Research Paper

A modified Tat peptide for selective intracellular delivery of macromolecules

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Abstract

Objectives The Tat peptide has been widely used for the intracellular delivery of macromolecules. The aim of this study was to modify the peptide to enable regulation of cellular uptake through a dependency on activation by proteases present in the local environment.

Methods The native Tat peptide sequence was altered to inhibit the initial interaction of the peptide with the cell membrane through the addition of the consensus sequence for urokinase plasminogen activator (uPA). uPA expression was characterised and semi-quantitatively rated in three cell lines (U251mg, MDA-MB-231 and HeLa). The modified peptide was incubated with both recombinant enzyme and with cells varying in uPA activity. Cellular uptake of the modified Tat peptide line was compared with that of the native peptide and rated according to uPA activity measured in each cell line.

Key findings uPA activity was observed to be high in U251mg and MDA-MB-231 and low in HeLa. In MDA-MB-231 and HeLa, uptake of the modified peptide correlated with the level of uPA expression detected (93 and 52%, respectively). In U251mg, however, the uptake of the modified peptide was much less (19% observed reduction) than the native peptide despite a high level of uPA activity detected.

Conclusions Proteolytic activation represents an interesting strategy for the targeted delivery of macromolecules using peptide-based carriers and holds significant potential for further exploitation.

Keywords drug targeting; macromolecular delivery; protease activation; proteolytic activation; Tat peptide

Introduction

Cell penetrating peptides (CPPs) have been considered as potential carriers for therapeutics that would otherwise be expected to display poor activity against intracellular targets.[1,2] The Tat peptide is one of the most widely studied CPPs and has been used as a carrier for the delivery of a wide range of substrates into cells, for example proteins, peptides, DNA and oligonucleotides.[3,4] Uptake of the Tat peptide is greatly reduced by substitution of positively charged residues within amino acids 49–57 of the original protein sequence, for example alanine substitution of positively charged residues reduced uptake of the new peptide by 70–90%.[5] It has since been shown that the positive charges help the Tat peptide bind avidly to the cell membrane before uptake.[6–8] This initial interaction is necessary for the peptide to cross the cell membrane and could potentially be interfered with if the charge interactions were perturbed.[9] Following the initial electrostatic interaction, the subsequent method of peptide internalisation is still a matter of some debate.[6,7,9–11]

A method to discriminate between healthy cells and tumour cells, and selectively target the latter in therapy, remains one of the main objectives of current cancer chemotherapy. Many solid tumours are characterised by unusual or up-regulated proteolytic activity in the area around the tumour to allow degradation of the extracellular matrix and invasion of the surrounding tissue.[12–14] As well as their known role in the degradation of the extracellular matrix, new roles for specific proteases are being uncovered in the earlier stages of tumourigenesis.[14,15] The idea of using tumour-associated enzyme activity as a strategy for unmasking pro-drugs at the tumour site has been investigated for carboxypeptidase G2,[16] β-glucuronidase[17] and plasmin,[18] among others.

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For CPPs, cellular uptake should be blocked by interfering with their normal membrane association mechanism.[5,8,9] Incorporation of peptide recognition motifs for tumour-associated proteases into the delivery construct may help prevent the appropriate interaction between the peptide and the membrane. In the absence of the necessary proteolytic activity, the additional peptide motif prevents the normal association of the CPP with the membrane and therefore limits uptake of the CPP–drug conjugate. However, in its presence, the additional peptide sequence is cleaved, leaving the CPP–drug conjugate free to enter the surrounding cells.

Proteolytic activation for site-specific drug delivery has been attempted for CPPs by different investigators, for example using matrix metalloproteases and prostate specific antigen.[19–21] In both cases, the authors used attenuating sequences attached by a protease recognition motif to form hairpin loops which upon processing of the recognition motif reveal the native carrier. In this study, a modified version of the straight chain Tat peptide was utilised where one of the lysine residue side chains was extended by another peptide sequence. It was envisaged that this would discourage the necessary interactions between the main chain guanidinium groups and negatively charged groups on the cell membrane, the first step in translocation.

