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Efficient Cellular Delivery of β-Galactosidase Mediated by NrTPs, a New Family of Cell-Penetrating Peptides

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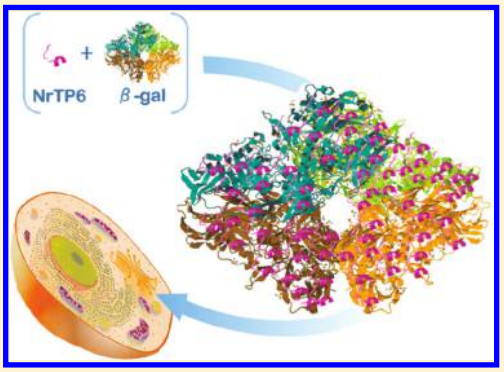
ABSTRACT: Nucleolar targeting peptides (NrTPs), a recently developed family of cell-penetrating peptides, have been shown to be very efficient in entering cells and accumulating in their nucleoli. In this work, we have used conjugates of NrTP6 (YKQSHKKGGKKGSG) covalently linked to β-galactosidase in order to demonstrate the capacity of NrTP for intracellular delivery of large molecules. NrTP6/β-galactosidase conjugates, prepared by maleimide-based chemistry, were stable and enzymatically active on the standard 4-methylumbelliferyl β-D-galactopyranoside substrate. Their translocation into HeLa cells, monitored by β-galactosidase activity as a readout of the uptake, showed efficient cellular entry and thus demonstrated the potential of NrTPs for intracellular delivery of large-size cargos with preservation of biological activity.



INTRODUCTION

Cell-penetrating peptides (CPPs) are short cationic sequences that, without disrupting membranes, can enter cells and carry over various types of cargo molecules.[1−3] Interest in these peptides started two decades ago with the observation that the HIV-1 accessory protein Tat is able to translocate into cells.[1] Since that time, CPPs have been shown capable of transporting biologically active cargos to the cell interior, both *in vivo* and *in vitro*.[4−6] Additionally, considerable effort has been invested in understanding the ability of CPPs to internalize various cargos [e.g., proteins, nucleic acids, fluorophores, radiolabels, magnetic resonance imaging (MRI) contrast agents, quantum dots, and so forth[7−10]], with the aim of developing CPPs into molecular tools with potential applications in basic research, diagnostics, or therapeutics. Relevant examples are pep-1 (Chariot), currently used as a commercial transfection reagent,[6,11,12] and KAI-9803, a selective δ-protein kinase C inhibitor conjugated to Tat that reduces injury associated with ischemia and reperfusion in animal models, presently undergoing phase IIb clinical trials.[13]

In principle, for any cargo intended to be ferried into a cell, a conjugate with a CPP can be envisaged. For instance, CPPs conjugated to either nucleic acids or proteins have been successfully used for transfection or antibody delivery.[14−16] Their eventual application in the therapeutic delivery of different drugs remains a challenging yet promising area of CPP research.[4,7,13] Studies of Tat-mediated protein translocation opened the way to CPP-facilitated delivery of proteins of different types and sizes, among which GFP (30 kDa), IgG (120−150 kDa), or β-galactosidase (465 kDa) have already been successfully tested.[2,4−7] β-Galactosidase (β-gal), in particular, is a frequent [2,6] and convenient cargo for CPP-mediated protein delivery, given its large size and its well-characterized enzymatic activity, for which different assays are available.[17−19] As to the conjugation of CPPs to the cargo of interest, whether a protein, a nucleic acid, or a nanoparticle, a number of approaches involving chemical conjugation, simple electrostatic interaction, or molecular fusion have been described.[2,4,6,20−22]

Nucleolar targeting peptides (NrTPs) were first described in 2008 as the result of structural minimization of the sequence of crotamine,[23] a 42-residue toxin that is one of the main components of the venom of the South American rattlesnake (*Crotalus durissus terrificus*).[24] The representative NrTP1 sequence (YKQCHKKGGKKGSG)[23] has been shown to possess remarkable translocating properties, namely, fast internalization (<15 min) and preferential nucleolar localization. For the purpose of this study, the original Cys at position 4 of NrTP1 has been mutated to Ser, and an extra Cys added to the C-terminal to facilitate conjugation. The resulting analogue, NrTP6 (YKQSHKKGGKKGSG), shows no detriment in CPP properties in the free form (unpublished), and we hereby demonstrate its ability to translocate large biomolecules into cells with full preservation of activity, a necessary condition

