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Cell Penetration, Herbicidal Activity, and *in-vivo*-Toxicity of Oligo-Arginine Derivatives and of Novel Guanidinium-Rich Compounds Derived from the Biopolymer Cyanophycin

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Abstract

Oligo-arginines are thoroughly studied cell-penetrating peptides (CPPs, Figures 1 and 2). Previous *in-vitro* investigations with the octaarginine salt of the phosphonate fosmidomycin (herbicide and anti-malaria drug) have shown a 40-fold parasitaemia inhibition with *P. falciparum*, compared to fosmidomycin alone (Figure 3). We have now tested this salt, as well as the corresponding phosphinate salt of the herbicide glufosinate, for herbicidal activity with whole plants by spray application, hoping for increased activities, *i.e.* decreased doses. However, both salts showed low herbicidal activity, indicating poor foliar uptake (Table 1). Another pronounced difference between *in-vitro* and *in-vivo* activity was demonstrated with various cell-penetrating octaarginine salts of fosmidomycin: intravenous injection to mice caused *exitus* of the animals within minutes, even at doses as low as 1.4 μmol/kg (Table 2). The results show that use of CPPs for drug delivery, for instance to cancer cells and tissues, must be considered with due care. The biopolymer cyanophycin is a poly-aspartic acid containing argininylated side chains (Figure 4); its building block is the dipeptide H-βAsp-αArg-OH (H-Adp-OH). To test and compare the biological properties with those of octaarginines we synthesized Adp_8-derivatives (Figure 5). Intravenouse injection of H-Adp_8-NH_2 into the tail vein of mice with doses as high as 45 μmol/kg causes no symptoms whatsoever (Table 3), but H-Adp_8-NH_2 is not cell penetrating (HEK293 and MCF-7 cells, Figure 6). On the other hand, the fluorescently labeled octamers FAM-$(Adp(OMe))_8$-NH_2 and FAM-$(Adp(NMe_2))_8$-NH_2 with ester and amide groups in the side chains exhibit mediocre to

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Author Contribution Statement

All syntheses were carried out by *M. G.* The other experiments were performed by *F. K.* (Fluorescence Microscopy), *S. W.* (determination of toxicity), *M.-O. E.* (NMR investigation). *D. H., P. S. D., F. K., M. R., C. S., P. M.*, and *A. K. B.* helped supervise the project and/or contributed to the final version of the manuscript. The research was conceived by *D. S.* who also wrote the manuscript.

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high cell-wall permeability (Figure 6), and are toxic (Table 3). Possible reasons for this behavior are discussed (Figure 7) and corresponding NMR spectra are presented (Figure 8).

Keywords

guanidinium-rich peptides; biopolymer cyanophycin; dipeptide Adp (H-β-Asp-α-Arg-OH); fosmidomycin; glufosinate; herbicidal activity; *in-vivo* toxicity; cell penetration; confocal fluorescence microscopy (CFM)

Introduction

Guanidinium-rich compounds (GRCs) are among the most extensively studied cell-penetrating substances. The guanidinium groups (GGs) are usually attached to backbones, which may be simple oligo-α- or -β-arginines or other oligopeptides with attached GGs, as for instance oligoprolines; other reported backbones include oligo-peptoids, -carbonates, -carbamates, -disulfides, -phosphates, -glycosides, peptide nucleic acids (PNAs), inositol, or dendrimers. As an entry into the field we refer to a review article with historical background by *P. Wender*, one of the discoverers[1][2] of oligo-arginine cell-penetration,[1 – 5] to an overview on cell-penetrating peptides (CPPs) by one of the experts in the field *Ü. Langel*,[6][7] to a recent article discussing the role of flexibility of attachment and of distance between the GGs on the backbone,[8] to the design and testing of a sophisticated disulfide polymer backbone with guanidinylated side chains that carries a fluorescent cargo into cell nucleoli,[9][10] and to clinical applications.[11] A schematic presentation of guanidinium-rich systems is presented in Figure 1,[2] together with an extreme case, in which the guanidinium moieties are actually part of the backbone.[15]

While a majority of CPP investigations deals with derivatives of *natural* products, such as Tat, Antp, and Penetratin, and with the *artificial* compounds oligo-L- and -D-arginines (**1**),[1 – 6][11 – 14] commonly specified as R_n and r_n, our work has focused on the unnatural oligo-β-arginines (**2**, **3** in Figure 2) and their fluorescently[16 – 24] or radioactively[21] labeled and covalently modified[22] derivatives.[3] They rapidly enter eukaryotic cells (3T3 mouse fibroblasts,[16][19] HeLa cells,[17] HEK293 cells,[20] human hepatocytes, fibroblasts, macrophages, infected but not 'healthy' erythrocytes,[19] and *Plasmodium falciparum*, a eukaryotic microorganism[19]), as well as prokaryotic cells (*Bacillus megaterium*, *Escherichia coli*);[18] they also penetrate deep into mouse skin,[17] just like the analogous α-peptidic oligo-arginines.[26] The most pronounced property of the oligo-β-arginines, which they share with all β-peptides, tested so far, is their peptidolytic and metabolic stability *in vitro* and *in vivo*. Thus, after *i.v.* administration to male albino rats (*Han Wistar*) all of the radioactively labeled octa-β-arginine **2b** remained chemically unchanged and was

[2]For a critical article about oligo-nucleotide cell delivery by CPPs with historical background, see a *short review* (*expert opinion*) in ref.[12] The well-considered comments about drug delivery, recently published by *J.-C. Leroux*, may be especially applicable to the field of cell penetration.[13] For those who are able to read German we recommend the recent review by *O. Avrutina*, *H. Kolmar*, and *M. Empting*.[14]

[3]For a comprehensive review article covering the literature on β-peptides up to 2004 see ref.[23] For a seminal full paper about preparation and properties of oligo-β-arginines see ref.[17] For independent work on this subject by the *Gellman* group see ref.[25] and earlier contributions cited therein.

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enriched in various tissues of the animals after 4 days (< 2% excretion), while with peroral administration almost the complete dose was recovered in feces within 24 h. No toxic effects were observed with the concentrations employed and under the conditions used[21] (*vide infra*). Like their α-peptidic counterparts the oligo-β-arginines do not enter anionic lipid-POPC/POPG vesicles.[20][24] Rather, they attach to the vesicle surfaces, disrupt the structure of the membrane and make it permeable, causing, for instance, calcein release from vesicles.[24][4]

4:1-Salts of Fosmidomycin and Glufosinate with Octaarginines and Test of Herbicidal Activity

Inspired by investigations of the *Matile* group[28] on the importance of polyion-counter-ion complexes for cell penetration[5] and remembering the *perfect fit* between guanidinium groups and so-called oxy-anions (Figure 3,a) that had been used in organic synthesis and in supramolecular chemistry,[30 – 36] we had prepared the 1:4 salt **1a**-4**Fos** of octaarginine amide and fosmidomycin to test its *in vitro* activity against *P. falciparum*, and we were able to report in 2013 that growth of this parasite (which causes malaria) was much more strongly reduced by the salt than by fosmidomycin itself (Figure 3,b and c).[37][6]

Fosmidomycin, a phosphonic-acid derivative, was first isolated as a natural product from *Streptomyces rubellomurinus*;[38] it is commercially available as the Na-salt[7] and was originally used as an herbicide; it inhibits the enzyme DOXP reductoisomerase (DXR) of the non-mevalonate pathway leading to isoprenoids in plants and in unicellular organisms, such as the eukaryotic *P. falciparum* or mycobacteria (causing malaria, toxoplasmosis, tuberculosis, or lepra, see the discussion and references in[37]). The *ca*. 40-fold decrease of *P. falciparum* growth rate caused by the salt **1a**-4**Fos** (Figure 3) shows that the cell-penetrating octaarginine with its fosmidomycin cargo passes the most complex cell wall of this eukaryotic microorganism for delivery of the inhibitor to the active site of the enzyme DXR.