Urokinase plasminogen activator (uPA) has been strongly linked to cancer development for some time.[22–25] Its roles are thought to include degradation of the extracellular matrix associated with tissue remodelling, angiogenesis and metastatic spread of tumours.[26] In addition, it has recently been shown to process a novel prodrug of doxorubicin that is water-soluble and albumin binding.[21] uPA generally occurs with low abundance and has a limited tissue distribution. The fully active enzyme is a two-chain protein that becomes localised to an uPA receptor and has an extremely limited substrate specificity cleaving the disulfide bridged loop formed from Cys-Pro-Gly-Arg560 Val561-Gly-Gly-Cys in plasminogen, making it unlikely to cleave the Tat peptide at any other site.[27,28] The limited distribution and activation of uPA around the sites of tumour invasion make it a good potential targeting mechanism for solid tumours. Finally, as the protease is associated with tumour invasion and extracellular matrix degradation, it is most likely to be found at the leading edge of a tumour and therefore at sites where drug therapy is most important to prevent or limit metastatic spread. The aims of this study were to assess whether it is possible to control uptake with a simpler, protease activated modification strategy that does not rely on hairpin loops to abrogate peptide–cell membrane interactions and to investigate the use of uPA as a potential activating enzyme.

Materials and Methods

Peptide synthesis

Pre-loaded resin and amino acids were from Protein Technologies Incorporated (Tuscon, AZ, USA) or Novabiochem (Nottingham, UK). Dimethyl formamide for peptide synthesis was obtained from Mallinckrodt Baker Incorporated (Phillipsburg, NJ, USA) and used solely for this purpose. Coupling reagents: diisopropylethanolamine was from Perseptive Biosystems (Framingham, MA, USA) and 2-(1H-9-azobenzotriazole-1yl-)-1,1,3,3,tetramethylaminium hexafluorophosphate, 2-(1H-benzotriazole-1yl-)-1,1,3,3,tetramethylaminium hexafluorophosphate and N-hydroxybenzotriazole were from Novabiochem and were used according to manufacturer's instructions. 5(6)-Carboxyfluorescein (CF) was purchased from Novabiochem. All other reagents were obtained from commercial sources and used without further purification.

Synthesis of CF-labelled tat$_{48-60}$ (CF-GRKKRRQRRRPPQ-NH$_2$)

CF-labelled Tat$_{48-60}$ (CF-Tat) was synthesised on a 0.025 mmol scale using standard Fmoc protocols and a 3-fold excess of amino acids on a Rink amide MBHA resin using a Synergy 432 Peptide Synthesiser (Applied Biosystems, CA, USA). C-terminal labelling was carried out using 5(6)-carboxyfluorescein prior to cleavage using a mix of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane and 2.5% H$_2$O. Following work up and freeze drying, a single peak was detected through absorbance at 214 nm using gradient elution reversed phase high-performance liquid chromatography (HPLC). Mass spectrometry analysis produced a peak at m/z 1039.0 ((M + 2H$^+$)$^{[2+]}$, predicted 1038.2).

Synthesis of CF-labelled lysine$_{50}$ modified tat$_{46-57}$ (CF-SYGRK(N-ε-Z-GGR)KRRQRRR-NH$_2$)

Lysine$_{50}$-modified Tat$_{46-57}$ (mCF-Tat) was synthesised on a PS3 Automated Solid-Phase Peptide Synthesiser (Protein Technologies Incorporated) using Rink amide MBHA resin and N-methylmorpholine as a base (0.1 mmol scale). Incorporation of Fmoc-Lys(Mtt) allowed for selective deprotection of the lysine side chain by 1% (v/v) TFA in dichloromethane. Upon completion of the main chain sequence and removal of the terminal Fmoc group, 5(6)-carboxyfluorescein was coupled to the N-terminal of the main chain. After rinsing and Mtt group removal, Cbz-Gly-Gly-Arg was coupled on the free lysine side chain manually. Following cleavage of the peptide from the resin (TFA/H$_2$O/thioanisole 95 : 2.5 : 2.5) it was filtered and washed with dichloromethane before being evaporated to a low volume and precipitated in ether. Reversed phase HPLC analysis at 214 and 494 nm showed a large number of products with the peptide of interest believed to be the major peak in the 494-nm trace. The retention time of the peptide absorbing at 494 nm was used as the basis for purification. Mass spectrometry analysis produced the base peak at m/z 1137.9, representing the (M + 2H$^+$)$^{[2+]}$ ion minus the Cbz protecting group (predicted m/z 1138.28). The fractions recovered were pooled and evaporated to dryness using an Eppendorf 5301 concentrator (Eppendorf UK Ltd, Cambridge, UK). Purified peptide was dissolved in double distilled water at a concentration of 10 mM before use.

