lipids, liposomes, controlled-release polymers) has been used for translocation of hydrophilic therapeutic agents through the membranes.

Cell penetrating peptides (CPPs) are one of the most widely used delivery systems. This group of compounds is very heterogeneous and has only one common denominator: these peptide vectors translocate through the cell membrane and can transport cargo molecules, covalently bound, into

the cell. The simplest peptides are short arginine-rich sequences (Tat 49–57: Vives *et al.*, 1997) and oligoarginines (Rothbard *et al.*, 2000; Futaki *et al.*, 2001; Suzuki *et al.*, 2002) that exhibit efficient intracellular import properties. Peptidomimetics such as oligoguanidine peptide derivatives (Wender *et al.*, 2000) are also effective CPPs. Hydrophobic sequences can also serve as import signals for crossing the membrane (Lin *et al.*, 1995; Zhang *et al.*, 1998; Morris *et al.*, 1999; Guiochon-Mantel *et al.*, 1994). Branched peptides with defined structure (lologomers) are non-natural peptide-based vehicles for drug delivery (Gariépy and Kawamura, 2001; Sheldon *et al.*, 1995).

Amphipatic peptides with cationic character form the most interesting group of CPPs. Transportan, a hybrid peptide of galanin and mastoparan (Pooga *et al.*, 2001) has been widely used as a drug delivery vector. Synthetic amphipatic model peptides like KLALKLALKALK-AALKLA proved to have good membrane translocation properties (Oehlke *et al.*, 1997, 1998; Scheller *et al.*, 1999). Recent advances in the field of cell penetrating peptides have been reviewed (Lindgren *et al.*, 2000; Plank *et al.*, 1998; Bogoyevitch *et al.*, 2002; Garcia-Chaumont

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**Abbreviations used:** CPP, cell penetrating peptide; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; FTC, fluorescein-thiocarbamoyl.

*et al.*, 2000; Gariépy and Kawamura, 2001; Morris *et al.*, 1999; Singh *et al.*, 1999; Fischer *et al.*, 2001; Langel, 2002).

Penetratin, a synthetic 16-amino acid peptide from the third helix of Antennapedia homeodomain (Derossi *et al.*, 1994, 1996, 1998; Fischer *et al.*, 2000) is a cationic, amphipatic peptide and might penetrate cell membrane via a postulated 'inverted micelle' pathway. However, the mechanism of membrane translocation is not well known. The main question is whether the peptide is internalized via endocytosis which is energy-dependent, or via direct transport? While the latter mechanism is largely unknown at present, it is believed to be a non-receptor-mediated process (Drin *et al.*, 2001; Derossi *et al.*, 1996).

Penetratin has been proposed as a universal intracellular delivery vehicle (Dupont *et al.*, 2002). A comprehensive analysis led to the conclusion that all three aromatic residues, two Trp (6 and 14) and one Phe (7) play crucial roles in the penetration process (Derossi *et al.*, 1994). A penetratin analogue containing only D-amino acids (D-penetratin) proved also to be an efficient vector (Rousselle *et al.*, 2000).