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[4]Interaction with the negatively charged surfaces of the vesicles is, of course, related to the mechanism of cell penetration: in non-endosomal mechanisms the oligoarginines first make contact with negatively charged phospholipids and glycans, such as heparin, on the cell surface (*cf*. Figure 3,a, below), before entering the cell, a process, which depends on the membrane potential across the cell wall maintained by ion pumps.[27] The composition of the cell surface changes when the cells are infected (*cf*. erythrocytes[19]) or when they are in an apoptotic state (*cf*. *HEK293* cells[20]).

[5]For delivery of an inositol pyrophosphate derivative by a guanidinium-rich transporter (with a polycarbonate backbone) into the cytoplasm of HeLa cells see ref.[29]

[6]It is important at this point to comment on the preparation of salts, like **1a**-4**Fos** and **1a**-4**Glufos**, and the concentrations of oligo-arginines used/administered herein and in reports of other groups. When preparing and purifying the oligo-arginines in the usual way, *i.e*. by Fmoc-technology and HPLC purification with trifluoro-acetic-acid (TFA)-containing solvent mixtures, an octaarginine amide, for instance, is isolated as the nona-triflate salt **1a**-**9TFA** (see Figure 2), thus containing *ca*. 45 wt-% of TFA. A sample purchased from one of the companies offering peptide-synthesis services consists of almost 50% TFA. We have confirmed this by elemental analyses of the F-content of various lyophilized samples. In the work described herein and in ref.[37] the oligo-arginine TFA-salts were treated with *Amberlyst* ion-exchange resin *A-26* (OH^- form, pK_a 12.13) to remove TFA before use, so the reported concentrations refer to *free* oligo-arginines, if not stated otherwise. Since other authors in the field (*vide infra*) do not mention corrections or precautions along these lines we have to suspect that the oligo-arginine concentrations given in their papers may actually be too high. The only paper we are aware of, in which $CF_3CO_2^-$ counterions of GRCs are explicitly shown with the molecular formulae, is the publication by *Wender et al.* in ref.[2].

[7]For preparing the 4:1-salts with oligo-arginines (Figure 3, b), the commercial fosmidomycin Na-salt and the glufosinate NH_4-salt were converted to the free acids by treatment with the ion-exchange resin *IR-120* (pK_a 2.2) (*cf*. footnotes 6 and 14).

The successful application of octaarginine for transporting an antibiotic compound into the parasite causing malaria drew our attention to another interesting phosphorous derivative: phosphinothricin, a phosphinic-acid (Figure 3), the L- or (*S*)-form of which was first isolated and identified by the *Zähner* group in Tübingen in 1972,[39][40] as a component of the tripeptide phosphinothricyl-Ala-Ala (from *Streptomyces viridochromogenes*). Phosphinothricin is a potent glutamate-synthetase inhibitor, the ammonium salt of the *racemic* form, glufosinate, is a widely used non-selective herbicide. Extensive use of glufosinate has led to reports of evolved weed resistance,[41] in which case much higher levels/amounts of the herbicide are required to control the resistant weeds. It would therefore be highly desirable to be able to increase the glufosinate activity, and thus reduce the necessary dosis, *cf.* the 40-fold *in vitro* activity increase when going from fosmidomycin to its octaarginine salt described above. Thus, we prepared the salt **1a**-4**Glufos** from the free phosphinic acid and octaarginine by the same procedure described above for the corresponding fosmidomycin salt and tested its activities in comparison with the corresponding ammonium salt and with **1a**-4**Fos**. The post-emergence herbicidal activity of all three compounds was compared at 13 days after application, using 0.5% *Tween 20* as the adjuvant. The plants species listed in Table 1 were assessed 13 days after herbicide application – 0% meaning no effect and 100% meaning complete kill. All samples were sprayed at 500, 125, and 60 g/hectare, with respect to the active glufosinate or fosmidomycin component. As can be seen from the data listed in Table 1, both **1a**-4**Glufos** and **1a**-4**Fos** salts of the cell-penetrating octaarginine peptide showed poor herbicidal activity. The glufosinate octaarginine salt was much weaker than glufosinate, and the fosmidomycin octaarginine salt was also much weaker than fosmidomycin (based on previous data[42]). Since it is known[8] that oligo-arginines penetrate plant cells very much the same way as other eukaryotic cells, it is possible that the low activity of both **1a**-4**Glufos** and **1a**-4**Fos** is instead due to poor foliar uptake. This is consistent with reports that very hydrophilic compounds cross a plant cuticle through a polar pathway that has molecular size limitations, which are likely incompatible with these large CPP-salts.[44][45]

Thus, the *in*-vitro plant-cell[43] penetration of oligo-arginine derivatives could not be exploited for delivery into whole plants of the herbicides glufosinate and fosmidomycin. We speculate that the limited biological activity we observe, especially with **1a**-4**Fos**, might be due to the foliar uptake of small amounts of the dissociated compounds (*i.e.* non CPP-salts).

In-vivo-Toxicity in Mice of the 4:1-Salts of Fosmidomycin with Octaarginines and of Other Oligo-Arginine Derivatives

In view of the observed lack of activity of the glufosinate and fosmidomycin salts **1a**-4**Glufos** and **1a**-4**Fos** in whole plants we worried about the activity of oligo-arginine derivatives in whole animals, *i.e.* their *in-vivo* activity. This appeared to be especially important because of recent reports about artemisinin resistance of *P. falciparum*,[46] which has caused a revival of interest in fosmidomycin as an anti-malarial drug; for an extensive review article about clinical trials see ref.[47] In order to find out whether the octaarginine salt **1a**-4**Fos** is more active than fosmidomycin itself, as it is *in vitro* (*vide supra*), we have

[8]See for instance, an article in which nonaarginine (R_9) was reported to carry a disulfide-attached protein into live plant cells.[43]

carried out *in-vivo* tests by intravenous tail-vein injection to *Plasmodium-berghei*-infected and to non-infected mice (*P. berghei* is a parasite that causes rodent malaria.[9]) There have been numerous *in vivo* studies involving oligo-arginines in drug delivery; for an extensive review we recommend an article by *Zaro* and *Shen*;[48][10] for two reports about acute toxicity of simple oligo-arginine derivatives determined by direct intravenous injection to mice we refer to papers by the groups of *Tsien*[55] and *Zhang*.[56][11] ,[12]