Cell lines and culture

HeLa cells were cultured in Eagle's minimum essential medium with 2 mM L-glutamine, 1% (v/v) non-essential amino acids and 10% (v/v) fetal calf serum.[29] U251mg cells, established from a human glioblastoma multiforme, were cultured in Dulbecco's modified Eagle's medium without phenol red, with sodium pyruvate, pyridoxine, 1000 mg/l glucose,

4 mM L-glutamine and 10% (v/v) fetal calf serum.[30] MDA-MB-231 cells, a human breast adenocarcinoma line, were kindly provided by Dr Bill Scott (School of Biomedical Sciences, Queen's University Belfast, Belfast, UK).[31] Cells were cultured in Leibovitz's L-15 media with 2 mM L-glutamine and 10% (v/v) fetal calf serum.

Detection and assay of uPA activity

Recombinant uPA was purchased from Calbiochem (Nottingham, UK). Human Lys-plasminogen in buffer was from Haematologic Technologies Incorporated (Essex Junction, VT, USA). The fluorogenic substrate Boc-Val-Leu-Lys-amidomethylcoumarin was supplied by Calibochem. uPA buffer (0.1 M phosphate buffer, pH 7.8) was prepared fresh and stored at −20°C until use. Anti-uPA IgG was obtained from Abcam Ltd (Cambridge, UK) as a mouse monoclonal antibody in phosphate buffered saline. The presence of uPA in cell conditioned media was assessed by Western blotting followed by immunodetection using a commercially available antibody to the active fragment of uPA.

A coupled assay involving the uPA-mediated conversion of plasminogen to plasmin was used to quantify uPA activity. The plasmin converted over 12 h by uPA was measured using the plasmin-specific fluorogenic substrate Boc-Val-Leu-Lys-amidomethylcoumarin. Although commercially available substrates for uPA, for example Cbz-Gly-Gly-Arg-amidomethylcoumarin, are available, a range of proteolytic species in media may display some activity against this substrate, leading to an overestimation of the level of uPA activity. The coupled assay substrate is very specific for plasmin and should therefore provide a more accurate representation of plasmin and uPA activity. Recombinant uPA (25 ng) or an appropriate amount of cell conditioned media was added to 0.1 M phosphate buffer in a 96-well black plate followed by plasminogen before a 12-h incubation at 37°C. Following plasmin substrate addition (final concentration 20 μM) the generation of fluorescence was measured at 460 ± 20 nm (λ_{ex} 395 ± 12.5 nm).

Peptide cleavage with recombinant uPA

uPA (56 ng) was added to an aliquot of mCF-Tat in phosphate buffer and the mixture was incubated at 37°C. Samples (200 μl) were collected at 3 h, and 1 and 3 days, flash frozen in liquid nitrogen and stored at −20°C before analysis. Analysis of the mixture by HPLC was carried out as described above.

Uptake studies

Cells were incubated with 50 μM CF-Tat or mCF-Tat in media for 6 h. Lysates of the replicates were pooled before analysis. Absolute fluorescence values for lysates were compared between CF-Tat and mCF-Tat. The values obtained were corrected for total protein content assessed using the BCA assay. Microscopy images were obtained using an ECLIPSE TE300 inverted microscope and were captured on a DXM1200 digital still camera. Fluorescence images were captured, processed and analysed using the LUCIA image analysis program (Nikon, Surrey, UK).

Statistical analysis

For the microscopy data, the percentage cell associated fluorescence measured with mCF-Tat relative to that measured with native CF-Tat was compared between the three cell lines using the Kruskal-Wallis test (GraphPad Prism, GraphPad Software, San Diego, CA, USA) (*n* = 5). Post-hoc comparisons of individual groups were made using Dunn's test where appropriate. The percentage cell uptake measured using a spectrofluorimeter with mCF-Tat relative to native CF-Tat was analysed in a similar fashion (*n* = 3). A *P* value less than 0.05 was considered significant in all cases.

Results

Synthesis of CF-labelled Tat and modified Tat

CF-Tat was synthesised in good yield. In the synthesis of mCF-Tat, the intended sequence for the Lys side chain modification was the consensus sequence for uPA (Pro-Gly-Arg). However, this would have required capping of the terminal Pro residue for stability on storage. A potential drawback was the problem of available hydroxyl groups on carboxyfluorescein which may have reacted with acetic anhydride to form an acetylate. Although this reaction normally requires heating, it is possible that it might have taken place to some extent at room temperature. Previous work indicates that the sequence Cbz-Gly-Gly-Arg is cleaved with an efficiency similar to Ac-Pro-Gly-Arg by recombinant uPA (k_{cat}/K_m of 20 900 M^{-1} s^{-1} for Cbz-Gly-Gly-Arg-amidomethylcoumarin).[32] Therefore, this sequence was used as the recognition motif for uPA, since Cbz-Gly is readily available commercially. Loss of the Cbz group during cleavage may have been due to the presence of thioanisole in the cleavage mixture, giving the product shown in Figure 1.[33,34]