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for NrTP-mediated delivery of therapeutically relevant cargos into cells and tissues. Our positive results offer obvious opportunities for NrTP application, given that the nucleolus has a key role in ribosome biogenesis and the control of the cell cycle and, more recently, has been reported to take part in the control of stress and viral infection.[25−27]

EXPERIMENTAL PROCEDURES

Chemicals. Fmoc-protected amino acids, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and Fmoc-Rink-amide (MBHA) resin were obtained from Iris Biotech GmbH (Marktredwitz, Germany). HPLC-grade acetonitrile, and peptide synthesis-grade *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), *N*,*N*-diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were from Carlo Erba-SDS (Peypin, France). 4-Methylumbelliferyl *β*-D-galactopyranoside (MUG), Triton X-100, *β*-galactosidase (EC 3.2.1.23) from *Escherichia coli* (grade VI), and 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) were obtained from Sigma (Madrid, Spain). DMEM, trypsin 0.05%, phosphate buffer saline (PBS), fetal bovine serum (FBS), penicillin, and streptomycin were from Invitrogen (Spain). All other chemicals were from Aldrich (Madrid, Spain)

Peptide Synthesis. The NrTP6 (YKQSHKKGGKKGSG) sequence was synthesized with an extra Cys residue at either the C- (i.e., NrTP6-C) or the N-terminus (C-NrTP6). Solid-phase peptide synthesis was done by Fmoc-based chemistry on Fmoc-Rink-amide (MBHA) resin (0.1 mmol) in a model 433 peptide synthesizer (Applied Biosystems, Foster City, CA) running FastMoc protocols. Couplings used 8-fold molar excess of both Fmoc L-amino acid and HBTU, and 16-fold molar excess of DIEA. Side chain protecting groups were *tert*-butyl (Ser, Tyr), *tert*-butyloxycarbonyl (Lys), and trityl (Cys, Gln, His). After chain assembly, full deprotection and cleavage of the N-deblocked resin were carried out with trifluoroacetic acid (TFA)-water-ethanedithiol-triisopropylsilane (94:2.5:2.5:1 v/v, 90 min, rt). Finally, peptides were precipitated by addition of chilled diethyl ether, taken up in H_2O, and lyophilized. Analytical reversed-phase HPLC was performed on a Luna C_{18} column (4.6 mm × 50 mm, 3 μm, Phenomenex). Linear gradients of solvent B (0.036% TFA in acetonitrile) into A (0.045% TFA in H_2O) were used for elution at a flow rate of 1 mL/min, with UV detection at 220 nm. Preparative HPLC runs were performed on a Luna C_{18} column (21.2 mm × 250 mm, 10 μm; Phenomenex), using linear gradients of solvent B (0.1% in acetonitrile) into A (0.1% TFA in H_2O), as required, with a flow rate of 25 mL/min. Matrix-assisted time-of-flight mass spectra were recorded in the reflector mode in a Voyager DE-STR workstation (Applied Biosystems, Foster City, CA) using *α*-hydroxycinnamic acid as matrix. Fractions of adequate HPLC homogeneity and with the expected mass were combined, lyophilized, and used in the cell uptake experiments.

Conjugation. The protocol for conjugation of NrTP6 to *β*-gal was adapted from Leteef et al.[28] Briefly, 1 mg of *β*-gal was dissolved in 2.1 mL of cold PBS and 4 mg of MBS were dissolved in 400 μL of DMF (1 μM and 32 mM *β*-gal and MBS concentrations, respectively). 160 μL of the MBS solution was then added to the *β*-gal solution under constant stirring and allowed to react for 30 min at 4 °C, to convert the protein Lys residues into maleimide units. The reaction mixture was then loaded to a Sephadex G-25 PD-10 desalting column (GE Healthcare) pre-equilibrated with 50 mM phosphate buffer, pH 6. 1 mL fractions were collected at 4 °C, and the three with highest absorbance at 290 nm were pooled and mixed with 1 mg (0.6 mmol) of either NrTP6-C or C-NrTP6, to assess the effect of the conjugation position on cargo internalization and preservation of bioactivity. The pH was adjusted to 7.5, and the mixture was incubated for 3 h and then dialyzed against 6 changes (30 min each) of 10 mM phosphate buffer pH 7.4, at 4 °C. The total protein concentration in the conjugates was determined by the Bradford method.[29] Conjugates with 100-fold lower concentrations of MBS (0.32 mM instead of 32 mM) were prepared likewise.