The results of our toxicity investigations of the fosmidomycin-octaarginine salts are collected in Table 2, which also contains some literature data, and, for comparison, parasitaemia-reduction values for the Na-salt of fosmidomycin. Clearly, all octaarginine salts are extremely toxic when administered intravenously, lethal down to 1.5 μmol/kg doses, and so are the previously reported oligo-arginine derivatives**1a** and **1b** themselves; it looks as if the TFA salt **1a**-9TFA of octaarginine is somewhat more toxic than peptide **1a** without this counter-ion. There is no difference between the octa-L-, -D- and -*β*-arginine salts **1a**-, *ent*-**1a**-, **2a**-4**Fos** or between infected and non-infected mice. Thus, the *in-vitro* increase of fosmidomycin activity against *P. falciparum* when used as the salt **1a**-4**Fos** cannot be confirmed in the *in-vivo* experiment with *P. berghei*. In contrast, the toxic activity of the salt turns out to be more or less identical to that of pure oligo-arginines **1**. Apparently, fosmidomycin found other counter-cations and octaarginine other counter-anions after injection into the blood stream, inspite of the perfect anion-cation fit between the two components of the salts (*cf.* Figure 3,a). The systemic toxicity in intravenous and intraperitoneal administration of polycationic CPPs is supposed to be associated with mast-cell degranulation.[55]

Suitably Protected Adp-Building Blocks (4 – 6) and Peptide Assembly to the Octamers (7 – 10) Derived from the Biopolymer Cyanophycin

Discovery, Physiological Function, and Structure of Cyanophycin—The guanidinium-rich compounds (GRCs, Figure 1,a) consisting of arginine (**1a** in Figure 2) must be considered *unnatural*; to the best of our knowledge no oligo-arginine has been identified as part of a natural CPP system. There is, however, a peptidic biopolymer carrying an arginine side chain with a terminal guanidine group on each and every residue: cyanophycin (Figure 4), largely unknown among chemists, biochemists, and biologists. In view of our interest in GRCs we have followed the research of *Alexander Steinbüchel* and his group (University Münster) in the field of cyanophycin for years.[58 – 64] This biopolymer has an intriguing structure: it is a poly-aspartic-acid *N*-argininylated on the carboxylic acid groups of the side chains (Figure 4). Cyanophycin was discovered as characteristic granules in blue-green algae by the Italian botanist *Antonio Borzi* in 1887 and chemically identified by *R. D. Simon* in 1971.[65] It is formed by cyanobacteria as a storage

[9]For *in vitro* tests with octaarginines and various *P. berghei*-infected human cell lines, see ref.[37]
[10]For 'life-cell' toxicities (cytotoxicities) of **1** and *ent*-**1** with $n = 9$ and 10, see ref.[49][50] For review articles with references to *in-vivo* tests with GRCs, including intravenous injections, see also ref.[51 – 53] Leucocyte toxicity of a taxol-octaarginine conjugate.[54]
[11]In the work by *Zhang et al.*[56] diastereomeric octaarginines consisting of L- and D-arginine moieties (called 'chimera') were tested, and it turned out that the toxicities differed substantially, depending on the number and position of D-entities in the chain of L-arginines. In independent work by our group[20] such 'mixed' octaarginine derivatives were found to have varying rates of cell penetration (up to a factor of 4) and varying stabilities in heparin-stabilized human plasma (from 5 min to > 7 days), depending upon the site and frequency of D/L-replacements in the octaarginine chain.
[12]For a non-toxic dosis administered *i.v.* to rats see **2b** in Table 2 and ref.[21] For application of nM doses to mice see ref.[57]

material for nitrogen and carbon under conditions of nutrient shortage; its molecular weight can be up to 130 kDa. Synthetases producing the polymer and cyanophycinases degrading it have been isolated and can be used for production of the polymer on technical scale. The building block of the polymer consisting of aspartic acid and arginin has been proposed as a di-aminoacid-nutrient additive; for leading papers on these subjects see the articles by the *Steinbüchel* group in refs.[58 – 64] and publications cited therein. Herein we refer to the building block of the cyanophycin polymer as H-Adp-OH (Figure 4). Adp may be considered a dipeptide of Arg and Asp, in which the aspartic acid is incorporated as a *β*-amino-acid building block.[23]

Interestingly, the side chains on the peptide backbone of an Adp-oligomer can be cationic, zwitterionic, or anionic, depending on the pH (Figure 4). For cell-penetration the guanidinium groups with their positive charges must be considered mandatory. Under physiological conditions the zwitterionic form (without a net charge) should be present to some extent; this could lead to reduced interaction of the guanidinium groups with negatively charged entities on the cell surface, believed to be an important initial step for cell penetration[4]. On the other hand, if Adp-oligomers would turn out to have cell-penetrating properties, they could possibly play this role in nature – hitherto unnoticed.

Synthesized Adp-Derivatives 4 – 6 and Octamers 7 – 10—To find out whether oligo-Adp-derivatives, segments of the guanidinium-rich biopolymer cyanophycin, behave like common GRCs, we have synthesized octa-Adp with (**7** – **9**) and without (**10** – **12**) *N*-terminal fluorescent FAM labels and with methyl ester (**8**, **11**) and dimethylamide groups (**9**, **12**) instead of COOH groups in the side chains (Figure 5).[66] The latter two compounds were chosen to probe a possible negative influence of zwitter-ion formation on cell-penetrating properties.[13]

All octa-Adp-derivatives were purified by preparative HPLC and identified by mass spectrometry. In this way 5 to15 mg quantities of the novel peptides were obtained. The lyophilized samples consist of TFA salts, which were treated with Amberlyst ion-exchange resin *A-26* (OH^- form, pK_a 12.13) for removal of the TFA counter-ions.[14] If not otherwise stated, the samples thus obtained were used for the biological experiments described in the following section.

Determination of *in-vivo*-Toxicity and of *in-vitro*-Cell permeability of Adp_8-Derivatives

***In-vivo*-Toxicity Measured by Intravenous Injection—**The salt of Adp_8 with four fosmidomycin counter-ions (**10**-4**Fos**), the octa-Adp (**10**), and its derivatives with ester (**11**) and amide groups (**12**) were tested for acute *in-vivo* toxicity as described above for the octaarginine derivatives (Table 2). Surprisingly, no obvious acute toxicity symptoms whatsoever were caused by the salt (**10**-4**Fos**) or octa-Adp (**10**), even at doses as high as 45 μmol/kg.[15] On the other hand, the octapeptides **11** and **12** with methylester and dimethyl-amide groups, respectively, in the arginine side chains are toxic. At doses of *ca*. 30 μmol/kg

[13]Interestingly, an Arg-methylester as in 8 was chosen by *Matile et al.*[9][10] to render poly(disulfide)s cell permeable.
[14]Elemental analysis of lyophilized H-Adp_8-NH_2 (**10**) showed that the sample had an F-content of 14.7%, a value that is calculated for [H-Adp_8-NH_2 · 8 CF_3CO_2H] (*cf.* footnote 6, above).

both derivatives are lethal, just like the octaarginines (Table 2). Detailed results are summarized in Table 3. Thus, the free carboxylic-acid groups in the side chains of octa-Adp prevent toxicity.