Characterisation of uPA production in cell culture

The presence of uPA in the cell conditioned media of three cell lines was detected by Western blotting. Recombinant uPA was detected using the anti-uPA antibody as a band at ~37 kDa (Figure 2a). The species found in cell lysates using a different lot of the same antibody displayed bands at 56 kDa, which was thought to be pro-uPA (Figure 2b). In contrast to the situation with MDA-MB-231 cells, it did not prove possible to detect the proteolytically active 37-kDa fragment of uPA in the U251mg or HeLa cell conditioned media (Figure 2a; U251mg and HeLa cell conditioned media data not shown).

Figure 3 shows the level of plasmin activity seen after 12 h incubation with plasminogen in the three cell lines. When corrected for protein concentration, a similar high level of activity was observed with MDA-MB-231 and U251mg cell lysates, whereas the HeLa cell lysates displayed almost no detectable activity. uPA activity measured against the plasmin substrate was also assessed but was found to be zero (results not shown).

Recombinant uPA activity against mCF-Tat

Both CF-Tat and mCF-Tat were incubated with uPA over at least 24 h to see if side chain hydrolysis could be observed
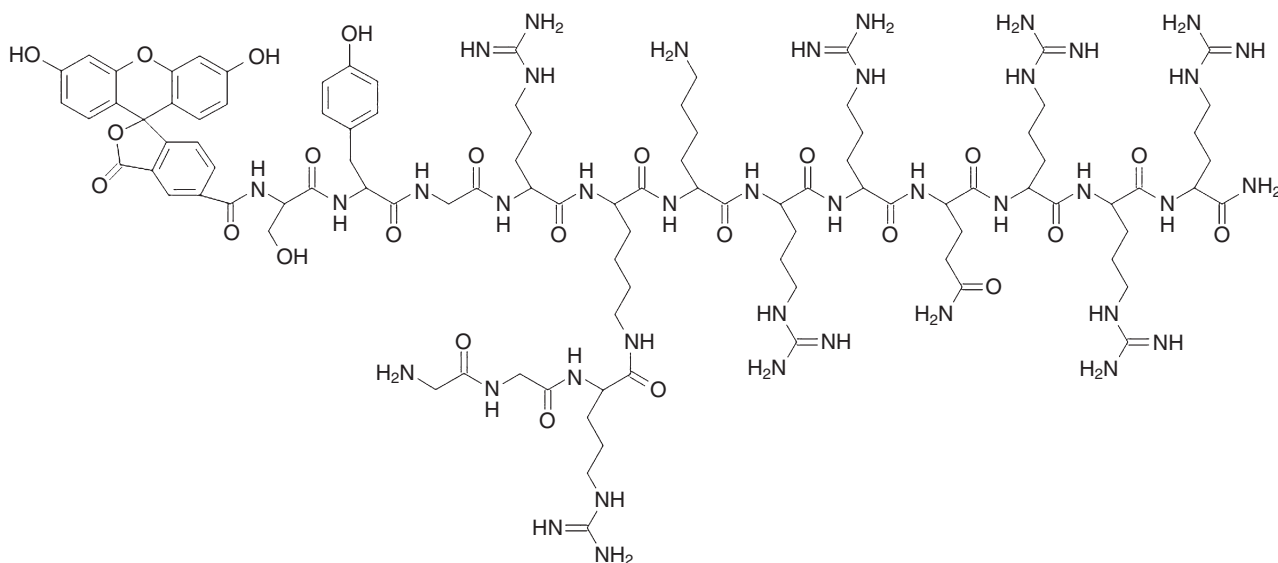
Figure 1 Carboxyfluorescein-Ser-Tyr-Gly-Arg-Lys(Gly-Gly-Arg-ε)Lys-Arg-Arg-Gln-Arg-Arg-Arg-NH₂ (modified CF-Tat). Synthesis was conducted on a PS3 Automated Solid-Phase Peptide Synthesiser. The core peptide was synthesised in one cycle. Carboxyfluorescein and side chain coupling were conducted on a manual bubbler system. Lys Mtt was incorporated in the main chain structure to allow for selective removal of the Mtt group using 1% trifluoroacetic acid in dichloromethane. Verification by mass spectrometry after work up and purification using HPLC (M + 2H⁺)[2+] m/z predicted 1138.3; observed 1137.9.
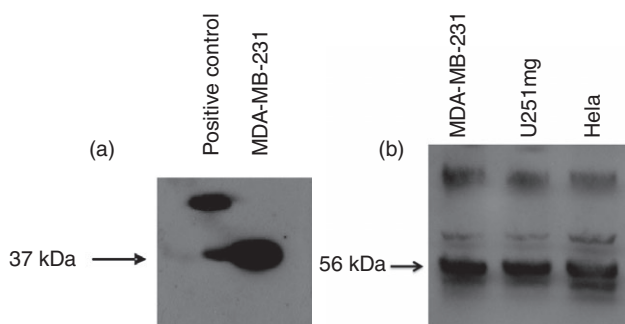