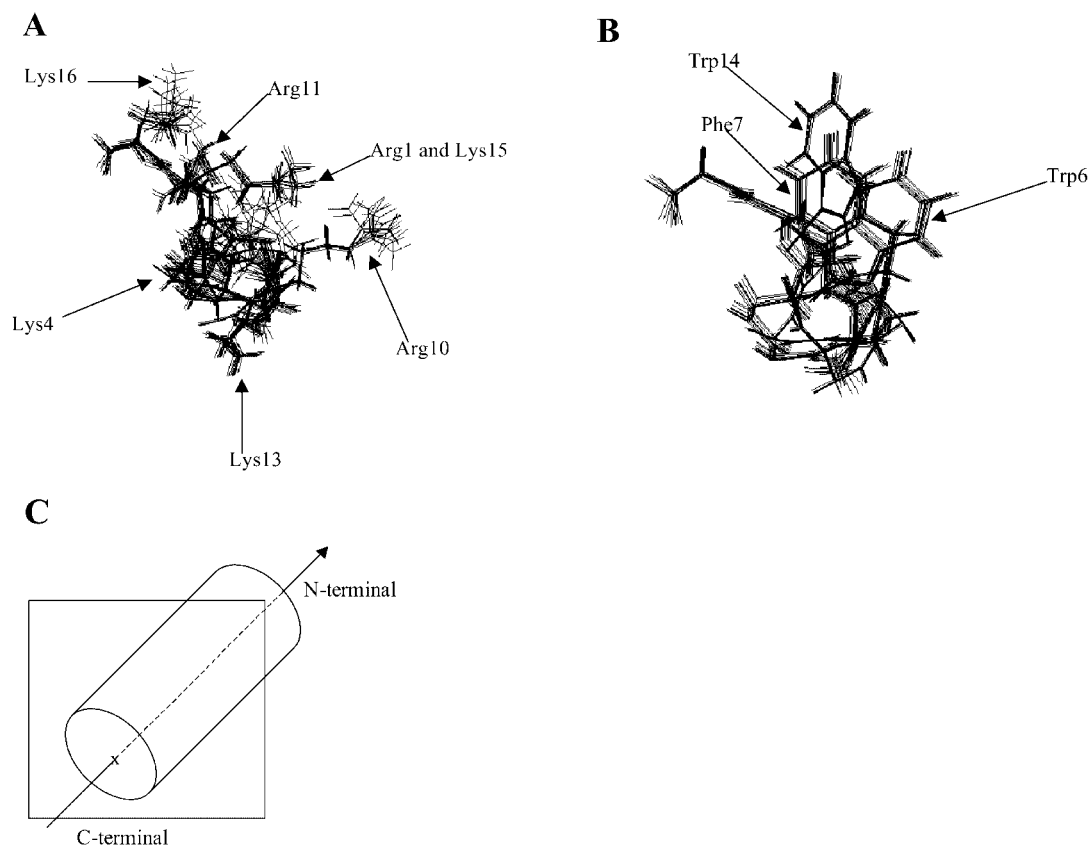
In order to examine the correlation between chemical structure and membrane translocation activity, penetratin and two analogs with the following structures were synthesized:

- |                    |                  |              |
|--------------------|------------------|--------------|
| (1) penetratin     | RQIKIWFQNRMRKWKK | (peptide 1); |
| (2) (6,14-Phe)     | RQIKIFFQNRMRKFKK | (peptide 2); |
| -penetratin,       |                  |              |
| (3) dodecapeptide, | RQIKIWF-R-KWKK   | (peptide 3); |

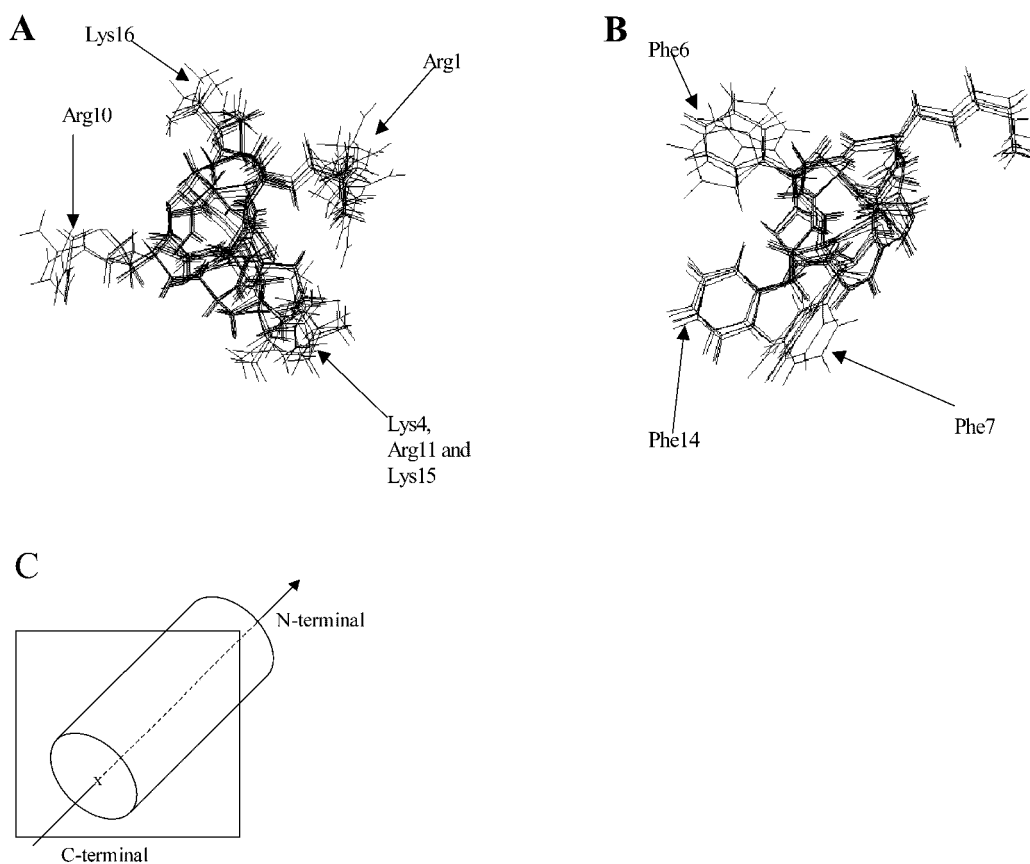
In peptide 2 the Trp-residues were replaced by Phe for investigating the effect of different aromatic side chains on the membrane translocation of the peptide. A third, shorter, biologically effective peptide (peptide 3) was also designed, in which two pairs of residues (Q<sup>8</sup>-N<sup>9</sup> and R<sup>11</sup>-M<sup>12</sup>) were simultaneously removed from the central turn of the helix. The consideration, that a peptide-based delivery vector should be as short as possible in order to reduce cost, guided the synthesis of this dodecapeptide. The conformation of the peptides was studied by <sup>1</sup>H-NMR and CD spectroscopy. Bioassay was performed using different cell lines. FITC-labeled derivatives of the three penetratin peptides were also synthesized for studying the membrane translocation using confocal microscopy.

