University of Szeged

Doctoral School of Pharmaceutical Sciences

PhD programme: Pharmaceutical Analysis

Programme director: Prof. Dr. István Ilisz

Institute: Department of Medical Chemistry

Supervisor: Prof. Dr. Tamás Martinek

Norbert Imre, Pharm.D.

Intracellular protein delivery with the use of endocytosis routing sequences

Final examination committee:

Chairman: Prof. Dr. György Dombi

Members: Dr. Gerda Szakonyi

Dr. Pál Szabó

Reviewer committee:

Chairman: Prof. Dr. Zsolt Szakonyi

Reviewers: Prof. Dr. Edit Buzás

Dr. Kata Horváti

Members: Dr. Dezső Csupor

Dr. Rita Ambrus

1. INTRODUCTION AND AIMS

One of the greatest challenges to overcome in drug development is the efficient translocation of protein-sized drugs into cells, because the mammalian cell membrane acts as a major obstacle to these hydrophilic large molecules, which could otherwise be highly specific, efficient, and tolerable pharmaceuticals. Internalization of these molecules can be achieved by clathrin-independent endocytosis (such as lipid-raft mediated/caveolar endocytosis), and this pathway is exploited by endogenous proteins, bacterial toxins (cholera and tetanus), and viruses (murine polyomavirus and echovirus 1), because this pathway tends to fuse with lysosomes only after a very long endosomal retention time, if at all. This endocytic mechanism is an attractive target to deliver functional proteins without degradation, and the leaky endosomes forming in the process allow direct escape for the molecules before moving to other cellular locations. The surface of these lipid rafts and caveolar pits are composed of various glycosphingolipids, especially of mono-, di-, and trisialotetrahexosylgangliosides (GM1, GD1a, GT1b), which are the major receptors for the natural cargoes. Binding and clustering the gangliosides induce an endocytic mechanism, where lysosomal fusion is negligible, allowing the proteins to reach the cytosol or undergo transcytosis. Many delivery systems fail to avoid lysosomal entrapment, or the molecule responsible for the internalization is required to be used at therapeutically irrelevant, high concentrations. Interpreting the glycan code by studying how gangliosides trigger endocytosis could be the key to solve these problems. Interest in binding gangliosides has already arisen; however, high-affinity molecular recognition is still a great challenge. The specific targeting of ganglioside GM1 is especially sought, because this ganglioside, while normally being expressed in many mammalian cell types, is highly abundant in cancerous cells. Therapeutic protein levels in the extracellular fluid yield 100–500 nM; therefore, a high-affinity interaction is needed to create a cell membrane enrichment that facilitates sufficient material flux in clinical applications.

Our main goal was to achieve nanomolar delivery of large proteins (up to the size of antibodies) via lipid raft-mediated endocytosis. We aimed to use a non-toxic peptide tag to mimic the ganglioside-mediated internalization of endogenous and exogenous proteins; therefore, we set out to find a minimal motif that can bind ganglioside GM1 with high affinity and specificity. By focusing on a structurally well-defined receptor and conducting a thorough biophysical characterization of the interaction, we aimed to open a way to structure-based design, which is rare in protein delivery approaches. We set out to investigate the ability of the characterized peptidic tag to deliver large proteins into the cells, while rigorously monitoring its toxicity,

mechanism of entry, and the tendency to fuse with lysosomes. Using a medicinal chemistry approach, we set out to establish a structure–activity relationship, to gain insight into the binding mechanism, and to improve the enzymatic stability while retaining high affinity.

2. METHODS

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetric experiments were performed with the synthesized peptides, peptide-conjugates and their target gangliosides (GM1, asialo GM1, GM3) using a MicroCal VP-ITC calorimeter. Ganglioside:dodecylphosphocholine 1:5 bicelles were titrated to peptides and their conjugates at pH 7.2, 35 °C using 15 μM ganglioside concentration.