Figure 2 Detection of urokinase plasminogen activator (uPA) in transformed cells. (a) Detection of the active proteolytic fragment of uPA in MDA-MB-231 cells. Cell-conditioned media (20 μl) were probed with an anti-uPA antibody (1 : 25 dilution) followed by a goat anti-mouse-HRP secondary antibody with subsequent detection using a chemoluminescent substrate. The 37-kDa molecular weight fragment is present in both the positive control lane and in the lane containing MDA-MB-231 cell-conditioned media. (b) Detection of the proform of uPA in MDA-MB-231, U251mg and HeLa cells. The proform of uPA (~56 kDa) was detected in cell-conditioned media as described above.

Analysis of CF-Tat uptake in cell culture

A proportional increase in fluorescence was detected in lysates prepared from cells exposed to increasing concentrations of CF-Tat for 6 h (Figure 4). MDA-MB-231 cells showed the most efficient uptake; the lowest uptake was observed in U251mg cells. The difference in the level of fluorescence between MDA-MB-231 and U251mg cells was approximately 4-fold at 50 and 100 μM peptide concentrations. At lower concentrations, the levels of uptake were similar to control values and did not vary significantly between cell types.

CF-Tat uptake was also assessed at different time points in the three cell lines. Figure 5 displays the level of fluorescence associated with cell lysates exposed to 50 μM CF-Tat for up to 24 h. In HeLa and MDA-MB-231 cells, the maximal uptake time was 6 h. U251mg cell lysates displayed maximal fluorescence 1 h after peptide incubation and the level of fluorescence dropped significantly at the 3-h time point. Subsequently, there was a general increase in fluorescence intensity over time to the 24-h time point. Again, a substantial difference in the level of fluorescence was observed between the three cell lines, with HeLa cells displaying the highest uptake and U251mg cells displaying the lowest uptake.

Comparison of uptake of native CF-Tat and mCF-Tat

Table 1 displays the percentage uptake of mCF-Tat (as fluorescence compared with native CF-Tat) in each of the three cell types alongside their uPA expression. In MDA-MB-231 cells, approximately equivalent levels of uptake of the two peptides were observed (93% of the uptake seen with the unmodified peptide). Conversely, HeLa cells displayed a significant difference in the level of fluorescence measured in
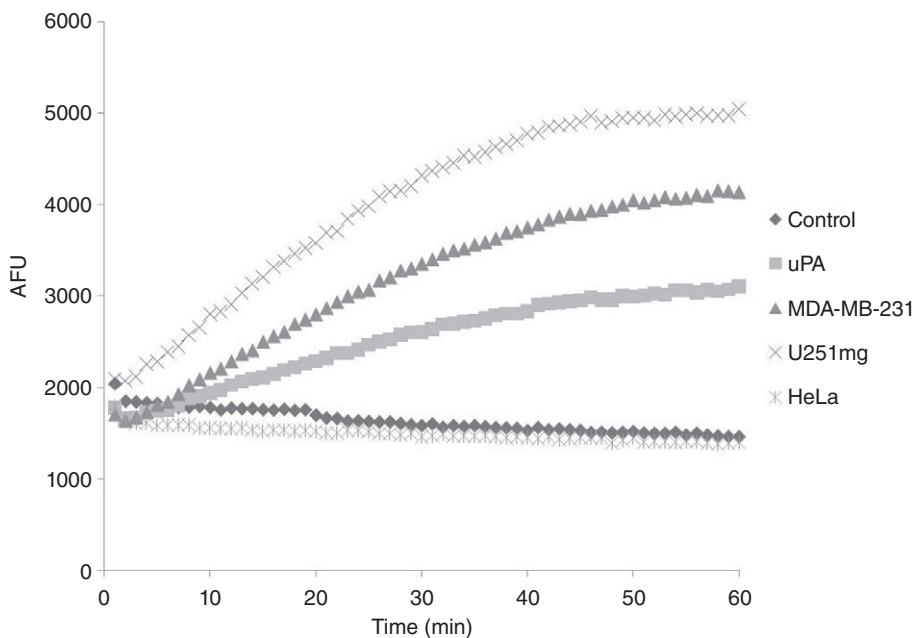
with the modified construct and to assess stability of the main chain. The mixtures were analysed by HPLC. No significant degradation of the CF-Tat main chain was observed. The mCF-Tat mixture produced several peaks over 3 days, indicative of hydrolysis. The peak considered most likely to contain the desired fragment was subjected to mass spectrometry analysis. Cbz-Gly-Gly-Arg-NH₂ was detected in small amounts as the Na⁺ ion adduct at m/z 445.1 (predicted 444.4). The fragment without the Cbz group was not observed. The other peaks could not be assigned.

