Final version published as:

Orosz, Á., Bősze, S., Mező, G., Szabó, I., Herényi, L., Csík, G.:

Oligo- and polypeptide conjugates of cationic porphyrins: binding, cellular uptake, and cellular localization.

Amino Acids, 49(7): 1263-1276 (2017)

DOI: 10.1007/s00726-017-2428-z

URL: https://link.springer.com/article/10.1007/s00726-017-2428-z

Oligo- and polypeptide conjugates of cationic porphyrins: binding, cellular uptake and cellular localization

*Ádám Orosz[1], Szilvai Bősze[2], Gábor Mező[2], Ildikó Szabó[2], Levente Herényi[1], Gabriella Csík[1]**

[1]Institute of Biophysics and Radiation Biology, Semmelweis University, 1444 Budapest POB 263 Hungary

[2]Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, Pázmány Péter sétány 1/A, Budapest H-1117, Hungary

*Corresponding author. 1444 Budapest POB 263 Phon/Fax: 36-1-266-6656, csik.gabriella@med.semmelweis-univ.hu

ORCID

G. Csik: 0000 0001 8464 2534;

L. Herenyi: 0000 0003 0535 0256

G. Mez: 0000-0002-7618-7954

A. Orosz: 0000-0002-2680-343X

I. Szabó: 0000-0002-9844-7841

Acknowledgements

This work was supported by research grant Országos Tudományos Kutatási Alapprogramok (OTKA) K104045 and K104275.

Abstract

Recently we have characterized the DNA and nucleoprotein (NP) binding of bis(4-*N*-methylpyridyl)-15,20-di(4-carboxyphenyl)porphyrin (BMPCP) and meso-tri(4-*N*-methylpyridyl)-mono(4-carboxyphenyl)porphyrin (TMPCP) and their tetra-peptide conjugates (BMPCP-4P_2 and TMPCP-4P, respectively). In this work we investigated the interaction of TMPCP conjugated to the tetra-peptide branches of branched chain polymeric polypeptide with poly-L-lysine backbone (AK) with DNA or NP using spectroscopic methods.

Analysis of absorption spectra revealed the external binding, but no intercalation of TMPCP-AK to DNA. There was no evidence for the interaction between TMPCP-AK and encapsidated DNA. Furthermore, we examined the cellular uptake of BMPCP and TMPCP and their tetra- or polypeptide conjugates by flow cytometry and analyzed how charge, size and structure of the compounds affect their incorporation. In comparison, liposomal association constants of these derivatives were determined. BMPCP-4P_2 accumulated the most, and porphyrins with two positive charges (BMPCP and BMPCP-4P_2) showed better accumulation than the tri-cationic TMPCP or TMPCP-4P.

Cellular uptake of polycationic TMPCP-AK was significantly lower than that of the free or tetra-peptide conjugated derivatives. The sub-cellular localization of all the five compounds was investigated in co-localization studies by confocal microscopy with special attention to their nuclear localization. Neither free nor conjugated BMPCP or TMPCP were co-localized with nuclear marker. Instead, these derivatives showed co-localization with lysosomal and mitochondrial fluorescent probes. TMPCP-AK conjugate had different localization pattern appearing mainly in mitochondria and cytoplasmic vesicles.

Our results may contribute to the further design of DNA targeting porphyrin based constructs.

Keywords

Cationic porphyrin, peptide conjugate, DNA binding, lipid association constant, cellular uptake, intracellular localization

1. Introduction

Porphyrins are best known for their anticancer applications in photodynamic therapy (PDT). PDT is being developed as a treatment for cancer (Brown et al. 2004; O'Connor et al. 2009). However, it was approved also in non-oncological applications, such as the treatment of age-related macular degeneration, polypoidal choroidal vasculopathy (Ziemssen and Heimann 2012) or dermatological diseases (Wan and Lin 2014) or as an alternative of antimicrobials. Porphyrinoid derivatives are also under investigation as fluorescent bioimaging agents in the early diagnosis of cancer, as delivery agents in boron capture therapy (Soloway et al. 1998) or peptide conjugates in regulation of genetic material.

Among others, meso-substituted cationic porphyrins gained special interest because of their interaction with nucleic acids. It was shown that out of water-soluble tetra-cationic 5,10,15,20-tetra(*N*-methylpyridinium-4-yl)porphyrin (TMPyP) naturally drawn to the DNA polyanion, tri- or dicationic derivatives also bind to DNA by intercalation or by external binding (Pasternack et al. 1983; Shui et al. 2000; Zupan et al. 2004; D'Urso et al. 2009; Gong et al. 2012; Accetta et al. 2015).

Recently, different bioactive moieties were introduced onto the periphery of cationic porphyrins to increase their cellular uptake or to utilize porphyrins as DNA targeting agent. Among others, peptide conjugates were also synthesized either to increase the cellular uptake of porphyrin derivatives or to deliver the peptide moiety to the nucleic acids (Orosz and Csik 2016; Sibrian-Vazquez et al. 2010, 2018; Dosselli et al. 2013).