Cell-Penetrating Properties of Adp-Derivatives 7 – 9 Studied by Confocal Fluorescence Microscopy (CFM)—At this point of our investigation the questions arose whether non-toxic Adp-derivatives with free carboxylic acid groups in the side chains have cell-penetrating properties and how they might differ in this respect from the compounds with ester and amide groups in the oligomer side chains. As candidates for cell-penetration studies we used the FAM-labeled octa-Adp-derivatives **7** (with free carboxylic acid groups) and **8** and **9** (with ester and amide groups, respectively).

To evaluate cell permeability, we monitored the permeation efficiency of the FAM-labeled peptides into HEK293 and MCF-7 cells by CFM. The fluorescent lipophilic membrane stain R18 was used to mark the plasma membranes of the cells.[16] The results are illustrated in Figure 6: *i*) FAM-Adp_8-NH_2 (**7**) does not enter the cells at all. *ii*) In contrast, the FAM-$(Adp(OMe))_8$-NH_2 (**8**) and FAM-$(Adp(NMe_2))_8$-NH_2 (**9**) derivatives exhibited mediocre to high cell permeability and yielded homogeneous cell loading accompanied by higher concentrations in the nuclear region.[17]

Thus, the FAM-labeled octamer **7** of the non-toxic cyanophycin-derived peptide **10** (*cf.* Table 3), with free carboxylic-acid groups in the arginine side chains of the Adp residues, turns out to have no cell-penetrating properties, while its toxic derivatives possessing ester and amide groups (**8** and **9**) are cell permeable and behave like conventional octaarginine CPPs such as **1b**.[20]

A simple *interpretation* of this result would be that there are no ‘free’ guanidinium groups on the backbone of peptides **7** and **10** under the physiological conditions (pH 7.4) of our experiment (*cf.* introduction and Figure 1). Rather than the cationic or zwitter ionic structures discussed above (see Figure 4) there could be a structure containing intramolecular salt moieties consisting of guanidinium cations and carboxylate anions. An example is shown in Figure 7,a, where the carboxylate group of Adp residue n forms an anion-cation complex with the guanidinium group of Adp residue $n + 2$. In such a structure the peptide backbone does not adopt an extended conformation that can present the attached side chains carrying guanidinium groups to enable interaction with cell surfaces. This intramolecular salt formation calls to mind the large decrease in cell-penetration observed for the peptides containing equal numbers of Arg and Glu residues shown in Figure 7,b,[68] as compared to their counterparts containing only Arg residues. Also, the so-called activatable cell-penetrating peptides (ACPPs) shown schematically in Figure 7,c[55][69 –

[15]The observation that the Adp-octamer **10** has no *i.v.*-toxicity matches well with the fact that the ‘monomer’ H-Adp-OH itself is being considered as a dipeptidic nutrient additive (*vide supra*), which means that it does not exhibit any *p.o.*-toxicity. See publications cited in ref.[58 – 63], especially those coauthored by *A. Sallam*; type in Google: ‘Zwei Aminosäuren als Geschäftsidee. Mit Cyanobakterien den Körper stäken’, https://www.n-tv.de/wissen.[63][64] See also: Cysal GmbH, Technologiehof Münster, Mendelstrasse 11, D-48149 Münster, Germany; http://www.cysal.de,[64] and footnote 18 below.

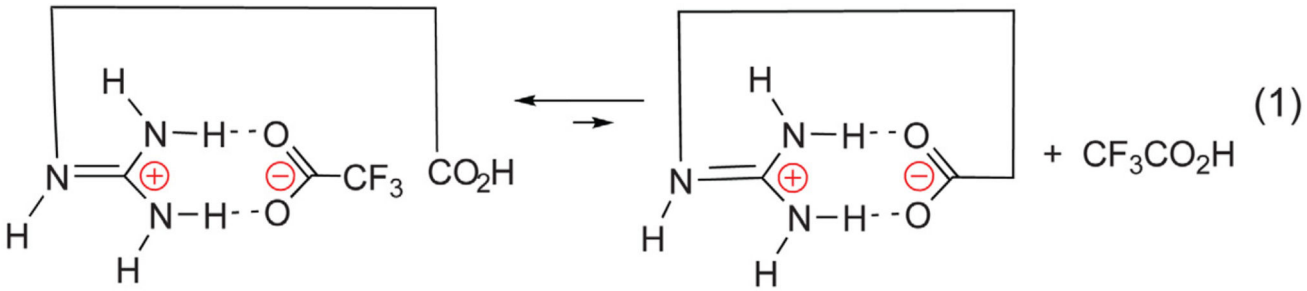
[16]In a previous investigation of octaarginine derivatives we employed the membrane-localization dye DiI.[20]

[17]Full experimental details and a quantitative analysis of the cell permeation considering the role of DMSO and of the membrane dye R18 is published in a separate paper.[67]

73] are not cell-penetrating by themselves, but contain a predetermined specific cleavage site, such as chemically (S–S bonds), photochemically or enzymatically labile entities, that are cleaved in the extracellular space, releasing cell-penetrating polycationic oligo-arginine derivatives with their cargo. A previously proposed example of guanidinium neutralization in an oligo-arginine by the phosphate dianion (HPO_4^{2-}) is shown in Figure 7,d; similar intermolecular salt-bridge formation with the phosphate-ester groups of membrane phospholipids has been suggested to facilitate transport through the membrane.[74]

Another *conclusion* from these results must be that the *in-vivo* toxicity observed with octaarginines and with the octa-Adp-ester and -amide derivatives is due to their poly-cationic structures.

NMR-Spectra of the Octa-Adp-Derivatives—If the Adp octamer with free carboxylic-acid groups would form intramolecular salt complexes, as indicated in Figure 7, a, we expected that its different backbone structure should give rise to a markedly different NMR spectrum in comparison with the spectra of the ester and amide derivatives. Thus, we measured the NMR spectra in water of the three octa-Adp-derivatives **10**-9**TFA**, **11**-9**TFA**, and **12**-9**TFA**, as obtained by preparative HPLC purification (using TFA-containing eluent) followed by lyophilization (*cf.* footnotes 6 and 14). The three spectra presented in Figure 8 are so similar that no evidence for a different backbone structure of octa-Adp **10** with its free carboxylic-acid groups can possibly be derived. This is actually not surprising, if we consider that formation of an intramolecular salt between the guanidinium and the carboxylate group would be energetically unfavorable for the triflate salt **10**-9**TFA** (see the accompanying Equation 1 and the pH-dependent structures of oligo-Adp shown in Figure 4). We are now in the process of synthesizing larger amounts of the octamer **10** to be able to measure its NMR spectra in the presence of different counterions in aqueous solutions at various pH values; the results of these investigations will be reported in due course.