Fluorescence activated cell sorting (FACS)

The internalization of peptides and peptide complexes was determined by flow cytometric analysis. Cells were incubated with peptides or peptide complexes at 37 °C for various times. The cells were then washed and harvested from the plates with trypsin-EDTA. Trypan blue and propidium iodide were added to the cells in phosphate buffered saline immediately before the cells were subjected to flow cytometric analysis (FACSCalibur flow cytometer, BD Biosciences). The data were evaluated using FlowJo™ software (FlowJo, LLC). For the in vitro competition assay, Jurkat cells were treated with peptide alone or with different concentrations of galectin-1. For the endocytosis inhibition experiments, HeLa cells were preincubated at 37 °C with methyl-β-cyclodextrin (MBCD), wortmannin, or chlorpromazine. The cells were then incubated with peptide complexes at 37 °C for 60 min, treated with trypan blue, and subjected to flow cytometric analysis as described above.

Confocal laser scanning microscopy (CLSM)

The cells were incubated with the studied complexes at different concentrations for different incubation times at 37°C. The cells then were washed with PBS; when antibody-complexes were used, they were also washed with β-lactose and the biotinylated peptide-NeutrAvidin complex without the primary and secondary antibody to remove surface-bound complexes. The cells were stained with Hoechst 33342 for 30 min at 37°C. In some experiments, after Hoechst staining, the cells were labeled with LysoTracker Red at 37°C according to the manufacturer's instructions. For the cholera toxin colocalization experiments, cells were co-incubated with FITC-labeled CTX-B subunit. For the structural test of the antibody complex, cells were treated with the complex, then the cells were fixed with paraformaldehyde at room temperature, permeabilized saponine and then cells were stained with Atto488-conjugated galectin-1 for 30 minutes at room temperature. For the IgG complex measurements, cell membranes were visualized with FITC-labeled WGA lectin after incubation

with the complex. FITC-NeutrAvidin complexes were treated with trypan blue to quench extracellular fluorescence. To observe the localization of the cargo, cell fluorescence was analyzed using a Leica SP5 AOBS confocal laser scanning microscope using the 405 nm UV diode (for Hoechst staining), the 488 nm argon laser line (for FITC and Atto 488 staining), the 543 nm HeNe laser line (for r-phycoerythrin and LysoTrackerRed staining) and the 633 HeNe laser line (for Alexa Fluor 647 staining). For emission detection, an appropriate spectral filter was used for each channel.

3. RESULTS AND DISCUSSION

1. We synthesized a lead molecule (peptide WYKYW) and measured its interaction with different gangliosides (GM1, asialo-GM1, GM3). We showed that the sequence had high affinity (K_D = 23.8 nM) and was specific towards ganglioside GM1. We showed that truncating the ganglioside GM1 resulted in decreased or completely abolished binding.

2. We found that fluorescent tagging in proximity of the peptide tag was detrimental to the ganglioside binding. We introduced and synthesized two linkers to lengthen the tag: a PEG-based one and a cell penetrating peptide (penetratin). We showed that both constructs retained high affinity towards ganglioside GM1.

3. We used a medicinal chemistry approach to set up a structure–activity relationship between the amino acids of the sequence and their binding efficiency. We demonstrated that every amino acid and their configuration was important in the binding. We identified several sequence analogues constructed with backbone homologation, which had comparable affinity with the parent sequence (Table 1).

Table 1. Binding affinities (K_D) of the first binding-step in nM of the original pentapeptide substituted in various manners. The binding stoichiometry (n_1) was 0.5 in all cases.

	^{N}**W**	**Y**	**K**	**Y**	**W**^{C}
original			23.8		
Ala-scan	not fittable	5755	10467	1694	1060
β-scan	4.3	60	332	40	86
D-scan	881	892	4523	3243	3926

4. We revealed the importance of the membrane components in the binding. We measured both the tryptophan insertion into the membrane and the decreased aromatic face-to-edge interactions during the binding.

5. We showed that the peptide tag was capable of triggering endocytosis at submicromolar concentrations of a 63 kDa protein, while avoiding the lysosomal pathways (Figure 1, 2a).