Figure 3 Hydrolysis of Boc-Val-Leu-Lys-amidomethylcoumarin by available plasmin in the cell-conditioned media of U251mg, MDA-MD-231 and HeLa cells after 12 h incubation with plasminogen. Plasminogen (1 µg) was added to cell conditioned media in 0.1 M phosphate buffer and incubated at 37°C for 12 h. The amount of plasmin generated was measured by hydrolysis of the substrate Boc-Val-Leu-Lys-amidomethylcoumarin (final concentration 20 µM; final volume 200 µl; excitation 395 ± 12.5 nm; emission 460 ± 20 nm). The uPA sample contained plasminogen with 25 ng uPA. AFU, arbitrary fluorescence units.
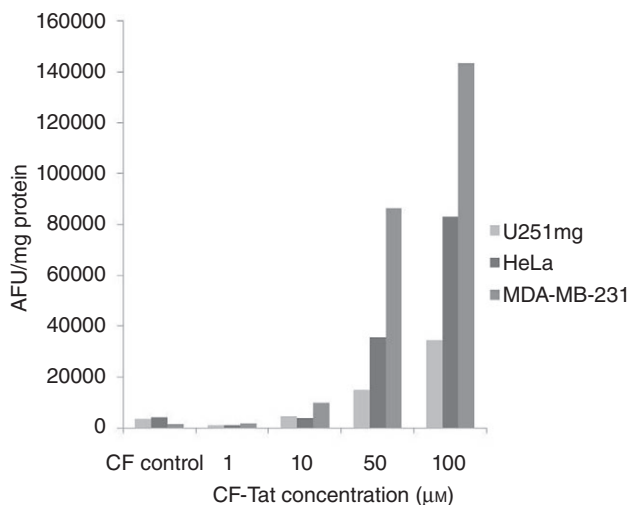

Figure 4 Uptake of different concentrations of carboxyfluorescein labelled Tat (CF-Tat) in HeLa, U251mg and MDA-MB-231 cells after 6 h. A total of 1×10^6 cells/ml were seeded in a 96-well plate and incubated with various concentrations of CF-Tat in media for 6 h; control cells were incubated with a solution of 100 µM carboxyfluorescein only. The cells were rinsed, trypsinised for 15 min and suspended in phosphate-buffered saline, pelleted, resuspended in fresh phosphate-buffered saline, pelleted again, resuspended in lysis buffer and frozen at −20°C. Lysates were analysed on a spectrofluorimeter (excitation 485 ± 10 nm; emission 530 ± 12.5 nm) and the data are expressed as the mean fluorescence corrected for protein levels (AFU per mg protein). AFU, arbitrary fluorescence units. Each value is the average of duplicates representing the sum of five wells with protein content of lysates assessed using the BCA assay.
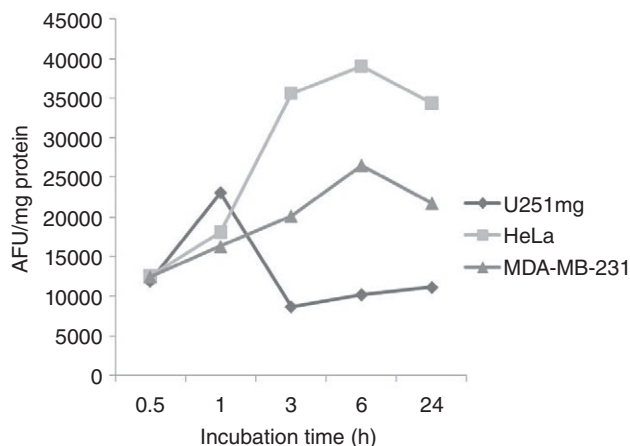

Figure 5 Uptake of 50 µM carboxyfluorescein labelled Tat (CF-Tat) in HeLa, U251mg and MDA-MB-231 cells over time. A total of 1×10^6 cells/ml were seeded in a 96-well plate and incubated with CF-Tat (50 µM) in media for up to 24 h. The cells were rinsed, trypsinised for 15 min and suspended in phosphate-buffered saline, pelleted, resuspended in fresh phosphate-buffered saline, pelleted again, resuspended in lysis buffer and frozen at −20°C. Lysates were analysed on a spectrofluorimeter (excitation 485 ± 10 nm; emission 530 ± 12.5 nm) and the data expressed as the mean fluorescence corrected for protein levels (AFU per mg protein). AFU, arbitrary fluorescence units. Each value is the average of duplicates representing the sum of five wells with protein content of lysates assessed using the BCA assay.