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Conclusion and Outlook

We have shown that the antiparasitic and herbicidal activities of fosmidomycin (a phosphonic acid) and of glufosinate (a phosphinic acid) are not increased when their salts with cell-penetrating octaarginines are administered *in vivo*, and we have demonstrated that intravenous injection of various octaarginine derivatives into the tail veins of mice is extremely toxic. These results contrast with those of *in-vitro* studies.

In search for new types of arginine-rich cell-penetrating peptides with improved properties we have synthesized for the first time and investigated an octamer segment [H–Adp_8–NH_2] of the biopolymer cyanophycin. This novel oligopeptide possessing arginines as side chains turns out to be neither toxic nor cell-penetrating. If, on the other hand, the COOH groups in the argininylated side chains of H–Adp_8–NH_2 are replaced by ester or amide groups, both the toxicity and the cell permeability, typical of oligoarginines, are restored. NMR spectra of the three triflate salts provide no evidence for different backbone structures of the octamer with COOH, as compared to the analogs with COOMe and $CONMe_2$ groups.

Future investigations of the cyanophycin-derived peptides will have to address, *inter alia*, the question of whether a possible intracellular enzymatic ester hydrolysis of the cell-penetrating compound H–$(Adp(OMe))_8$–NH_2, converting it into the non-cell-penetrating compound H–Adp_8–NH_2, could lead to unexpected effects in CPP applications. Furthermore, the toxicity of H–$(Adp(OMe))_8$–NH_2 might be reduced, and its cell-permeability modulated by having ester groups in the side chains only at certain positions of the peptide chain (‘mixed’ Adp/Adp-ester oligomers). Another aspect in connection with the use of olig-Adp derivatives for CPP investigations is their enzymatic degradation. Oligo-L-arginines are degraded rapidly; the half-life of the octamer in human plasma is 0.5 min, oligo-D-arginines are much more stable under these conditions[20] and are therefore the commonly preferred enantiomers in CPP studies (r_n instead of R_n). It will be important to determine the half-lifes of the cell-penetrating octa-Adp derivatives with ester and amide groups in the side chains.[18]

Acknowledgements

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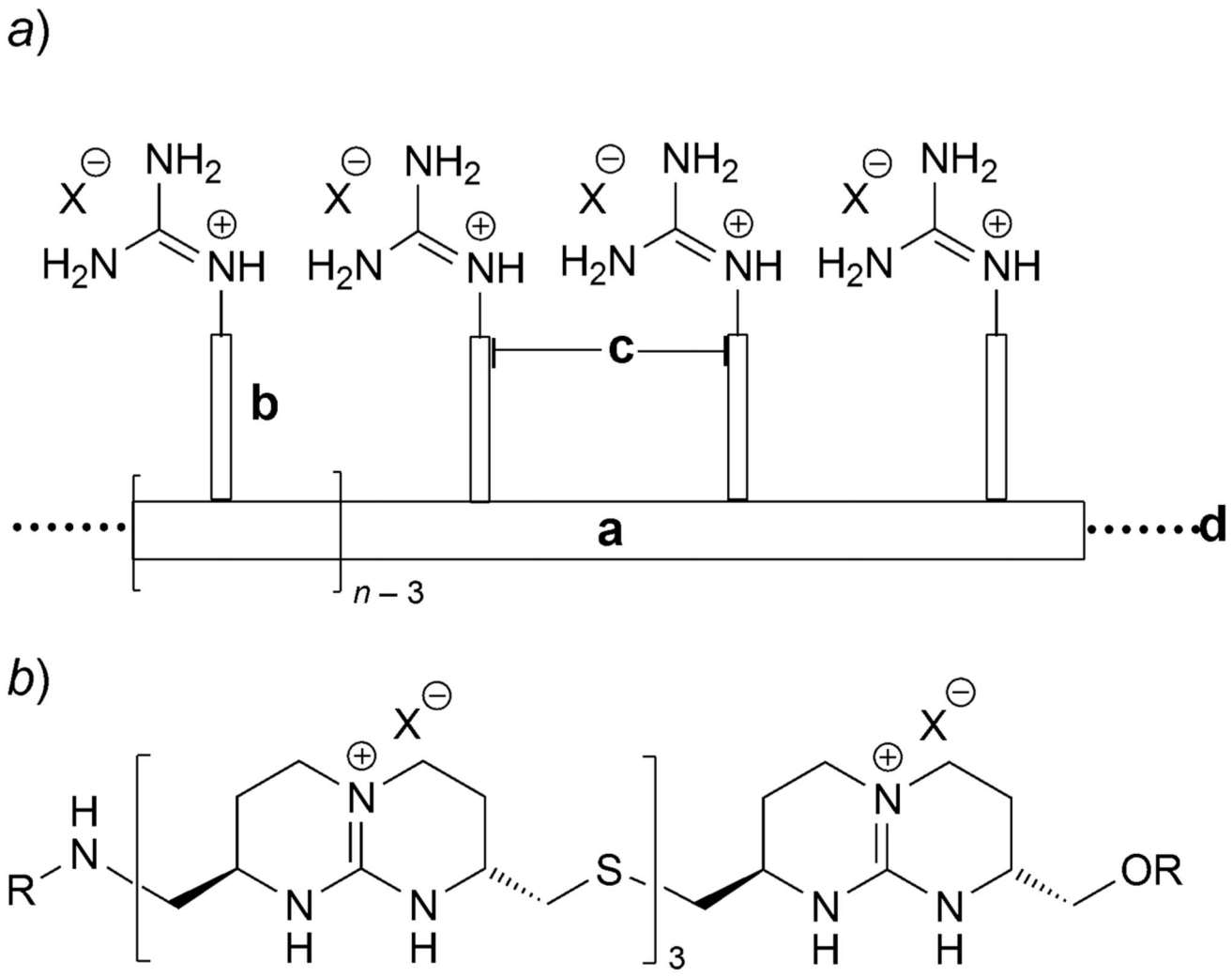

Figure 1.

Guanidinium-rich structures. *a*) The guanidinium groups (GGs) are attached to various backbones, mostly oligo-peptides. The rate of cell penetration and the peptidolytic stability depend upon the nature of the backbone (**a**), the length and flexibility of the connecting units (**b**), the distance (**c**) between the GGs, and the number (*n*) of GG-bearing units. Cargoes (**d**), such as fluorescent groups or bioactive moieties to be delivered into cells are attached to the backbone. **b**) A cell-penetrating oligomer, in which GGs are part of the backbone.[15]