***β*-Gal Enzyme Assay.** The colorless 4-methylumbelliferyl-*β*-D-galactopyranoside (MUG) substrate is hydrolyzed by *β*-gal into 4-methylumbelliferone (4-MU), whose formation was monitored by its bright blue fluorescence (λ_{exc} = 360 nm, λ_{exc} = 440 nm, pH 7.4). Briefly, MUG was dissolved at 0.5 mM concentration in PBS containing 5 mM $MgCl_2$ and the enzyme reaction was started with the addition of either free *β*-gal (0−6 μg/mL) or conjugate (0−10 μg/mL), following a procedure adapted from previous reports.[6,30] The kinetics of 4-MU production was monitored by fluorescence intensity measurements, for 90 min, on a TECAN Infinite 200 Multimode microplate reader (Männedorf, Switzerland).

Uptake of NrTP6-C/*β*-gal (or C-NrTP6) Conjugate. Having established the activity of the NrTP6/*β*-gal conjugates in cell-free conditions, their internalization by human adenocarcinoma epithelial (HeLa) cells (gently supplied by Dr. Xavier Mayol, Cancer Cell Line Repository, Municipal Institute of Medical Research, Barcelona) was next studied. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) of heat inactivated fetal bovine serum (FBS), 10 U/mL penicillin, and 100 μg/mL streptomycin, at 37 °C in a 5% CO_2 atmosphere. Cells were used for internalization experiments when they reached ca. 80% confluence. Experiments were performed either in Petri dishes (35 mm × 10 mm) or in 4-well plates. Briefly, conjugate (0−12 μg/mL) was incubated with cells for 60 min at 37 °C and 5% CO_2 atmosphere. Trypsin was added after incubation to detach cells and hydrolyze extracellular nonincorporated (membrane and nonmembrane bound) conjugate. Next, cells were harvested, centrifuged twice at 500 *g* for 5 min (with an intervening PBS wash), and permeabilized with 0.1% (v/v) Triton X-100 for 15 min at 25 °C. A last centrifugation step was done also at 500 *g* for 5 min and supernatant was assayed for enzymatic activity by fluorescence spectroscopy accordingly with the procedure described above. For these samples, 4-MU end point concentrations were also precisely quantified by UV absorbance after stopping the reaction by alkalinization with 50 mM NaOH to pH 9. UV absorbance measurements (ε_{360} = 1.9 × 10^4 M^{-1} cm^{-1}) were also done on a TECAN Infinite 200 Multimode microplate reader. The efficiency of internalization was studied either with a fixed cell density (2 × 10^5 cells/mL) at different conjugate concentrations or, alternatively, with a fixed amount of conjugate (7 μg/mL) and variable cell densities, in the (2−12) × 10^5 cells/mL range. Cell viability was controlled throughout the 60 min incubation both by the trypan blue exclusion assay and by differential interference contrast microscopy (DIC) in a Leica TSC-SP2 microscope (Wetzlar, Germany). Controls using nonconjugated enzyme and peptide, either combined or separately, were also performed. Moreover, an extensive study of *β*-gal residual activity was done, testing the enzyme in the 0−170 μg/mL concentration range.