Branched chain polymeric polypeptides with poly-L-lysine backbone (e.g. poly[Lys-(DL-Ala_m)] (AK), developed in our laboratory (Hudecz and Szekerke 1980) are efficient carrier to target drugs into the infected cells by fluidic endocytosis (Hudecz 1995). The increased endocytotic properties of tumor cells provide some selectivity for the cellular uptake of the polypeptide – drug conjugates. The core of these branched chain polypeptides with poly(Lys)

backbone possess oligo-DL-Ala branches (in average 3 racemic alanine residues). We initially designed porphyrin conjugates of a tetrapeptide (Ac-Lys(H-Ala-D-Ala-Ala)-NH_2) as representative monomeric unit of branched polypeptide AK.

In our previous studies, we presented the design and synthesis of porphyrin-tetrapeptide conjugates. In these conjugates a tetrapeptide (Ac-Lys(H-Ala-D-Ala-Ala)-NH_2) (Mező et al. 2011; Orosz et al. 2013) was attached to meso-tri(4-*N*-methylpyridyl)-mono-(4-carboxyphenyl)porphyrin (TMPCP) or meso-5,10-bis(4-*N*-methylpyridyl)-15,20-di-(4-carboxyphenyl)porphyrin (BMPCP).

It was shown that peptide conjugates of di- and tricationic porphyrins, like their unconjugated forms, bind to naked DNA by two distinct binding modes which can be identified as intercalation and external binding. Tri-cationic structure and elimination of negative charges in the peptide conjugates is preferable for the improved binding. Investigation was extended to the binding of porphyrin conjugates to nucleoprotein complexes. We found that the viral capsid protein does not inhibit the binding of cationic species to DNA; tetrapeptide-conjugates of cationic porphyrins with two or three positive charges bind to encapsidated DNA in T7 phage nucleoprotein complex (NP).

In order to extend our studies, here we report on the synthesis of a conjugate in which TMPCP is attached to branched chain polypeptide with polylysine backbone (TMPCP-AK), and we characterize the binding properties of this conjugate to isolated DNA, as well as to nucleoprotein complex (NP).

However, based on the binding of cationic porphyrins to naked DNA or nucleoprotein complex it is not possible to predict their binding to intracellular targets. A prerequisite of intracellular drug-DNA binding is the cellular uptake of the drug, and its accumulation in the nuclear region or in mitochondria. It was shown before that the nature of the electric charge of porphyrin derivatives plays an important role in the interaction with biological targets.

Positively charged porphyrins proved to accumulate more readily in cultured cells, suggesting a significant electrostatic contribution (Lambrechts et al. 2004; Ricchelli et al. 2005; Jensen et al. 2010).

Further in this study, we examined the cellular uptake of di- and tri-cationic porphyrins BMPCP and TMPCP, and their tetra- or polypeptide conjugates, and analyzed how charge, size and structure of the molecules affect their incorporation. Their sub-cellular localization was also investigated with special respect to their nuclear localization.

2. Materials and Methods

2.1. Materials

meso-Tri(4-*N*-methylpyridyl)-mono(4-carboxyphenyl)porphyrin (TMPCP) and meso-5,10-bis(4-*N*-methylpyridyl)-15,20-di(4-carboxyphenyl)porphyrin (BMPCP) were purchased from Frontier Scientific (Carnforth, UK). Porphyrins were stored at 4° C in powder form, or as a stock solution in distilled water or in methanol. Before experiments the porphyrin stock solutions were diluted into methanol or into a buffer solution.

Bacteriophage DNA was prepared from T7 phage (ATCC 11303-B7) grown on Escherichia coli (ATCC 11303) host cells. The cultivation and purification were carried out according to the method of Strauss and Sinsheimer (Strauss and Sinsheimer 1963). The phage suspension was concentrated on a CsCl gradient and dialyzed against buffer solution composed of 20 mM Tris - HCl and 50 mM NaCl, (pH=7.4).

Nucleoprotein complex was incubated with SDS (1,4 μg SDS for 1 μg protein) at 65^{o}C for 30 min; followed by precipitation with 1M KCl on ice for 10 min. The precipitate was centrifuged twice for 10 min in an Eppendorf microcentrifuge at 13000 rpm. The DNA was precipitated with ethanol from the supernatant. The pellet was washed with 70% ethanol, and resuspended in buffer solution 20 mM Tris - HCl , 50 mM NaCl, (pH=7.4). The amount of

DNA was determined spectrophotometrically at λ=260 nm. The quality of the DNA was checked by gel electrophoresis and by its absorption spectrum.

2.2 Synthesis of branched chain polypeptide – porphyrin conjugate

Poly[Lys-(DL-Ala)$_m$] (AK) branched chain polymeric polypeptide with polylysine backbone was prepared as described before (Hudecz and Szekerke 1980; Hudecz et al. 1985). Briefly, poly[L-Lys] was synthesized by the polymerization of N$^{\alpha}$-carboxy-N^{e}-benzyloxycarbonyl-lysine anhydride under conditions that allowed a number average degree of polymerization [DP$_n$] approximately 250. Protecting groups were cleaved by HBr and DL-alanine oligomers (DL-Ala$_m$) were grafted to the ε-amino groups of polylysine by polymerisation of N-carboxy-DL-alanine anhydride to produce poly[Lys(DL-Ala$_m$)] (AK). Samples were dialyzed against distilled water, freeze-dried. From viscometry analysis of polylysine, the number average degree of polymerization (DP$_n$) and the average molar mass were calculated. The average molar mass of the branched polypeptide (AK) was estimated from DP$_n$ of polylysine backbone and from the amino acid composition of the side chains determined by quantitative amino acid analysis.