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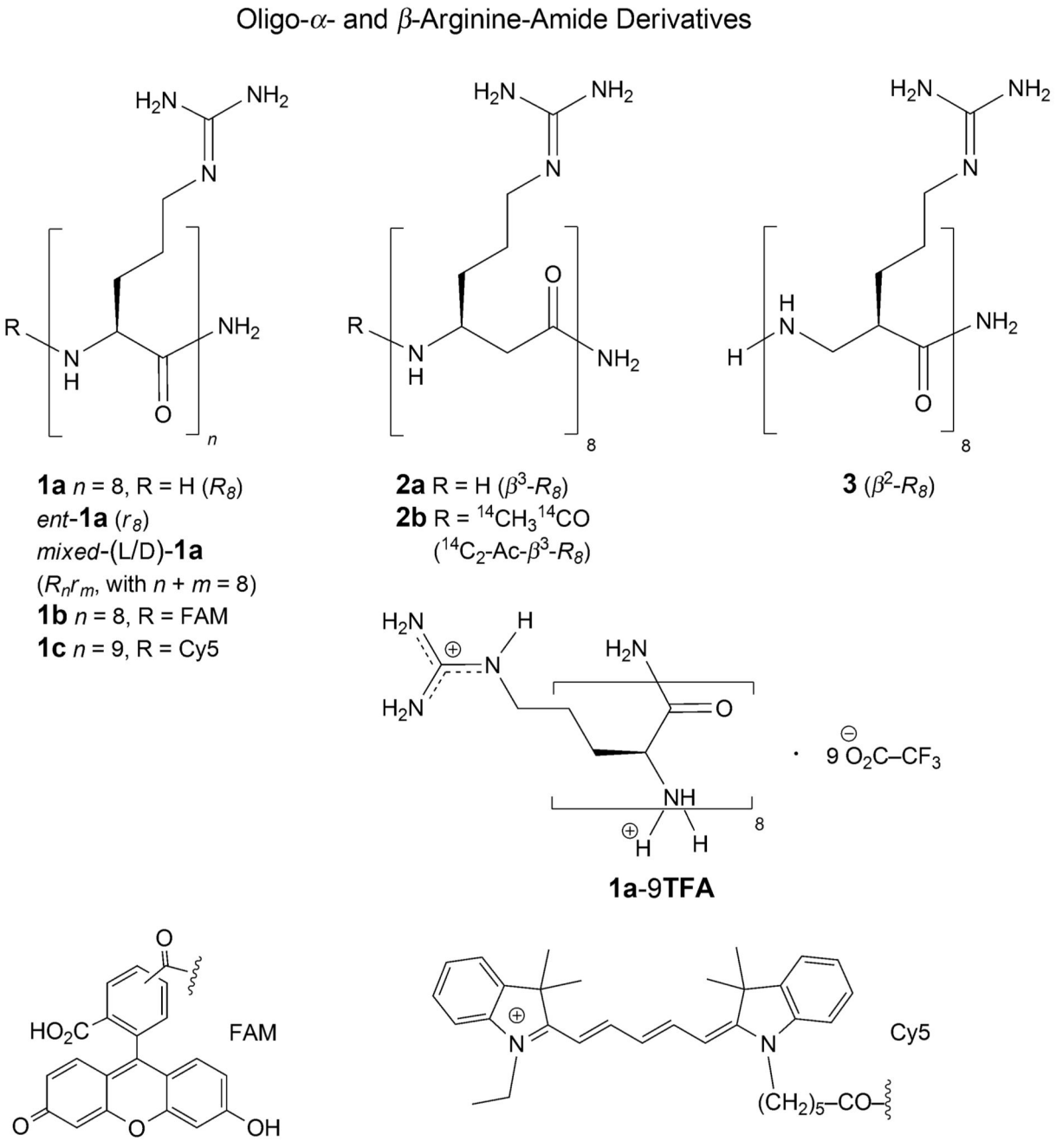

Figure 2.

Frequently investigated oligo-arginines (**1**, R_n), their enantiomers (*ent*-**1**, r_n) and β-octaarginine derivatives (**2**, β^3-R_n and **3**, β^2-R^n). The *C*-terminal group is NH_2, due to the solid-phase peptide synthesis (SPPS) by the Fmoc technology on Pal-PEG-PS-type resins. The *N*-terminal group R may be a hydrogen atom, a fluorescent marker, or a cargo moiety with biological activity to be carried into cells. As pointed out in footnote 6 below, the product obtained by an Fmoc-octaarginine synthesis, followed by HPLC purification and

lyophilization is actually the nona-triflate salt **1a**-9**TFA**. For FAM derivatives see Figure 5 and the section on CFM; for a Cy5 derivative see Table 2, below.

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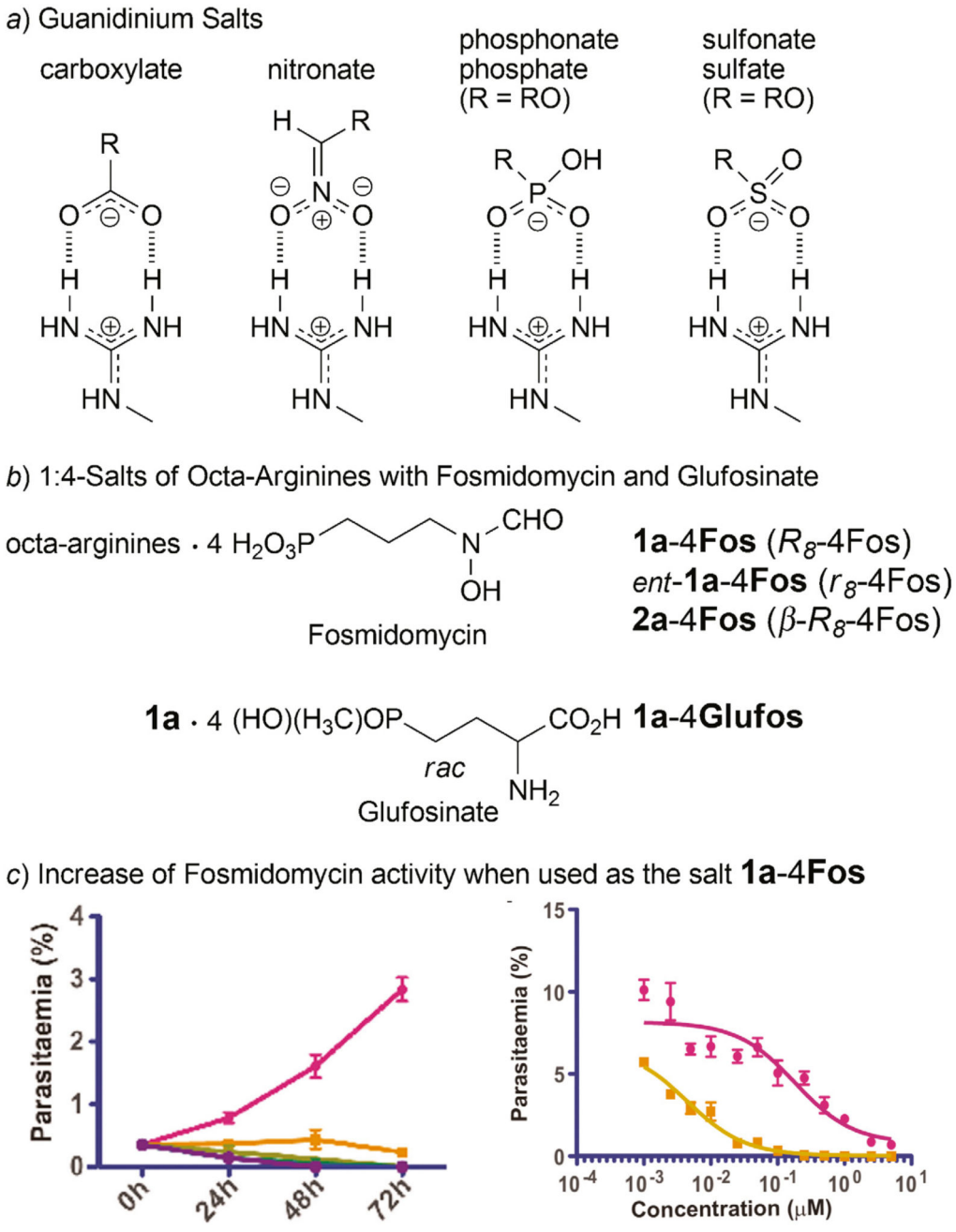

Figure 3.