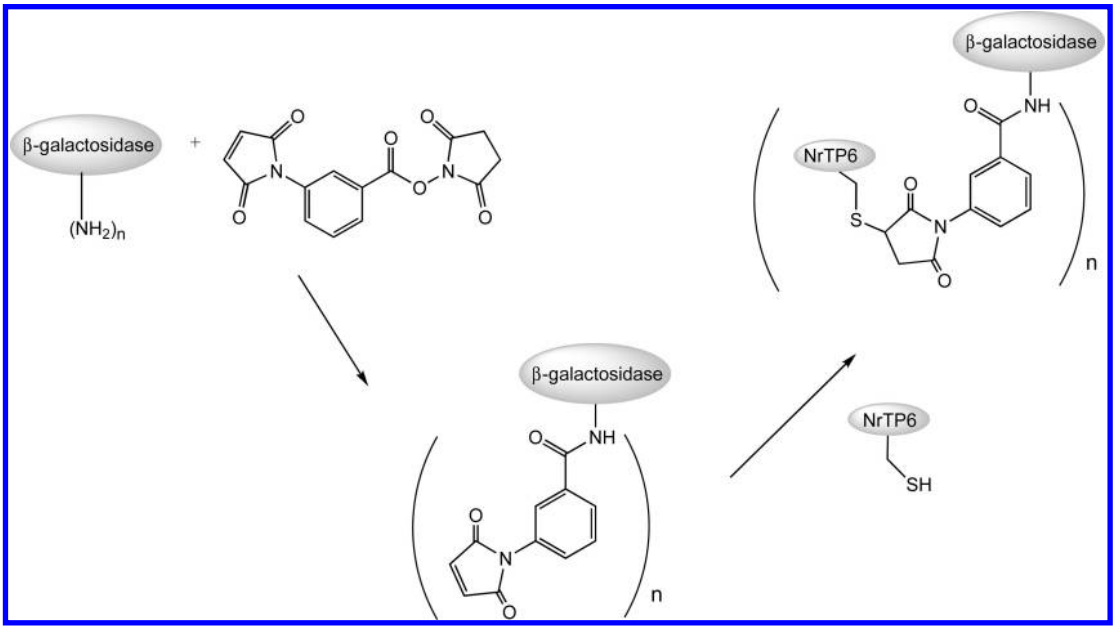

Figure 1. Maleimide-mediated conjugation of NrTP6-C and C-NrTP6 to *β*-galactosidase. The Cys residue was either on the C- or on the N-terminus of the peptide (NrTP6-C or C-NrTP6, respectively).

RESULTS AND DISCUSSION

Chemical conjugation, either protein (or peptide)-protein[13,16,28] or protein (or peptide)-nucleic acid,[20,31,32] is a well-established procedure for covalent attachment of two molecules. Figure 1 illustrates the conjugation between NrTP6 and *β*-gal, the latter chosen as a model to test CPP-mediated uptake of large cargos.[1,2,6] *β*-Gal activity was used as a readout of the uptake, with the fair assumption that activity and internalization are directly related. *β*-Gal is a large homotetramer, with 1023 amino acid residues per subunit, often used as a reporter of product transcription. The enzyme catalyzes the hydrolysis of lactose and other *β*-D-galactosides into monosaccharides. Although very specific for the galactose moiety, it is rather promiscuous regarding the rest of the substrate, which has allowed the development of different colorimetric assays for measuring activity.[33] For our study, we chose as probe 4-methylumbelliferyl-*β*-D-galactoside (MUG), hydrolyzed by *β*-gal to fluorescent 4-methylumbelliferone (4-MU).[6,30] *β*-Gal activity was measured by means of progression curves of fluorescent product formation (Figure 2). The enzymatic assay was conducted under substrate excess conditions, ensuring quasi-steady-state enzyme activity. In this way, initial rates can be assumed as V_{max} and, hence, k_{cat} is defined by the slope of the v_0 vs conjugate concentration variation (Figure 2). Fluorescence data were normalized by dividing by the fluorescence intensity of a 1 μg/mL solution of free *β*-gal after 90 min. Prior to cell uptake experiments, the enzymatic activity of various NrTP6/*β*-gal conjugates was confirmed (Figure 3A), a linear relationship being found between v_0 and conjugate concentration up to 2.5 μg/mL. The enzymatic activity was also compared with that of free *β*-gal at different stages of the protocol to ensure that no loss of activity was occurring. Results revealed a linear relationship between enzymatic activity and concentration from 0 to 5 μg/mL of free *β*-gal, with a k_{cat} of 26.3×10^{-3} μg^{-1} mL min^{-1} and r^2 = 0.995.