32 mg AK was dissolved in 500 μl distilled water then the solution was diluted with 5 mL DMF. Tri-cationic meso-tri(4-*N*-methylpyridyl)-mono(4-carboxyphenyl)porphyrin (TMPCP) (20.3 mg, 0.25 equiv of the polymer) and 44.2 mg (4 equiv of TMPCP) BOP reagent were dissolved in 4.5 mL DMF. 34 μl DIEA (8 equiv of TMPCP) was added to the solution. Then the two solutions were mixed and stirred for overnight at RT. Afterwards, the unreacted TMPCP and side products were removed by dialysis (cut off 10000 Da) was continued for two days followed by the lyophilization of the conjugate. 29 mg of TMPCP-AK conjugate was yielded. TMPCP content was determined spectrophotometrically (Hudecz et al. 1993), using the molar absorptivity of TMPCP-4P at λ=436 nm (ε=4.62E5 M^{-1} cm^{-1}) (Mező et al.

2011). It was assumed that the TMPCP coupled to the α-amino group of terminal alanine of the branch, has the same molar absorptivity as TMPCP coupled to α-amino group of alanine in tetrapeptide Ac-Lys(H-Ala-D-Ala-Ala)-NH_2.

2.3. Synthesis of Ac-Lys(H-Ala-D-Ala-Ala)-NH_2

The synthesis of Ac-Lys(H-Ala-D-Ala-Ala)-NH_2 model tetrapeptide was described previously (Mező et al. 2011). Briefly, the Ac-Lys(H-Ala-D-Ala-Ala)-NH_2 was prepared by solid phase peptide synthesis on 4-methylbenzhydrilamine resin (MBHA) using standard Boc-strategy. The peptide was cleaved from the resin with liquid hydrogen fluoride (HF) in the presence of *p*-cresol as scavenger (0.5 g / 10 ml HF) at 0^oC for 1h. The crude product was precipitated with dry ether and it was purified by RP-HPLC. The purified tetrapeptide was characterized by analytical HPLC and ESI-MS.

2.4. Conjugation of porphyrin derivatives to Ac-Lys(H-Ala-D-Ala-Ala)-NH_2

Conjugation of porphyrin derivatives was performed as described previously (Mező et al. 2011). Briefly, the porphyrin derivatives were attached to the tetrapeptide in DMF solution by the aid of coupling agents, either BOP reagent or a water soluble carbodiimide EDC. BOP reagent was preferred to develop TMPCP conjugate, however, better yield was observed in the preparation of BMPCP conjugate when EDC was applied. One tetrapeptide was conjugated to the porphyrin in the case of TMPCP, while BMPCP cross-linked two tetrapeptide chains. The conjugates were purified by RP-HPLC and characterized by ESI-MS.

2.5. Absorption spectroscopy

Ground-state absorption spectra of porphyrin and porphyrin-peptide conjugate solutions were recorded with 1 nm steps and 2 nm bandwidth by use of a Cary 4E (Varian, Mulgrave, Australia) spectrophotometer at various DNA concentrations. In DNA containing samples, the

composition of solutions was expressed in terms of an *r* number representing the molar ratio of DNA base pairs to porphyrin molecules.

In the applied concentration range free porphyrins were in monomeric state. Spectral changes due to the adsorption of porphyrins on the cuvette wall were less than 5%.

2.6. Decomposition of absorption spectra

The spectral decomposition was performed for absorption spectra [$A(\lambda)$, absorbance versus wavelength] of the series of TMPCP-AK–DNA-complex solutions with various base pair/porphyrin molar ratios (*r*). All the spectra were analyzed in the λ=370-490 nm wavelength range.

For fitting we used the Gaussian multi-peaks fit routine from the Microcal Origin software. The error of the fit was determined as:

$$\chi^2 = \frac{\sum_{\lambda=370}^{490}\left[A(\lambda)_{measured} - A(\lambda)_{calculated}\right]^2}{\sum_{\lambda=370}^{490} A(\lambda)_{measured}} \qquad (1)$$

We did not apply the usual wavelength-frequency conversion, because the maximum errors of the fitting parameters due to the absence of this conversion were not higher than 0.5 nm. It was assumed that all the measured spectra [$A(\lambda)$] can be considered as a sum of the component spectra. These components can be fitted by Gaussians, thus:

$$A(\lambda) = \sum_{i=1}^{n} \frac{A_i}{w_i\sqrt{\pi/2}} \exp\left(\frac{-2(\lambda-\lambda_i)^2}{w_i^2}\right) + y_0 \qquad (2)$$

where A_i is the total area under the i^{-th} band; λ_i is the center, w_i is the full widths of the same band. For this fitting we used the Gaussian multi-peaks fit routine from the Origin software. We did not use any preconceptions for the fitting, just a general purpose was applied: the least

number of possible components for an acceptable fit. The only parameters changing with the concentration of the porphyrin are A_i-s, and all other parameters are constant for such a series