## NMR AND CD-STUDIES OF PENETRATIN PEPTIDES

NMR spectra were typically recorded at 500 MHz (Bruker Instruments) and were processed with the Insight II-Felix97 software package. Structure determination was performed in TFE d<sub>2</sub>/water = 9:1. Homonuclear COSY and TOCSY spectra were used for identification of the spin systems. An atomic level comprehensive analysis of penetratin and its two analogues was performed (Czajlik *et al.*, 2002). Increase of the polarity of the molecular environment



**Figure 1.** The best 10 structures of penetratin from the plane of the helix, superimposed on the heavy atoms of residues 1–16 in TFE–water 9:1 system. The packing of basic side chains (A) and the aromatic side chains (B); the approximate orientation of the helical cylinder (C).



**Figure 2.** The best structures (6,14-Phe)-penetratin from the plane of the helix, superimposed on the heavy atoms of residues 1–16. The packing of basic side chains (A) is on the left and the aromatic side chains (B) is on the right; the approximate orientation of the helical cylinder (C).

was performed up to highly aqueous system (TFE  $d_2$ /water = 1:9).

CD spectra were recorded on a Jobin Yvon micrograph Mark IV. Typically, three scans were acquired with a cell path length of 0.02 cm, between 185 and 280 nm. CD data were analysed via the recently updated version of Convex Constraint Analysis Plus (CCA+).

### Peptide structure in highly aqueous medium

CD results have shown that penetratin has a considerable population of non-random structures ( $\sim 40\%$ ), even in aqueous solution. The NOESY spectra of the penetratin indicate a threshold population with ordered structures. Locally ordered structural segments are present in the molecule and short-range order can be traced: the peptide assumes a nascent helical conformation (Dyson *et al.*, 1988).

The penetratin analogues (peptides 2 and 3) exhibited multiple structures unresolved at the NMR timescale. This conclusion is supported by CD-data.

### Peptide structure in a low dielectric environment

The conformation of penetratin involves a helix-like structure in the 4–12 region, which begins and ends with a  $\beta$ -turn.