Salts with guanidinium groups. *a*) Perfect match of counter-ions in guanidinium salts with symmetrical dibasic anions; carboxylates, phosphonates, phosphates (see also *d*) in Figure 7, below), sulfonates, and sulfates are common in biological systems. *b*) Salts of the octaarginines **1a** and **2a** with the physiologically active compounds fosmidomycin (herbicide, antimalarial drug) and glufosinate (herbicide). c) left side: Enhanced efficacy of fosmidomycin against blood-stage *Plasmodium falciparum*, when used as the salt **1a**-4**Fos** (*in-vitro* test); red: course over 72 h of parasitaemia when untreated; other colors:

parasitaemia when treated with 0.1 – 5.0 μM of the 1:4 salt **1a**-4**Fos**. *c*) right side: IC_{50} for growth inhibition in the presence of fosmidomycin alone (181.4 nM, red) and in the presence of the salt **1a**-4**Fos** (4.4 nM). The two diagrams are taken from ref.[37]

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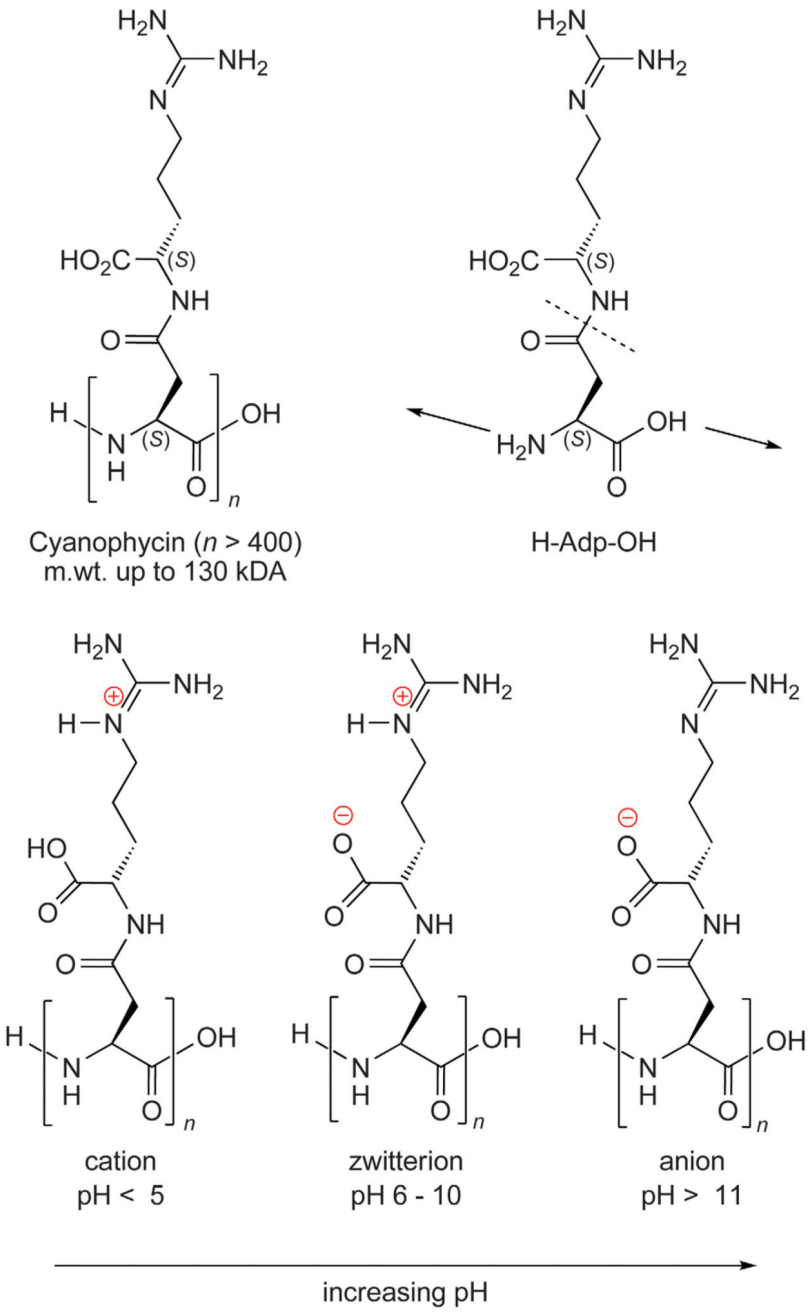

Figure 4.

The biopolymer cyanophycin and the monomeric argininylated aspartic-acid derivative *β*-**A**sp-*α*-Arg-**dip**eptide (H-Adp-OH), from which the polymer is built. In the literature cyanophycin is commonly referred to as ‘cyanophycin granule polypeptide’ (CGP). The side chains of an Adp-oligomer contain pH-dependent charges (positive and negative charges on the *N*- and *C*-termini are not considered in this presentation).