Table 1 Uptake of carboxyfluorescein labelled modified Tat as a percentage of uptake of native carboxyfluorescein labelled Tat in HeLa, U251mg and MDA-MB-231 cells related to urokinase plasminogen activator expression

Cell type	Urokinase plasminogen activator activity	Uptake (%) of mCF-Tat
HeLa	Low	52 ± 8
U251mg	High	19 ± 7
MDA-MB-231	High	93 ± 25

CF-Tat, carboxyfluorescein labelled Tat peptide; mCF-Tat carboxyfluorescein labelled modified Tat peptide. Uptake of mCF-Tat is the percentage total fluorescence measured compared with that measured with CF-Tat. Cells incubated with 50 μM CF-Tat or mCF-Tat in media for 6 h were trypsinised and rinsed before the lysates were assessed for total fluorescence corrected for total protein content. Data represent the mean ± SD of a minimum of three replicates in each case.

cells incubated with normal and modified CF-Tat (52% of the uptake seen with the unmodified peptide). U251mg cells displayed a significant reduction between the amounts of fluorescence detected in cells incubated with CF-Tat compared with those incubated with mCF-Tat (19% of the uptake seen with the unmodified peptide). The difference in this case was larger than that seen in HeLa cells. In all cases, the level of fluorescence observed with the modified peptide was substantially less that that observed with the native peptide via microscopy (results not shown). Again, substantial differences in the level of fluorescence were observed between different cell types. Statistical analysis of the percentage uptake measured with mCF-Tat compared with native CF-Tat using the Kruskal-Wallis test showed no significant differences between any of the cell lines. Similarly, no significant difference was seen when the microscopy data were compared between cell lines (percentage of cell associated fluorescence).

Discussion

The potential of proteolytic activation of peptide carriers as a means of localising and regulating the activity of an otherwise non-specific carrier was investigated. A modified Tat peptide was designed, aimed at reducing uptake into cells in the absence of the appropriate proteolytic activity. In the presence of the active protease, cleavage of the modified peptide would convert the peptide back to its native form, restoring cell penetrating activity. This approach has been further validated by similar studies in the field.[19–21]

Significant uPA activity was detected in MDA-MB-231 and U251mg cell conditioned media, with little or no activity detected in HeLa cell conditioned media. Despite several attempts, it was not possible to accurately quantify the level of uPA activity using the coupled assay. Hence, a semi-quantitative rating of the level of uPA activity in the three cell lines was used based on the results displayed in Figure 3.

Incubation of mCF-Tat with recombinant uPA and subsequent analysis by HPLC suggested hydrolysis of the substrate sequence. However, the peak representing the Na$^+$ adduct of Cbz-Gly-Gly-Arg at m/z 445 was only present in small quantities compared with the total number of peaks, and a peak representing the side chain without the Cbz group (the majority of which was removed during synthesis) was not detected. In addition, we could not detect the main chain native peptide from the hydrolysis products. The presence of other salts in small quantities may have reduced the sensitivity of the analysis, or the turnover of the substrate may have been lower due to the iso-peptide bond of the side chain attachment.[35] Further investigation of the optimal time required to process the side chain modification would be useful as the hydrolysis may be slower than that seen with other straight-chain peptide substrates. However, other prodrug peptide modifications have been reported as being recognised and cleaved by uPA.[21]

Despite maximal uptake in U251mg cells being seen after 1 h, a 6-h incubation was used for the comparative experiments to allow sufficient time for proteolytic activation of the carrier at the cell surface and subsequent internalisation, while maximising the level of fluorescence seen in each cell line. In agreement with others, a dramatic increase in the total level of cell-associated fluorescence was detected without a trypsin rinse (approx. 10-fold) over that seen when a trypsin rinse was included at both the 3- and 6-h time points (data not shown).[8,36]

Table 1 displays the percentage levels of fluorescence for mCF-Tat for each of the three cell lines tested along with the level of uPA expression for each cell line. No significant difference was observed when the percentage uptake of mCF-Tat was compared with native CF-Tat. This lack of a difference is likely to be due to the small number of replicates available for analysis. No substantial difference between the uptake of normal and modified peptide was seen in the amount of fluorescence detected in the lysates of MDA-MB-231 cells (mCF-Tat displaying 93% of the fluorescence of native CF-Tat). This suggests that either modification of the peptide does not have any substantial influence on uptake or, more likely, that the significant quantity of uPA expressed by MDA-MB-231 cells is capable of processing the modified peptide to its native form, allowing it to penetrate the cell.