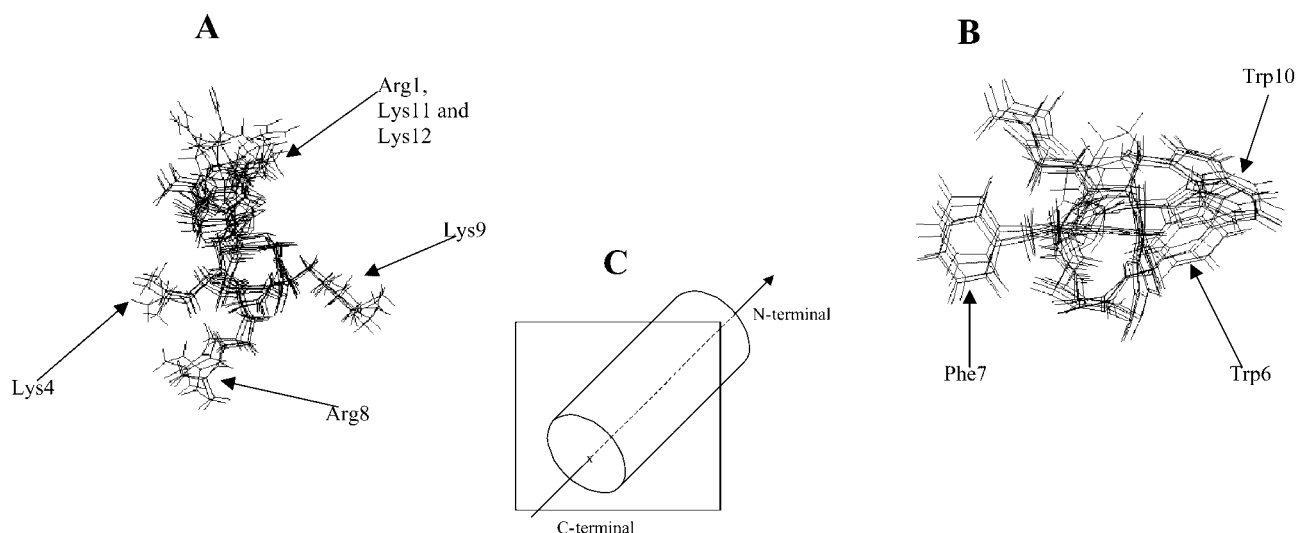
The aromatic rings of the Trp and Phe in positions 6 and 7 are close to each other (Fig. 1). The aromatic side chains, together with the two Ile in positions 3 and 5, form a hydrophobic core. The molecule is stabilized by interactions between the hydrophobic amino acids and by multiple hydrogen bonds. Penetratin can only be described in a hydrophobic environment by one well-defined conformation corresponding to a bent helical structure in the 4–12 region. Our data show that penetratin is a non-ideal helix which looks like a  $3_{10}$  helix with  $\beta$ -turns at the two ends.

The (6,14-Phe)-penetratin (peptide 2) adopts a helical structure, interrupted by a  $\beta$ -turn (Fig. 2). Both the 3–9 and 12–14 regions display helical properties on the basis of dihedral angles.

The dodecapeptide (peptide 3) has a helical conformation only in the central region including residues 5–9 (Fig. 3). The peptide does not form a hydrophobic core. Since this peptide is very short, the molecule cannot be described by a single well-determined structure, and is characterized by several somewhat similar conformers.

### Structure of penetratin peptides based on CD-spectra

CD-spectroscopy is sensitive to small conformational changes in molecules. For the three penetratin peptides, a



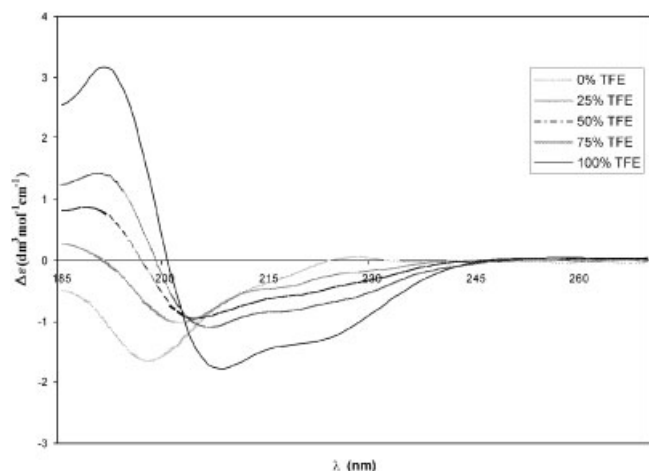
**Figure 3.** The best 10 structures of penetratin dodecapeptide from the plane of the helix, superimposed on the heavy atoms of residues 1–12. The packing of basic side chains (A) is on the left and the aromatic side chains (B) is on the right; the approximate orientation of the helical cylinder (C) is in the middle.

total of 21 CD-curves were acquired at TFE percentages of 0, 10, 25, 50, 75, 90, 94 and 100%. One representative CD-spectrum of penetratin is shown in Fig. 4.