Next, HeLa cells were incubated with the conjugate and activity was measured in the cell-free extract upon addition of

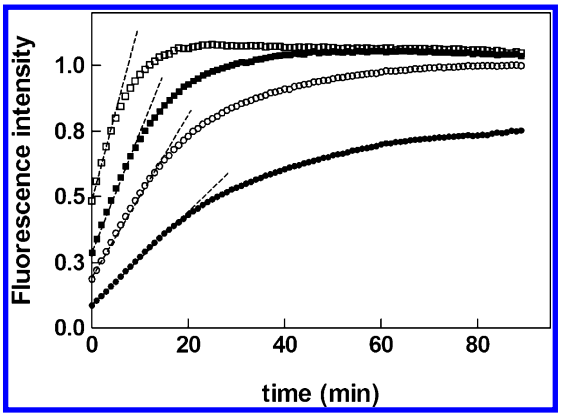

Figure 2. Time-course of *β*-galactosidase enzymatic activity. Fluorescence intensity, proportional to the 4-MU product concentration, was recorded at 440 nm (excitation at 360 nm) over 90 min after addition of enzyme to MUG substrate. The above kinetic curves correspond to 0.5 μg/mL (●), 1 μg/mL (○), 1.5 μg/mL (■), and 2.5 μg/mL (□) free *β*-gal concentrations, respectively. Fluorescence intensity values were normalized to the intensity for 1 μg/mL of *β*-gal at 90 min. Initial rates, v_0, were determined from the initial slope of the curves. The same procedure was used for *β*-gal conjugate samples (alone or after cell incubation).

MUG. Figure 3B shows a clear dependence of product formation on conjugate concentration, hence supporting the efficient delivery of NrTP6-conjugated *β*-gal into HeLa cells. Incubation with nonconjugated *β*-gal (Figure 3B) resulted in no product formation, in further support of the inability of *β*-gal to enter cells unless conjugated to an efficient CPP. The fact that the tetrameric form of *β*-gal is required for enzymatic activity clearly demonstrates that the conjugate not only enters cells, but does so while preserving its proper folding and oligomerization, i.e., with minimal interference, if any, from NrTP on the cargo structure. A control experiment with a mixture of nonconjugated NrTP6 and *β*-gal gave negative results that ruled out the possibility of NrTP6 electrostatic binding to *β*-gal, forming conjugates with translocating activity.

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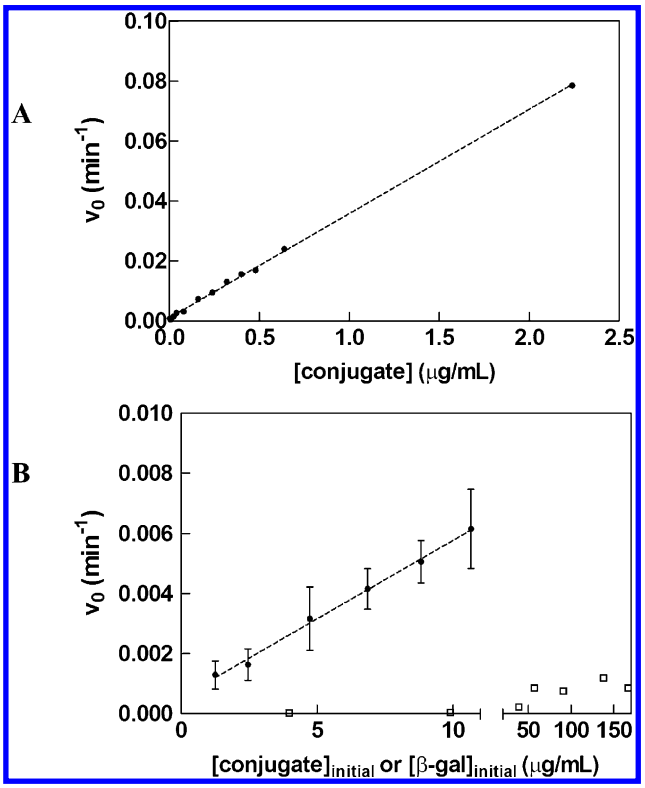

Figure 3. Enzymatic activity of the NrTP6-C/β-gal conjugate. (A) Standard activity in the absence of cells. (B) Activities of conjugate (circles) and β-gal (open squares) incubated with a cell-free extract (initially 2×10^5 HeLa cells/mL) for 60 min. Initial rates, v_0, were determined as indicated in Figure 2. k_{cat} values of 34.7×10^{-3} and 0.52×10^{-3} μg^{-1}.mL.min^{-1}, for conjugate alone and for the cell-free extract, respectively, were obtained. Results are from duplicates of a representative experiment.