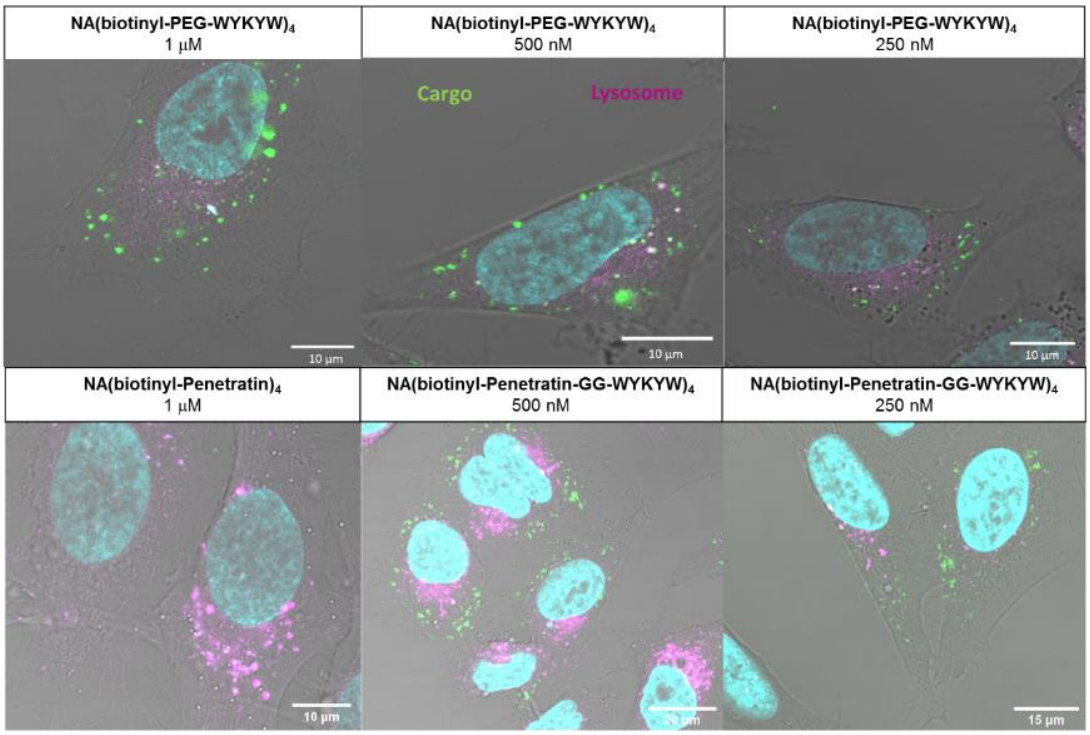

Figure 1. Internalization of the constructs at different concentrations by HeLa cells after 6 hours as determined by live confocal laser scanning microscopy. FITC-labeled NeutrAvidin is shown in green, Hoechst 33342-stained nuclei are shown in cyan, and LysoTracker Red-stained lysosomes are shown in magenta.

6. We proved that the translocation was energy dependent, and that the process could be blocked with methyl-β-cyclodextrin, which showed that the endocytosis was lipid raft-mediated (Figure 2b).