The level of uptake of CF-Tat and mCF-Tat was substantially different in HeLa cells. Uptake of mCF-Tat was about half of that recorded for CF-Tat (52% of the fluorescence detected with native CF-Tat). This result suggests that modification of the native peptide structure did negatively affect cell uptake, offering confirmation that the equivalent uptake by MDA-MB-231 cells was due to proteolytic processing of the mCF-Tat to its native form prior to uptake. The differential observed between cell types also infers that uptake was dictated by the amount of protease expressed by the cells as we previously observed almost no uPA activity in HeLa cells (Figure 3). Due to synthetic considerations outlined previously, it is possible that other proteases present may have been able to process the modified peptide to its native form. This may explain the partially improved uptake seen with HeLa cells compared with that seen with alanine substituted variants (approx. 50% reduced compared with 70–90% for the alanine substituted peptide).[5]

U251mg cells displayed the greatest difference in the extent of the uptake measured for CF-Tat and mCF-Tat, approximately 19% of the fluorescence detected for mCF-Tat compared with native CF-Tat. This is contrary to our hypoth-

esis that the level of peptide uptake into cells should correlate with the level of uPA activity. It is not clear why there was such a difference in uptake within this cell line. It is possible this may be due to cell-specific differences in Tat uptake. For example, genetic variation may contribute to differences between cells in both the amount of macropinocytosis undertaken within a defined period of time and also in the expression of cell surface molecules which may aid or hinder uptake, for example cell surface proteoglycans. Other groups have reported differences in the extent of uptake of native Tat between different cell lines with very poor uptake seen in some cells.[37–39] In comparison with the uptake seen for Tat with HeLa and MDA-MB-231 cells, that seen with U251mg cells was much lower for both the normal and the mCF-Tat. Alternatively, there may be a difference in the nature of uPA expression and activity in U251mg cells that negatively impacts on its ability to process the modified form of the peptide. For example, we were unable to detect the active fragment of uPA in either HeLa or U251mg cells by Western blotting.

Previous work has demonstrated that, in the absence of a trypsin rinse, membrane-bound peptide represents a significant proportion of the total peptide population.[38] Using fluorescence microscopy, the level of fluorescence observed with mCF-Tat was significantly lower than that seen with native CF-Tat in each case. None of the cell lines examined displayed fluorescence levels above 30% of those seen with the native peptide when using mCF-Tat (results not shown). This suggests that the side chain modification does discourage the initial interaction between the peptide and the membrane constituents. However, statistical analysis failed to reveal any significant differences between the percentage cell-associated fluorescence in any of the cell lines. This may be due to saturation of the cell surface with peptide or the small sample size available for analysis.

This study provides further evidence that the modification of a CPP through the addition of a protease activatable sequence can retard cellular uptake while offering selective uptake into cells expressing the activating protease and confirms this strategy as a potential approach to distinguish between normal and malignant cells in cancer drug therapy. The use of FACS analysis and confocal microscopy will assist in the characterisation of the cellular uptake of modified and native peptides. A more detailed examination of the differences in uptake between cell lines may allow for a more accurate quantification of the effects of proteolytic activation of the carrier.

Conclusions

An attempt was made to use proteolytic activity as a means to control entry of a modified CPP into cells. The lysine side chain modification reduced the binding of the peptide to the cell membrane in all cases. The level of uptake of mCF-Tat was comparable with that observed with native CF-Tat in MDA-MB-231 cells and was substantially reduced for both HeLa and U251mg cells. The uptake of the modified peptide could be linked to the activity of uPA in MDA-MB-231 and HeLa cells but not in U251mg cells. This may represent a cell-specific effect. The level of native peptide uptake varied

substantially between cells, however statistical analysis failed to reveal any significant differences between the percentage uptake in each cell line. The use of peptide modification to limit uptake of CPPs into cells solely with the appropriate proteolytic activity represents an interesting strategy for increasing the selectivity of cell transduction technology.

Declarations

Conflict of interest

Funding

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