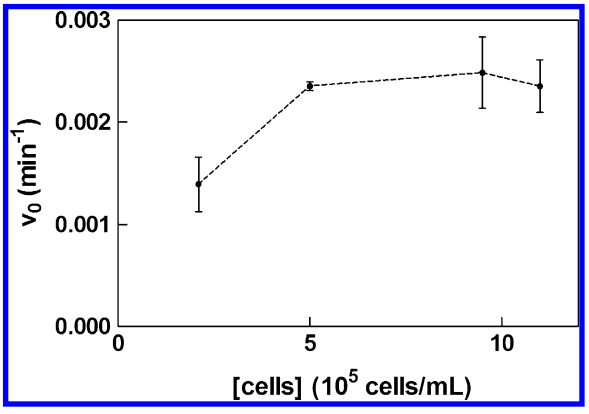

Figure 4. Effect of cell number on the enzymatic activity of the NrTP6-C/β-gal conjugate. Conjugate (7 μg/mL) was incubated with various cell densities for 60 min, at 37 °C. Initial rates, v_0, were determined as indicated in Figure 2. Results are from duplicates of a representative experiment.

The apparent decrease in the k_{cat} of β-gal from 34.7×10^{-3} to 0.52×10^{-3} μg^{-1} mL min^{-1} upon HeLa cell internalization (Figure 3 B) may be explained by several factors: The first, and possibly most relevant, is the fact that not all the conjugate is internalized. This is supported by Figure 4, where the fraction of internalized conjugate is shown to increase until a threshold level is reached. Another important factor for the apparent lower activity of the conjugate in the cell-free extract is competition for β-gal between MUG and endogenous substrates, mainly membrane components such as ganglioside GM1, lactosylceramides, glycoproteins, and keratin sulfate.[34,35] On the other hand, no significant competition for MUG appears to exist between endogenous and conjugated β-gal, as observed by the nonsignificant amounts of 4-MU detected in control experiments using nonconjugated NrTP (data not shown). In the above scenarios, CPP-to-cell ratio, rather than absolute CPP concentration, would appear to be influential in the uptake (or delivery) capacity of NrTP6, with the possibility of improving the delivery by increasing the volume of incubating peptide.[36]

Assuming no change in the intrinsic activity of NrTP6-delivered β-gal upon entering the cell, given that the apparent decrease in activity is essentially due to the equilibrium between the conjugate inside and outside the cell, the fraction of conjugate that penetrates the cell can be calculated from the above k_{cat} data to be 1.51%. Even if that value is underestimated, it is within the range reported for the uptake of other conjugates (0.07% to 5%).[37−39] Cargo size, even if CPP-mediated, is known to affect the efficiency of translocation, as observed, e.g., for the best-studied Tat.[40] Indeed, the highest reported uptake efficiency (5%) corresponds to a oligodeoxynucleotide−doxorubicin conjugate,[37] much smaller than the present NrTP6/β-gal conjugates (6.5 kDa vs 465 kDa). Our results reveal an intracellular conjugate concentration in the 3−57 μg/mL range, depending on the initial conjugate concentration and assuming a volume of 2.6 pL for a HeLa cell.[41] Assuming 116 NrTP6 copies bound to each β-gal tetramer (one per Lys residue), and from the respective molecular weights of β-gal and NrTP6 (465 and 1.6 kDa), it follows that approximately 72% and 28% of conjugate weight correspond to β-gal and NrTP, respectively. From this, the concentration of internalized β-gal can be estimated to be in the 2−41 μg/mL range. This rather high local concentration, entirely due to CPP-mediated internalization, clearly envisages relevant results with other more application-driven cargos.