For all three peptide models, an increase of the TFE concentration in water, clearly increases the helical and/or  $\beta$ -turn content and decreases the atypical or random part of the backbone conformation. In pure TFE, the structures of both penetratin and peptide 3 (dodecapeptide) appear to be assemblies of type I and III  $\beta$ -turns, while the conformation of (6,14-Phe) penetratin is more like that of an  $\alpha$ -helix. The backbone structure of penetratin peptides is shown in Fig. 5.

### Study of penetration of penetratin-peptides into different cell types

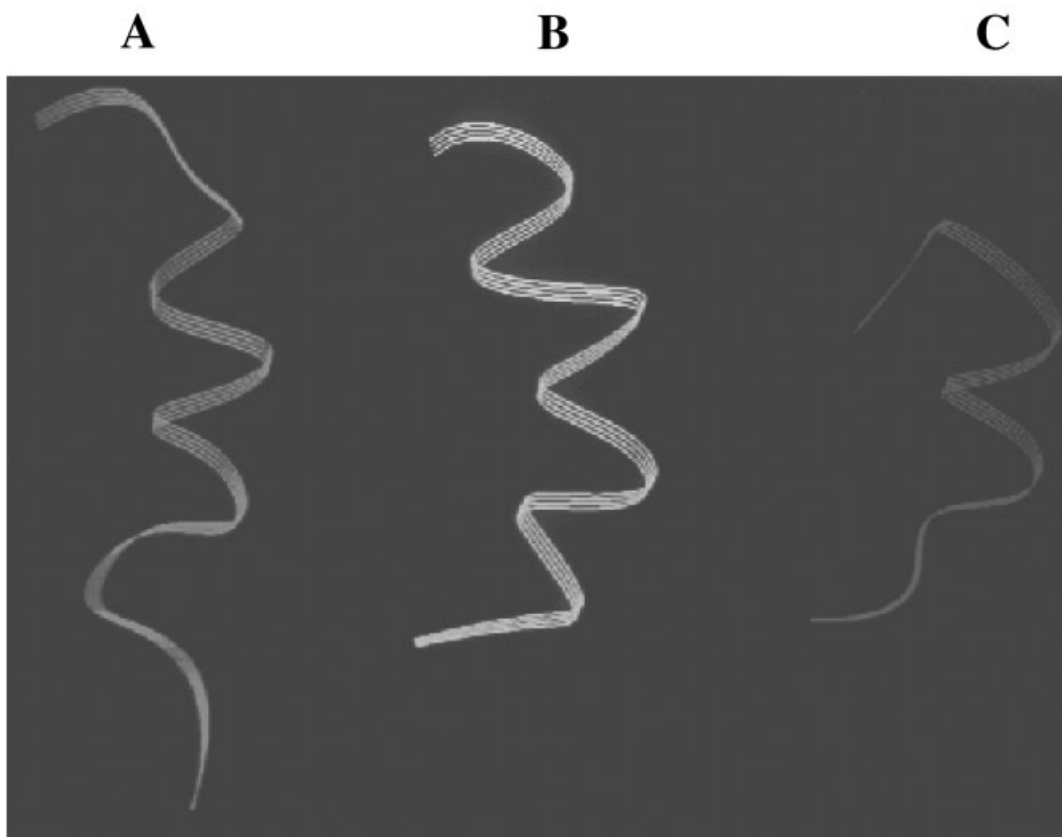
Penetratin and the two model peptides were fluorescently labeled for studying their penetration into cells. FITC was used for labeling the N-terminal amino group of the peptide



**Figure 4.** CD spectra of penetratin recorded in different TFE-water systems.

resulting in fluorescein-thiocarbamoyl (FTC) peptide derivatives. Membrane translocation of FTC-penetratins was studied by confocal fluorescence microscopy on living cells using the following cell lines: chicken monocytic cells (HD-11), human fibroblasts (SV-80), HeLa cells and L929 murine fibroblast cells. For quantitative measurement of peptide translocation fluorescence activated cell sorter (FACS) measurements were used on L-929, SV-80, human monocytic (MonoMac 6), chicken fibroblast (CEC-32) and WEHI 164 murine fibrosarcoma (WC/1) cells. Temperature dependence of the uptake kinetics of FTC-penetratin was also studied on SV 80 cells at 37, 20 and 4°C using FTC-pentaglycine as negative control. The following results were obtained:

- (1) FTC-penetratin enters the cell in a relatively short time (30–60 min) and will be concentrated in the cell nucleus in 90 min (Fig. 6).
- (2) The membrane translocation is temperature-dependent. Using SV-80 cells, FTC-penetratin shows practically no translocation activity at 4°C. At 20°C penetration is a very slow process. FTC-pentaglycine not can penetrate into SV-80 cells. At 37°C, FTC-penetratin translocates rapidly into the cells and concentration in the cells is time-dependent (Fig. 7). Lack of peptide penetration at low temperature is interpreted in the literature as proof of an active, energy-dependent transport mechanism. We believe that this interpretation is incorrect since at 0 or 4°C mammalian cell membranes are rigid corresponding to a frozen state! This allows no direct translocation via physicochemical interaction between peptide vectors and membranes. Therefore the lack of peptide translocation at 0 or 4°C in mammalian cell cultures cannot be taken as evidence for an energy-dependent transport mechanism.
- (3) Cell membrane fluidity affects the translocational activity of penetratin (Fig. 8). Cell lines with different membrane fluidity were incubated with FTC-penetratin and FTC-pentaglycine. Translocation was measured at

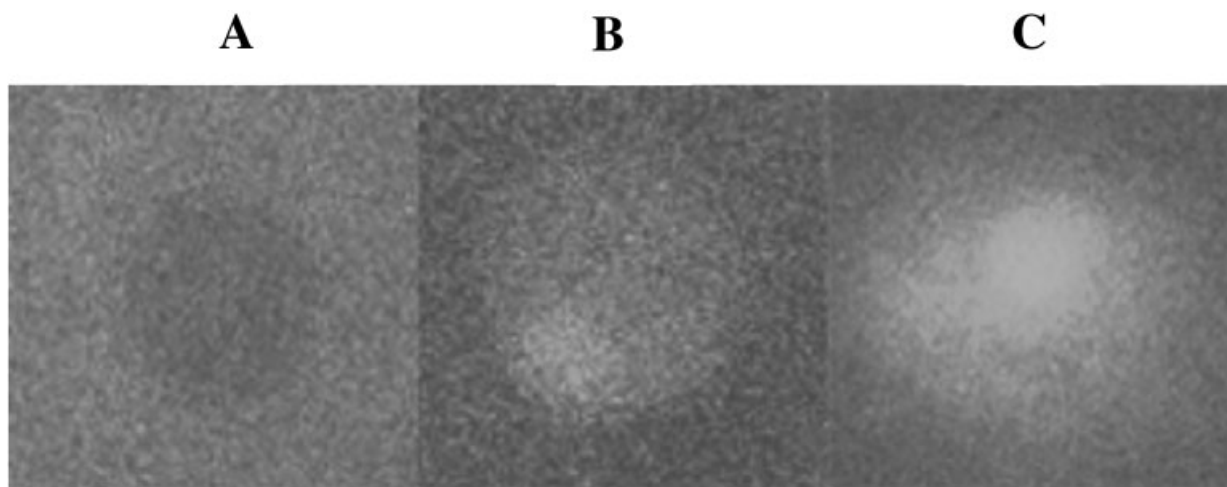


**Figure 5.** The backbone structure of penetratin (A), (6,14-Phe)-penetratin (B), and the shorter dodecapeptide (C).

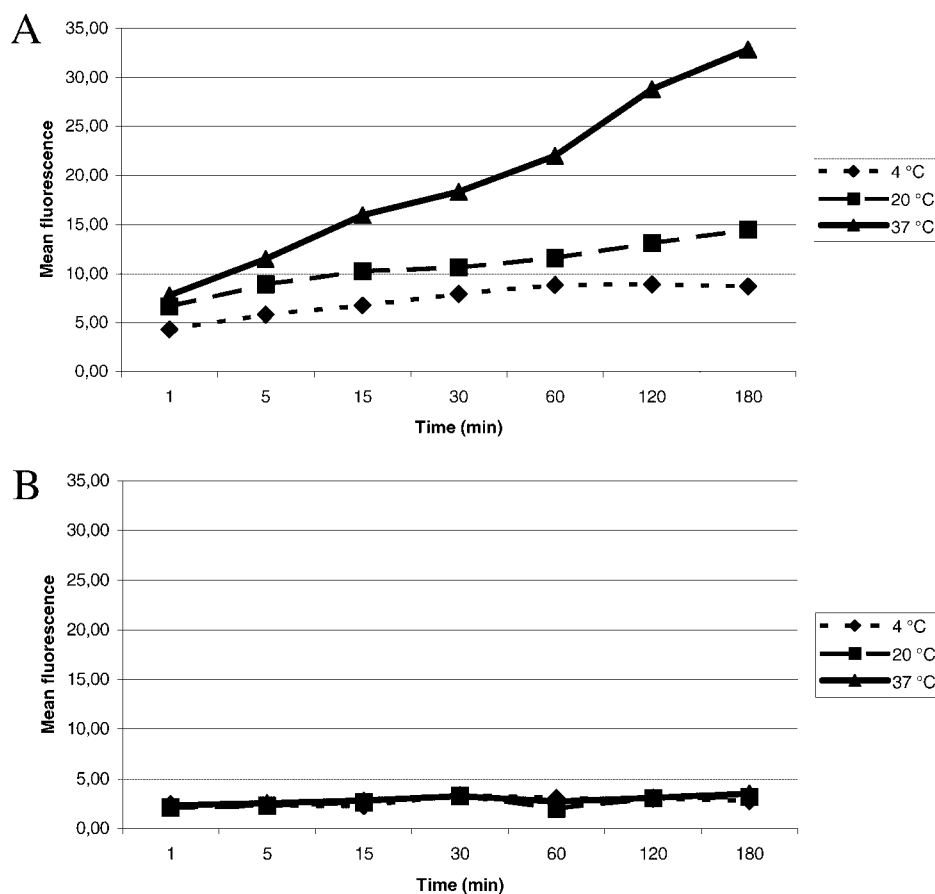