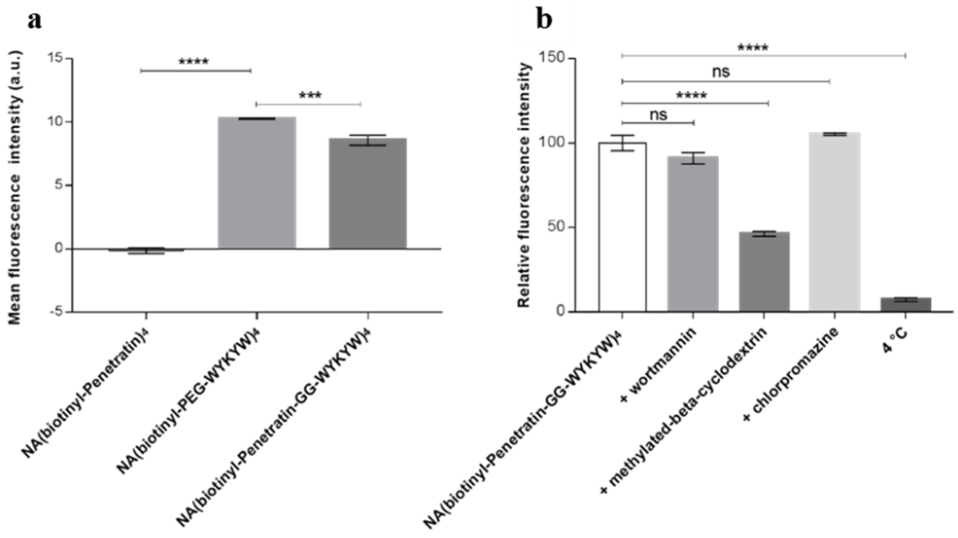

Figure 2. (**a**) Internalization of the constructs at 1 μM by HeLa cells after 1 hour as determined by flow cytometry. (**b**) Influence of endocytosis inhibitors on cellular uptake.

7. We demonstrated that the internalization was in correlation with the ganglioside GM1 content of the cells.

8. We carried out an in vivo titration with galectin-1, which could decrease the uptake of our complex to the base level displayed by our linker alone, strengthening the biomimetic behavior of our carrier. We confirmed this finding by observing co-localization of our complex and cholera toxin B subunit.

9. We showed that the WYKYW-tagged sequence could efficiently internalize antibody complexes (ca. 580 kDa) into human cells at low nanomolar concentrations. We observed a diffuse fluorescence throughout the cytoplasm, which we confirmed with artificial intelligence aided quantitative analysis (Figure 3).

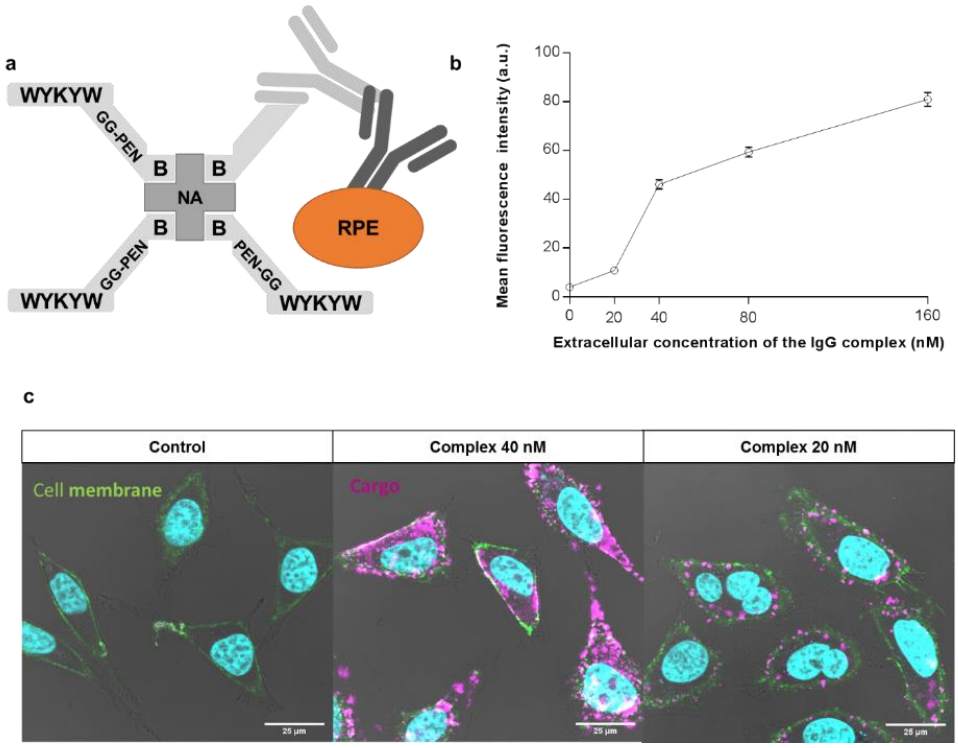

Figure 3. (**a**) Schematic representation of the bottom-up designed modular carrier–hub–antibody cargo–secondary antibody–r-phycoerythrin construct. (**b**) Artificial intelligence-aided quantitative analysis of the live CLSM images. HeLa cells were incubated for 6 hours with various concentrations of the IgG complex. (**c**) Delivery of the IgG complex into HeLa cells at various concentrations after 3 hours. R-phycoerythrin-conjugated secondary antibody is indicated in magenta; green staining defines cell membranes (WGA-FITC). Nuclei are indicated in cyan. Control cells were treated with r-phycoerythrin-labeled secondary antibody at 160 nM for 3 hours.