Additional aspects explored in the present study included cell viability, as well as the influence of the position (N- or C-terminal) and extent of conjugation upon NrTP6/β-gal uptake. With regard to cell viability, both trypan blue exclusion and DIC live imaging after 60 min incubation with either type of conjugate (NrTP6-C or C-NrTP6-derived) revealed over 98% viability and no detectable signs of morphological changes or cellular stress over that period. Further comparison between both types of conjugates also showed similar uptake levels, either in cell-free assays (Figure 5) or upon incubation with HeLa cells (Figure 6), suggesting that positioning of the Cys residue at either the N- and C-terminal end has similar impact on both the conjugation and translocation efficiencies. Finally, results in Figure 6 also show that no significant differences exist between conjugates made with amounts of the bifunctional MBS reagent differing by a factor of 100.

The experimental results of the present report offer a necessary proof-of-concept for the intracellular delivery of large molecules mediated by NrTPs. It is worth noting that, two decades after introduction of the CPP concept, studies on delivery of drugs and other cargos by the most extensively studied CPPs (e.g., Tat, Ant, pep-1, and oligo-arginines) are still ongoing and, for the more recently discovered CPPs, insights on their translocation abilities and the underlying mechanisms are still scarce. In this regard, the present results are a valuable contribution to establishing the role of NrTPs as

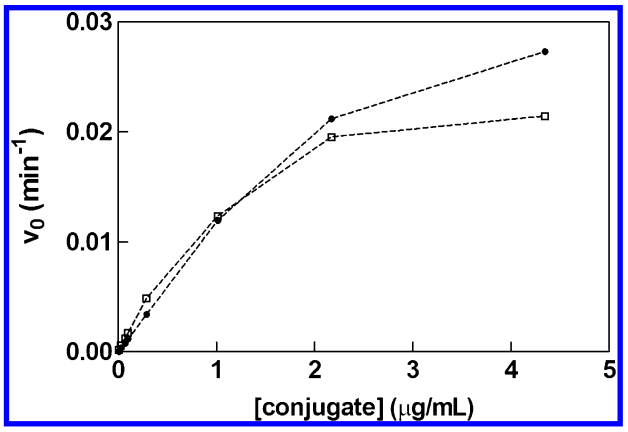

Figure 5. Effect of N- vs C-terminal conjugation on enzymatic activity. NrTP6-C (open squares) and C-NrTP6 (circles) derived β-gal conjugates assayed in the absence of cell extract. Initial rates, v_0, were determined as indicated in Figure 2.

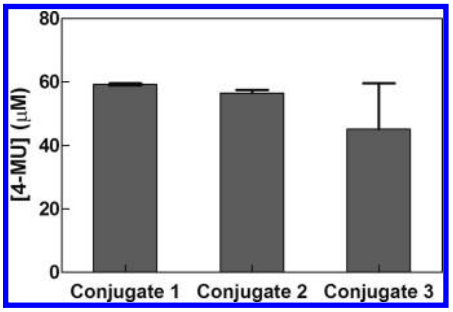

Figure 6. Effect of conjugation position and NrTP6/β-gal density on uptake efficiency. Conjugates **1**−**3** are, respectively, NrTP6-C/β-gal, C-NrTP6/β-gal, and NrTP6-C/β-gal prepared with 100-fold less MBS. [4-MU] was determined spectrophotometrically ($\varepsilon_{360\ nm}$ = 1.9 × 10^4 M^{-1} cm^{-1} at pH 9), after incubation with HeLa cells for 60 min, at 37 °C, and 90 min reaction in the cell-free extract, stopped with NaOH 50 mM. Results are duplicates from a representative experience. Scale bars represent the range of variation.

useful biotools. β-Gal is a rather large protein (size estimated as 17.4 × 13.0 × 7.5 nm^3 from Protein Data Bank (entry 1DP0) data, using the UCSF Chimera software, http://plato.cgl.ucsf.edu/chimera/), far exceeding the thickness of cell membranes. The fact that it is also well above the size of many therapeutically relevant cargos (e.g., antibodies are ∼150 kDa), in conjunction with the preferential nucleolar localization of NrTPs, allows to envisage promising applications for NrTPs in areas such as immunotherapy or cell cycle regulation.

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