2.7. Optical Melting Measurements

Thermal denaturation curves of bacteriophage solutions were recorded by absorbance at 260 nm on a Cary 4 E spectrophotometer (Varian, Mulgrave, Australia) equipped with a Peltier thermoregulator. The heating rate was 0.5 °C/min in the temperature range 25 – 98 °C. Five samples were measured in parallel using an automatic cell changer; the sixth sample holder was used to measure the temperature in an identical quartz cell filled with buffer. The cell holder was insulated to ensure that the temperature did not vary more than 0.1 °C between cells, even above 90 °C. The initial absorbance of the samples was adjusted to approximately 0.3 at λ=260 nm in quartz cells of 1 cm path length. The absorbance data were collected at every 0.5 °C. Data were treated using the program Origin7. The curves were normalized to the absorbance at room temperature, smoothed in five point intervals; derivative melting curves were calculated from the differences between adjacent points and once more smoothed for five points. The peak of derivative melting curve was accepted as the corresponding melting temperature (T_m).

2.8. Fluorescence spectroscopy

Corrected steady-state emission and excitation spectra were obtained using Fluorolog 3 spectrofluorometer (Jobin Yvon, France). Samples were excited at the corresponding excitation maxima at room temperature. If the absorbance of the sample exceeded 0.05, the observed fluorescence intensity was corrected for inner filter effect of the solution.

2.9. Incorporation of porphyrin derivatives in vesicles

Small unilamellar vesicles (SUV) of L-α-phosphatidylcholine dipalmitoyl ((DPPC) (Sigma Chemicals Co.) were prepared by sonication. Stock solution of DPPC in chloroform was dried to a film using a rotary evaporator. Lipids were hydrated with phosphate buffer. Sonication was performed using an MSE Ultrasonic Desintegrator (frequency 20 kHz; wave-amplitude 8 μm). The power output was approx. 150 W. Clear solutions were obtained after 15-20 min sonication. The mean diameter of the liposomes was about 80 nm as measured by dynamic light scattering. The final phospholipid concentration in the stock solution was 1.5 mM.

Porphyrin derivatives of 0.1 μM concentration were incubated with DPPC liposomes in phosphate buffer solution pH=7.4. The phospholipid concentration was varied from 1 μM to the required concentration for the total incorporation of the porphyrin. If the phospholipid concentration is large compared to that of the dye it can be assumed that the state of occupancy of a vesicle does not influence the interaction with further molecules and the system can be described by focusing on the dye in its various environment (Clarke and Appel 1989). In these conditions the association constant (K_L) can be defined as

$$K_L = \frac{[P_i]}{[P_f][DPPC]} \tag{3}$$

where P_i and P_f denote the incorporated and free porphyrin, respectively. At the porphyrin concentration used the fluorescence intensity emitted by each form can be assumed to be directly proportional to its concentration (Margalit and Rotenberg 1984). The total fluorescence intensity (F) is the sum of all contributions. Thus, at fixed porphyrin concentration, the association constant can be determined from the fluorescence intensities of the dye at a given wavelength (Brault et al. 1986)

$$F = F_0 + (F_\infty - F_0)\frac{K_L[DPPC]}{1 + K_L[DPPC]} \tag{4}$$

where F_0 and F_∞ are the fluorescence intensities corresponding to nil and full incorporation of the porphyrin in the vesicle, respectively.

2.10. Cell cultures for in vitro studies

HL-60 (ATCC: CCL-240) (Gallagher et al. 1979) and HT-29 (ATCC: HTB-38) (von Kleist et al. 1975) cells were used for determination of *in vitro* cellular uptake profile of the compounds. HL-60, human acute promyelocytic leukemia cells, were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich), L-glutamine (2 mM) and gentamicin (160 μg/ml; Sigma-Aldrich). HT-29, human colon adenocarcinoma cells, were grown in RPMI-1640 (Sigma-Aldrich) supplemented with 10% FCS, L-glutamine (2 mM) and gentamicin (160 μg/ml). Cells were maintained in plastic tissue culture dishes (Sarstedt, Nümbrecht, Germany) at 37°C with a humidified atmosphere containing 5% CO_2 / 95% air.

2.11. Determination of the in vitro cellular uptake by flow cytometry

Measurements of cellular uptake of the compounds were evaluated by flow cytometry (BD LSR II, BD Biosciences, USA) on HL-60 human acute promyelocytic leukemia cells. Cells were harvested in the logarithmic phase of growth and plated on a 24-well tissue culture plate (10^5 cells/1 ml medium/well) 24 h prior to the experiment. Compounds were dissolved in serum free RPMI-1640 medium, and running dilutions were prepared. Cells were treated with the compounds at 2.5-20 μM concentration range for various time periods between 0,5 – 5 hours. Cells, treated with serum-free medium for similar time period were used as controls. After incubation, treatment solutions were removed, and the cells were washed twice with serum-free medium and trypsinized (Estrada and Gitler 1974; Ramos 1983; Madani et al. 2011). After washing steps, the supernatant was completely removed, and 100 μL of 1 mM trypsin (Sigma) was added to the cells. The effect of trypsin was stopped by 900 μL Hepes

buffer (HPMI; 100 mM NaCl, 5.4 mM KCl, 0.4 mM MgCl2, 0.04 mM CaCl2, 10 mM Hepes, 20 mM glucose, 24 mM $NaHCO_3$ and 5 mM Na_2HPO_4 at pH = 7.4 (Kapus et al. 1994;) containing 10% FCS, and the cells were transferred from the plate to FACS-tubes. Cells were centrifuged (1000 rpm, 5 min, 4 °C) and the supernatant was removed. After this procedure, cells were re-suspended in 500 μL HPMI. Intracellular fluorescence intensity of cells was monitored by BD LSR II (BD Biosciences, UK) flow cytometer. Fluorescence intensity of cells relative to the autofluorescence of controls, was measured, and/or fluorescence positive cells were counted. Samples were excited at 488 nm (Coherent Sapphire, 22 mW), and fluorescence intensity was detected in the 670-735 nm range. Data were analyzed with FACSDiVa 5.0 software. All measurements were performed in duplicate.