10. We verified that the molecular recognition between the primary and the secondary antibodies is functional. We showed that the delivered primary antibody is structurally intact.

11. We showed that substituted sequences could internalize the IgG complex. We demonstrated that weaker binder sequences could be sufficient when increasing the avidity of the construct. We showed that decreasing the tryptophan content of the peptide tag was beneficial to the uptake of the construct.

FULL PAPER AND PATENT RELATED TO THE THESIS

I. N. Imre, A. Hetényi, E. Szabó, B. Bodnár, A. Szkalisity, I. Gróf, A. Bocsik, M. A. Deli, P. Horvath, Á. Czibula, É. Monostori, T. A. Martinek (**2020**). Routing Nanomolar Protein Cargoes to Lipid Raft-Mediated/Caveolar Endocytosis through a Ganglioside GM1-Specific Recognition Tag. *Advanced Science*, 7, 190262

IF (2019): 15.84

II. N. Imre, A. Hetényi, E. Szabó, B. Bodnár, A. Szkalisity, I. Gróf, A. Bocsik, M. A. Deli, P. Horvath, Á. Czibula, É. Monostori, T. A. Martinek. ENDOCYTOSIS ROUTING SEQUENCE PEPTIDE FOR CELL DELIVERY SYSTEMS, Patent Application, Patent Number: P1900205, IP share: 20%

OTHER FULL PAPERS

III. R. Ismail, T. Sovány, A. Gácsi, R. Ambrus, G. Katona, N. Imre, I. Csóka (**2019**). Synthesis and Statistical Optimization of Poly (Lactic-Co-Glycolic Acid) Nanoparticles Encapsulating GLP1 Analog Designed for Oral Delivery. *Pharmaceutical Research, 36,* 99

IF: 3.242

IV. A. Bocsik, I. Gróf, L. Kiss, F. Ötvös, O. Zsíros, L. Daruka, L. Fülöp, M. Vastag, Á. Kittel, N. Imre, T. A. Martinek, C. Pál, P. Szabó-Révész, M. A. Deli (**2019**). Dual Action of the PN159/KLAL/MAP Peptide: Increase of Drug Penetration across Caco-2 Intestinal Barrier Model by Modulation of Tight Junctions and Plasma Membrane Permeability. *Pharmaceutics*, *11*, 73.

IF: 4.421

V. Zs. Hegedüs, I. Makra, N. Imre, A. Hetényi, I. M. Mándity, É. Monostori, T. A. Martinek (**2016**). Foldameric probes for membrane interactions by induced β-sheet folding. *Chemical Communications*, *52*, 1891

IF: 6.319

SCIENTIFIC LECTURES RELATED TO THE THESIS

1. N. Imre, A. Hetényi, E. Szabó, B. Bodnár, A. Szkalisity, I. Gróf, A. Bocsik, M. A. Deli, P. Horvath, Á. Czibula, É. Monostori, T. A. Martinek

   IgG bejuttatása specifikus GM1 gangliozid felismerő szekvenciával

   MTA Gyógyszerkémiai és Gyógyszertechnológiai Szimpózium

   Kecskemét, 2019. szeptember 5-6.

2. N. Imre, A. Hetényi, E. Szabó, B. Bodnár, A. Szkalisity, I. Gróf, A. Bocsik, M. A. Deli, P. Horvath, Á. Czibula, É. Monostori, T. A. Martinek

   IgG bejuttatása specifikus GM1 gangliozid felismerő szekvenciával

   MTA Peptidkémiai Munkabizottság Tudományos Ülése

   Balatonszemes, 2019. május 27-29.

3. N. Imre, B. Bodnár, E. Szabó, É. Monostori, T. A. Martinek

   Gangliozid-mediált sejtpenetráció

   MTA Peptidkémiai Munkabizottság Tudományos Ülése

   Balatonszemes, 2018. május 28-30.