37°C after 60 min. One of the cell types used (WC/1 cells) possesses a gene which gives the cell membrane a high fluidity. Figure 8 shows that penetratin translocates very rapidly and effectively also in these cells. High membrane fluidity increases peptide translocation.

- (4) Peptide structure strongly influences the translocation activity. Figure 9 shows the peptide uptake of different cell lines at 37 and 0°C, using quantitative flow cytometry

measurements. FTC-pentaglycine shows practically no translocation into any cells at any temperature. The (6,14-Phe) penetratin analog enters the different cell types very slowly. Interestingly, the short penetratin dodecapeptide is almost as active in membrane translocation as the mother compound. High membrane fluidity enhances penetration of all the peptides. The most interesting feature is the behaviour of MonoMac cells that



**Figure 6.** Peptide visualization by confocal microscopy. HeLa cells were incubated with FTC-penetratin for 30 (A), 60 (B) and 90 (C) min at 20°C.

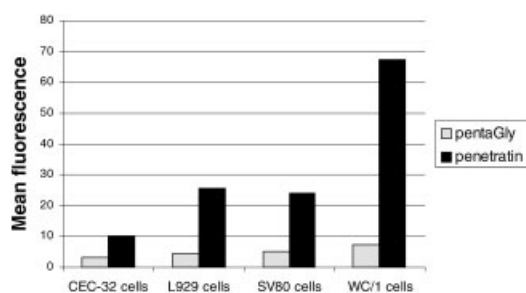


**Figure 7.** Temperature dependence of the uptake kinetics of FTC-penetratin. SV80 cells were incubated at 37, 20 and 4°C, respectively, with FTC-penetratin (A) and FTC-pentaglycine (B) at a concentration of 1  $\mu$ M for various periods of time (1, 5, 15, 30, 60, 120 and 180 min).

possess high endocytotic capacity: these cells show the highest cellular uptake of penetratin and its analogues.