2.12. Confocal microscopy

Intracellular localization studies were conducted with a Zeiss 710 (Carl Zeiss Microscopy, Jena, Germany) confocal microscope, using its 63x oil immersion objective. 30000 HT-29 cells/well were seeded onto Lab-Tek II 8-chamber Slide (Thermo Fisher Scientific Inc. NYSE: TMO) and they were allowed to grow for 24 h before the experiment. The porphyrin derivatives were then added to a final concentration of 20 μM in RPMI medium and incubated for 3 hours.

For colocalization experiments organelle specific fluorescent labels were obtained from Molecular Probes (Eugene, Oregon, USA). For nuclear DNA staining the nucleic acid binding fluorescent dye SYBR GreenI was used at an incubation concentration of 400 nM, lysosomes were traced with Lyso Tracker Green DND-26 at 50 nM, mitochondria were stained using MitoTracker Deep Red FM at 100 nM. The organelle specific dyes were diluted in RPMI medium and the cells were incubated for 30 min concurrently with the porphyrin derivatives before washing twice with HPMI. Then the cells were immediately analyzed with confocal microscopy. Samples containing the porphyrin derivatives were excited at λ=488 nm, while

detecting the fluorescence emission between λ=650-750 nm. SYBR Green was excited with the same 488 nm laser, but the emission was registered at λ=520 nm. In the case of LysoTracker Green and MitoTracker Deep Red FM the excitation wavelengths were λ=488 nm and λ=510 nm, detection wavelengths were λ=633 nm and λ=665 nm, respectively. As control samples, cells incubated with one dye only, or dye-free medium, were measured simultaneously. The acquired images were elaborated with the ImageJ software.

3. Results

3.1. Synthesis and characterization of TMPCP- branched chain polypeptide conjugate.

In order to investigate DNA/NP binding and cellular localization of TMPCP – conjugate, TMPCP was attached to branched chain polypeptide with polylysine backbone. Branched chain polymeric polypeptides (poly[Lys-(DL-Ala_m)] (AK) was synthesized as described above. The average degree of polymerization (DP_n) of poly[Lys] was 250 as determined by viscometry. According to the amino acid analysis DL-Ala/Lys ratio was 2.7 (M_{Ala}/M_{Lys}). The average molecular mass of the monomer unit [Lys-(DL-$Ala_{2,7}$)] of the polymer was 320 Da.

The coupling of TMPCP to branched polypeptide was achieved by the carbodiimide strategy: one carboxyl group of the TMPCP was linked to the α-amino group of the terminal amino acid to provide covalent α-amide bonding. Average degree of substitution was 17.8 %($M_{TMPCP}/M_{monomer}$%). Schematic representation of TMPCP-AK conjugate is presented in Fig. 1.

3.2. Binding of TMPCP-AK to DNA and NP

Absorption spectra of TMPCP-AK were recorded at constant porphyrin and various DNA and NP concentrations. Concentration of TMPCP-AK was determined from the absorbance of TMPCP using Lambert-Beer's law.

Fig. 2a and 2b show two series of TMPCP-AK absorption spectra recorded at increasing base pair/porphyrin ratio (*r*) in the presence of naked DNA and NP. In insets the corresponding normalized Soret bands are presented.

At increasing concentration of DNA or NP, the overall changes of the spectra can be characterized by hypochromism. In the case of DNA binding, a few nanometer red shift and the widening of Soret band can be also observed.

The absorption spectra of the conjugate were fitted by component Gaussian function as described above. It is interesting to note that around the center of Soret band two Gaussian components were resolved (λ=418 and λ=421 nm) already without the presence of DNA. The reason for the splitting of Soret band can be that in polymeric AK-conjugate the environment of porphyrin moieties is not perfectly identical. Besides the main component bands two additional components were found in the shoulder region at λ=372 and λ=407 nm. These results fit to the results received before for cationic porphyrins and their tetrapeptide conjugates (Mező et al. 2011; Orosz et al. 2013). Decomposition of the spectra recorded in the presence of DNA, showed one additional Gaussian component centered around λ=435 nm.

The relative areas under the Gaussians components were determined at various base pair/porphyrin ratios (*r*). The change in relative area of a component indicates the change of relative concentration of the corresponding porphyrin population. In Fig. 3, A_i-s are presented as the function of *r*. Component bands of the free porphyrins are diminished by the increasing base pair/porphyrin ratios and, in parallel to that, the relative area of the band with a maximum at λ=435 nm, induced by the presence of DNA, increases.

These results indicate that the porphyrin moiety of TMPCP-AK binds to the nucleic acid, and out of the non-bound porphyrin molecules one distinct population of bound TMPCP-AK can be formed in the presence of DNA.

The presence of phage nucleoprotein complex (NP) induces a moderate hypochromicity of TMPCP-AK, but neither red shift nor increase or presence of a new band within the Soret region can be recognized.

3.3. Optical melting measurements

The thermal stability changes of DNA, or NP, induced by porphyrin derivatives can provide further information about the existence of DNA-porphyrin interaction(s). In order to identify possible structural changes caused by the porphyrin binding, the thermal stability of DNA and NP was followed by optical melting method, i.e., the thermal denaturation of the whole T7 phage and isolated DNA was monitored via the changes in their absorbance at λ=260 nm.

The derivative melting curves of isolated DNA and NP, recorded at various TMPCP-AK and constant base pair concentrations, are presented in panels a and b of Fig. 4.

All curves in Fig. 4a and 4b show the structural transitions typical for DNA and NP, respectively. These are the hyperchromic transitions around 80 - 85 ºC both in DNA and NP and a hypochromic change between 50 and 60 ºC for NP (Csik et al. 2009). The hyperchromic transition is attributed to the denaturation of the DNA double helix: the opening of the H bonds, weakening of the stacking interaction and the separation of the two single strands. The low-temperature transition is due to the disruption of the phage capsid.

It can be seen in Fig. 4a that the strand separation temperature of DNA is slightly shifted in the presence of TMPCP-AK conjugate. This means about 1 ºC increase even at 0.8 porphyrin/base pair molar ratios (*1/r*). Strand separation temperature of encapsidated DNA (NP) is not influenced by TMPCP-AK (Fig. 4b). Also, melting parameters of phage capsid disruption remain unchanged up to 0.8 porphyrin/base pair molar ratios; that is, the presence of this compound does not influence the stability of phage protein capsid and/or DNA-protein interaction.

3.4. Interaction of porphyrin derivatives with liposomes

Unilamellar liposomes can be considered as simple model of the lipid region of biological membranes. Partition of compounds between lipid and water phase based on their relative hydrophobicity can predict the relative permeability of biological membranes for those molecules. Small unilamellar liposomes (SUV) were prepared from phospholipids with neutral head groups (DPPC) and the association of porphyrins was investigated by fluorimetric technique. The liposomal interactions were studied at 0.1 μM concentration of porphyrins, in order to assure the predominance of monomeric species.

On mixing with vesicles TMPCP and BMPCP and their tetra-peptide conjugates exhibit an increase in their fluorescence intensity as it is shown in Fig. 5. These observations indicate an interaction between liposomes and cationic porphyrin derivatives. The equilibrium constants for the association of porphyrins to liposomes (K_L) were obtained using equation 2, and are presented in Table 1.

3.5. Cellular uptake

HL-60 cells were incubated with c=0–200 μM porphyrin derivatives for 0–24 h. Incorporated fluorescent species were detected by flow cytometry.

Prior characterization of the uptake, the cellular toxicity of compounds was investigated. It was found that neither of the compounds studied is toxic up to 50 μM concentration and up to 5 h incubation period (data not shown). Over 50 μM, toxicity of TMPCP-AK gradually increased with the concentration (data not shown).

Percentage of stained cells increases in correlation with incubation period and porphyrin concentration as it is shown for TMPCP in Fig. 6. At c=20 μM TMPCP, the ratio of porphyrin positive cells was almost 100 % after 3 hours incubation. This kinetic is typical for several porphyrins investigated by other authors (Moret et al. 2015). Similar pattern was received for

BMPCP, TMPCP, TMPCP-4P, however, the saturations were detected at different porphyrin concentrations.

In Fig. 7 ratios of porphyrin positive cells incubated with the five derivatives are compared after 3 hours incubation period at various incubation concentrations. Highest ratio was found always for BMPCP-4P_2, followed by the others, in the next order: BMPCP > TMPCP > TMPCP-4P >> TMPCP-AK.

3.6. Intracellular localization

Intracellular localization of the porphyrin derivatives was studied by confocal microscopy after 3 h incubation period at 20 μM drug concentration. Nucleus and selected cytoplasmic, organelles, lysosomes and mitochondria were stained with specific fluorescent probes.

None of the investigated derivatives, neither free cationic porphyrins nor their conjugates were co-localized with nuclear binding SYBR Green I. As an example, BMPCP-4P_2 localization in SYBR Green I stained cells are shown in Fig. 8.

BMPCP, TMPCP and their tetra-peptide showed co-localization with lysosome specific fluorescent dye LysoTracker Green DND-26 and mitochondrium specific probe MitoTracker Deep Red FM. As an example, in Fig. 9 subcellular localization of BMPCP-4P_2, LysoTracker Green DND-26 and MitoTracker Deep Red FM in HT-29 cells is presented. The porphyrin localization is shown by red, and that of lysosome and mitochondrium specific dyes is indicated by green; therefore, yellow color represents common loci in the overlay images. In corresponding images, BMPCP-4P_2 seems to be co-localized in a greater extent with lysosomal stain, while showing only limited coincidence with the typically fibrillar cytoplasmic pattern of the mitochondria. The dominancy of lysosomal localization was the highest in the case of un-conjugated TMPCP (data not shown).