## DISCUSSION AND CONCLUSION

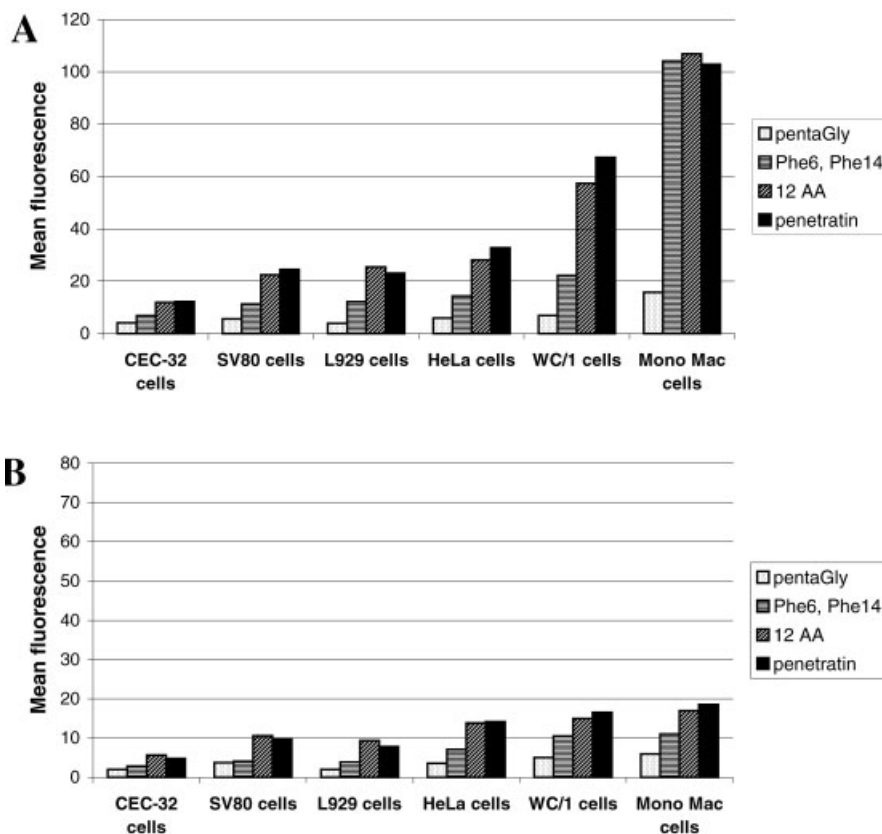
Internalization of peptide vectors has been investigated by a chemical approach. A well-known hypothesis states that tilted peptides can destabilize membranes (Brasseur, 2000). Conformational and topological requirements of cell-permeable peptide functions have been studied (Burton



**Figure 8.** The effect of membrane fluidity on the translocational activity of penetratin. CEC-32, L929, SV80 and WC/1 cells were incubated with 1  $\mu$ M of FTC-penetratin and pentaglycine at 37°C for 60 min.

*et al.*, 1996; Du *et al.*, 1998). Cargo delivery kinetics of CPPs were also measured (Hällbrink *et al.*, 2001). Peptide-lipid interactions were studied (Magzoub *et al.*, 2001) also by penetratin (Persson *et al.*, 2001). Translocation of penetratin through artificial lipid bilayers was directly observed (Thorén *et al.*, 2000) and the position of the peptide in SDS micelles was determined by NMR (Lindberg and Gräslund, 2001). Conformational and associative behaviors of penetratin in membrane-mimetic environments (Berlose *et al.*, 1996) led to the hypothesis that penetratin forms inverted micelles in which the peptide is trapped (Dupont *et al.*, 2002). In this model, penetratin is always kept in hydrophilic environment, included in the cavity of the micelle. Peptide helicity seems to be not important for the translocation (Derossi *et al.*, 1996). This hypothetical mechanism favors a direct transport of penetratin which is fully receptor independent. Recently the mechanism of penetratin uptake has been re-evaluated and this led to the conclusion that penetratin translocates primarily by an energy-dependent endocytotic pathway (Richard *et al.*, 2003).

Our results demonstrate that peptide helicity plays no decisive role in penetratin translocation and that the amphiphilicity of the peptide is much more important. Our working hypothesis is that penetratin peptides interact with the cell membrane presumably with electrostatic forces



**Figure 9.** Comparative flow cytometric assay of the cellular uptake of penetratin and its analogs. Various cell-lines (CEC-32, SV80, L929, HeLa, WC/1 and MonoMac) were incubated at 37°C (A) and 4°C (B) with FITC-labeled penetratin and its analogs at a concentration of 1  $\mu$ M for 60 min. FITC-pentaglycine, a membrane-impermeable peptide, was used as a negative control.

between positively charged Arg/Lys and negatively charged phospholipids. Another important interaction includes the apolar interaction between hydrophobic side chains and the apolar part of the lipid bilayer. Tryptophans play a crucial role in this interaction and are poorly replaceable by phenylalanine. A conformational change ( $\alpha$  helix  $\rightarrow$   $\beta$ -structure  $\rightarrow$   $\alpha$  helix) occurs during membrane translocation. In addition to this direct translocation mechanism, the

experiments with MonoMac cells demonstrated that penetratin enters the cell also via an endocytotic pathway.

### Acknowledgements

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