The AK-conjugated porphyrin on the other hand exhibited a different subcellular distribution (see Fig. 10), staining the membranous compartments of the cytoplasm, therefore

showing more affinity to mitochondria than to lysosomes, while also significantly accumulating in other subcellular membranes.

4. Discussion

Recently we synthesized two new porphyrin-tetrapeptide (Ac-Lys(H-Ala-D-Ala-Ala)-NH_2) conjugates which can be considered as a typical monomer unit corresponding to the branch of porphyrin-branched chain polymeric polypeptide conjugate. One of the new compounds was derived from tri-cationic TMPCP with the conjugation of a single tetrapeptide, while in the other compound two identical tetrapeptides were linked to the bi-cationic BMPCP. DNA binding of porphyrin derivatives, and their peptide conjugates was investigated earlier with comprehensive spectroscopic methods. It was found that both porphyrin derivatives and their peptide conjugates can bind to DNA and reflect on at least two distinct binding modes, i.e., intercalation and external binding (Mező et al. 2011; Orosz et al. 2013). Among others, these binding forms were identified by their absorption bands, since typical absorption spectra of bound forms show characteristic features relative to the free porphyrin: about 20 nm red shift and about 40% hypochromicity of the Soret band for intercalation, and a fewer red shift and about 5% hypochromicity for external binding (Zupan et al. 2004; Pasternack et al. 1993; Lee et al. 2001). For example, in the case of TMPCP-4P the new bands were centered around 429 nm (for external binding) and 446 nm (for intercalation).

In order to complete our binding studies, we have synthesized TMPCP conjugates of branched chain polymeric polypeptide with poly(L-Lys) backbone (TMPCP-AK) and its binding to naked DNA and T7 nucleoprotein complex was investigated.

Absorption spectra of TMPCP-AK show significant changes at increasing concentration of DNA. These changes reflect the decrease of free porphyrin and increase of bound porphyrin concentration with increasing base pair/porphyrin ratios. However, the alterations of absorption spectra are significantly different from the changes experienced in the case of

TMPCP or TMPCP-4P under similar conditions (Mező et al. 2011; Orosz et al. 2013), i.e., the results of spectral decomposition show clearly the presence of only one type of bound chromophore. The position of the band, the week hypochromicity and the lack of the energy transfer between nucleotide bases and porphyrin (data not shown) rather suggest an external binding. Although, the absorption maximum of this binding form does not match perfectly with neither maxima received before for TMPCP-tetrapeptide conjugates. This could be explained by the possibly different molecular environment of the conjugated porphyrins in the polymeric chain. Such a way the absorption maximum received from the fitting procedure is the result of absorption maxima of different populations of bound porphyrins even if the binding mode is the same in each case.

The lack of intercalation is also supported by the optical melting results. The strand separation temperature of DNA is slightly increased by the presence of TMPCP-AK. However this temperature increase is significantly smaller than that was measured before for cationic porphyrins or their tetrapeptide conjugates at similar porphyrin/base pair ratios. Moreover, there is no linear correlation between phase transition temperature and porphyrin/base pair ratio, that would be an indication of intercalation.

As it was shown before, presence of protein capsid in T7 phage does not oppose the binding of unconjugated or tetrapeptide conjugated cationic porphyrins to DNA. In the case of TMPCP-AK the absorbance of the compound is also reduced in the presence of NP indicative of porphyrin binding. However, there is no evidence proving that this interaction takes place between porphyrin and nucleic acid.

Cationic porphyrins and their investigated conjugates can be taken up by HT-29 cells in a time and concentration dependent manner. Several authors showed before that the cellular uptake of cationic porphyrins varies with the number and distribution of positive charges, the

size and the hydrophilic/lipophilic character of the compounds (Jensen et al. 2010; Rajaputra et al. 2013; Ezzeddine 2013).

Among our series BMPCP-4P_2 accumulated the most, and derivatives with two positive charges (BMPCP and BMPCP-4P_2) showed better accumulation than tri-cationic TMPCP or TMPCP-4P. Cellular uptake of polycationic TMPCP-AK was significantly lower than that of the free or tetra-peptide conjugated derivatives. This finding are in harmony with the result of Jensen et al. (2010) reporting that two positive charges in mezo-position are the most beneficial for the cellular uptake.

In order to investigate the role of relative hydrophobicity in the cellular incorporation, the association constants for the studied compounds, to liposomes composed of neutral phospholipids, were determined. These values are typical for cationic porphyrins (Angeli et al. 2000). Parallel to the cellular uptake, higher association constants were found in case of di-cationic then tri-cationic derivatives. For the polycationic TMCP-AK significantly smaller K_L value and lower uptake was received.

Cellular uptake and association constants are not uniformly influenced by the presence of tetra-peptide moiety in di- and tri-cationic derivatives. Both of them are increased in the case of BMPCP-4P_2, but cellular uptake is smaller for TMPCP-4P than for TMPCP. These findings indicate that amphiphilic character is better for the cellular uptake (Ezzeddine et al. 2013) but other factors like charge distribution (Rajaputra et al. 2013) size and geometry of the compound could also influence the interactions with the cell.

The weaker performance of TMPCP-AK in these studies can be explained by its polycationic nature and significantly different size. These parameters can lead also to different uptake mechanism. As it was shown by Szabo et al. free AK is predominantly incorporated by macropinocytosis and caveole/lipid raft mediated endocytosis, and the efficacy of the internalisation of its derivatives is markedly influenced by the charge properties.

Based on their well characterized binding to DNA, cationic porphyrins have been recommended as potential targeting vehicles of oligo- or polypeptides to the close vicinity of nuclear DNA (Biron and Voyer 2008). This possibility was tested when we investigated the cellular distribution of cationic porphyrins and their peptide conjugates with confocal laser microscopy.

Our co-localization studies demonstrated the presence neither of free nor conjugated BMPCP or TMPCP in the nuclear region. Instead, these compounds proved to be localized in cytoplasmic organelles.

Considerable amount of interest has already focused on the subcellular distribution of various porphyrin derivatives. Recent results indicate that hydrophilic, amphiphilic or zwitterionic compounds tend to accumulate in the lysosomes while hydrophobic structures prefer the endoplasmic reticulum, Golgi and mitochondria, although the latter can also attract cationic moieties (Jensen et al. 2010). Porphyrin peptide conjugates seemed to be present predominantly in the lysosomes, and the number and sequence of the amino acids have no marked effect on subcellular localization (Sibrian-Vazquez et al. 2005).

In accord with earlier findings di-cationic BMPCP and tri-cationic TMPCP and tetra-peptide conjugates showed co-localization with lysosomal and mitochondrial fluorescent probes. Here the charge distribution can be also a crucial factor which may shift the emphasis from lysosomal to mitochondrial localization (Jensen et al. 2010; Kessel et al. 2003; Odeh et al. 2014). TMPCP-AK shows a different localization pattern appearing mainly in different cytoplasmic vesicles. For the better understanding of these differences the cellular uptake mechanisms of these compounds will be analyzed in the future.

Conflict of Interest statement: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure Legend

Fig. 1. Schematic representation of TMPCP-branched polypeptide conjugate (TMPCP-AK).

Fig. 2. Absorption spectra of TMPCP-AK at various concentration of DNA (a) or NP (b) measured at room temperature in Tris-HCl buffer at pH=7. Base pair/porphyrin molar ratio (*r*) varies between 0 and 26 (a) and 0 and 38 (b). Insets show the normalized Soret bands of the spectra.

Fig. 3. Relative area of the components of the absorption spectra of TMPCP-AK as a function of base pair/porphyrin molar ratio (r) in DNA. Absorption bands are identified by the position of corresponding maxima indicated in the figure.

Fig. 4. Derivative melting curves of DNA (a) and NP (b) in the presence of TMPCP-AK. Porphyrin / base pair ratios (*1/r*) are indicated in the figure.

Fig. 5. Relative fluorescence intensity of porphyrin derivatives at their emission maxima as a function of DPPC/porphyrin molar ratios: (x) BMPCP, (Δ) TMPCP, (o) BMPCP-$4P_2$, (□) TMPCP-4P. Samples were excited at 422 nm.

Fig. 6. TMPCP positive HL-60 cells at various TMPCP incubation concentrations and incubation periods.

Fig. 7. Comparison of cellular uptake of porphyrin derivatives at various incubation concentrations. The incubation period was 3 h.

Fig. 8. Subcellular localization of BMPCP-$4P_2$ and nucleus specific fluorescent dye SYBR Green I in HT-29 cells. (1) Transmission image; (2) Fluorescence of overlay image of BMPCP-$4P_2$; (3) Nucleus stained with SYBR Green I; (4) overlay image of BMPCP-$4P_2$ and SYBR Green I. Incubation period was 3 h for BMPCP-$4P_2$ and 30 min for SYBR Green I. BMPCP-$4P_2$ concentration was 20 μM, SYBR Green I concentration was 400 nM.

Fig. 9. Subcellular localization of BMPCP-4P_2, lysosome specific fluorescent probe LysoTracker Green DND-26 and mitochondrion specific probe MitoTracker Deep Red FM in HT-29 cells. (1) Transmission image; (2) fluorescence of BMPCP-4P_2; (3) lysosomes stained with LysoTracker Green DND-26; (4) overlay image of BMPCP-4P_2 and LysoTracker Green DND-26; (5) fluorescence of BMPCP-4P_2; (6) mitochondria stained with MitoTracker Deep Red FM; (7) overlay image of BMPCP-4P_2 and MitoTracker Deep Red FM. Incubation period was 3 h for BMPCP-4P_2 and 30 min for fluorescent probes. BMPCP-4P_2 concentration was 20 μM, fluorescent probes concentration was 50 nM.

Fig. 10. Subcellular localization of TMPCP-AK, lysosome specific fluorescent dye LysoTracker Green DND-26 and mitochondrion specific probe MitoTracker Deep Red FM in HT-29 cells. (1) Transmission image; (2) fluorescence of TMPCP-AK; (3) lysosomes stained with LysoTracker Green DND-26; (4) overlay image of TMPCP-AK and LysoTracker Green DND-26; (5) fluorescence of TMPCP-AK; (6) mitochondria stained with MitoTracker Deep Red FM; (7) overlay image of TMPCP-AK and MitoTracker Deep Red FM. Incubation period was 3 h for TMPCP-AK and 30 min for fluorescent probes. BMPCP-4P_2 concentration was 20 μM, fluorescent probes concentration was 50 nM.