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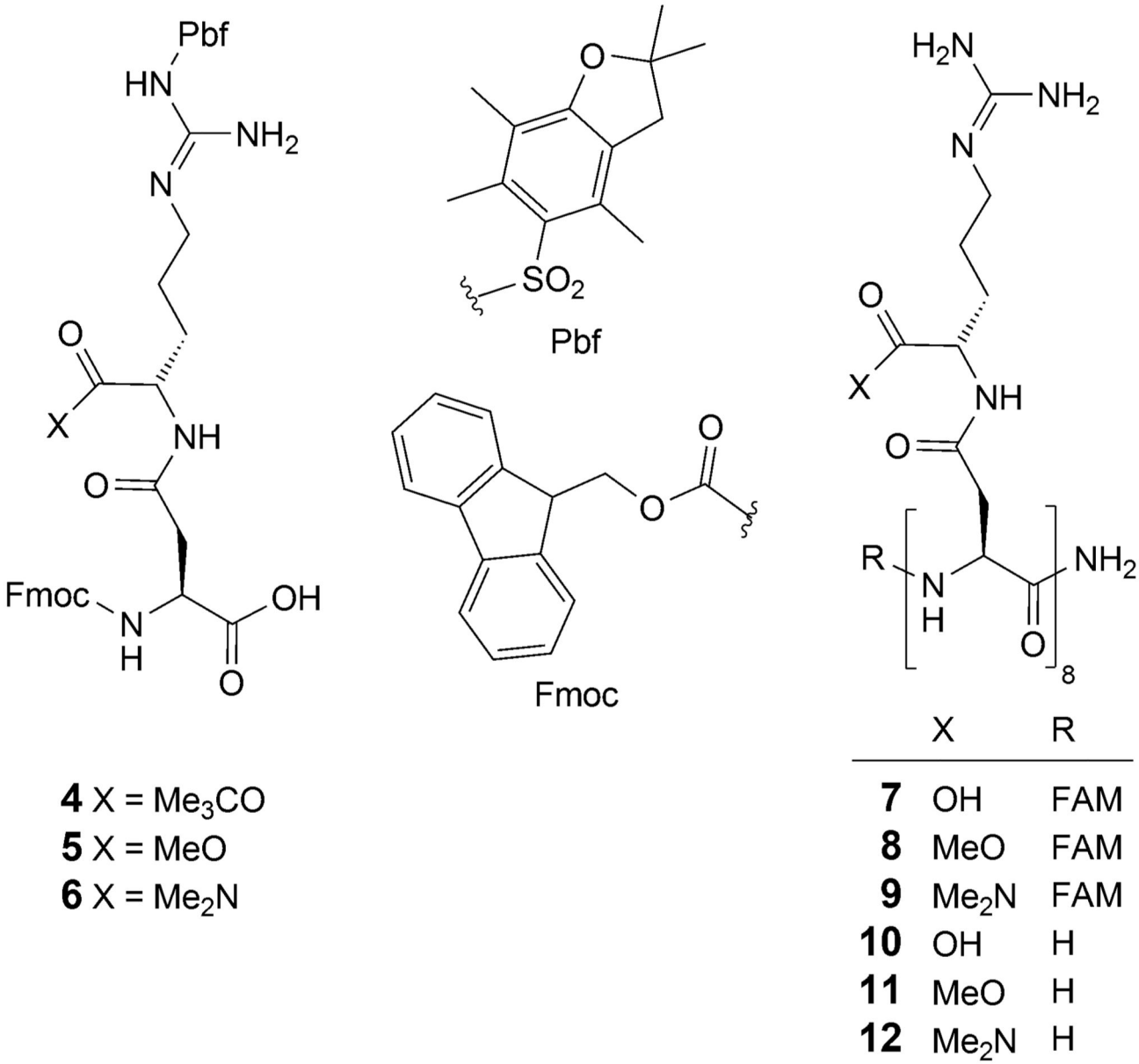

Figure 5.

Synthesized building blocks **4** – **6** for solid-phase-peptide synthesis by the Fmoc-technology on Fmoc-Pal-PEG-PS resin. SPPS leads to the Adp-octamers **10** – **12**, and their FAM-derivatives **7** – **9**. For the formula of the fluorescein-derivative FAM see Figure 2. For details of the syntheses see ref.[66]

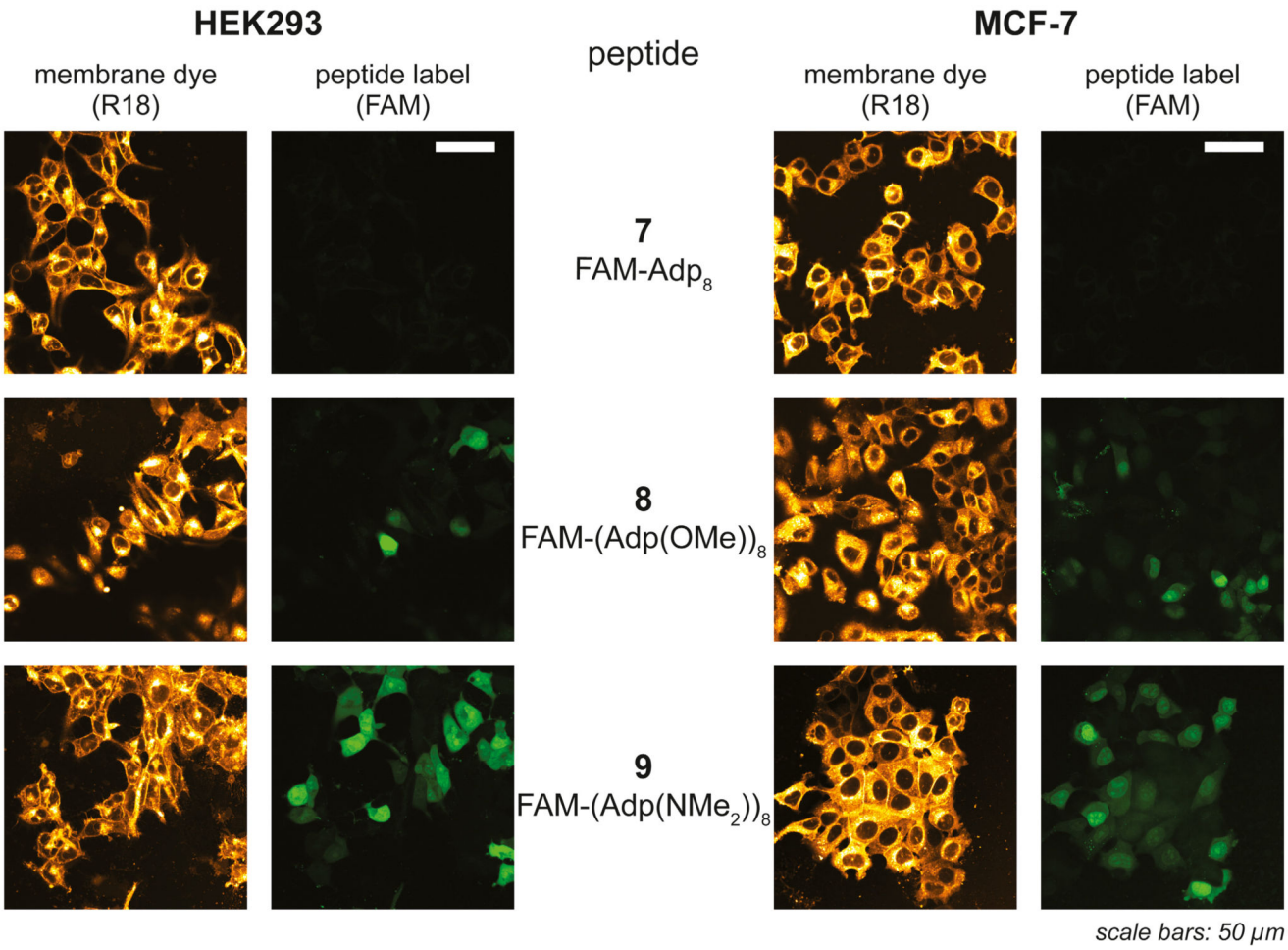

Figure 6.

CFM Images for determining penetration into HEK293 and MCF-7 cells by the octa-Adp-derivatives **7** – **9**. Yellow fluorescence: R18-marked cell walls; green fluorescence: FAM-labeled octa-Adp-derivatives. Clearly, compound **7** with free carboxlic-acid groups in the peptide side-chains does not enter the cells. For details see ref. [67]

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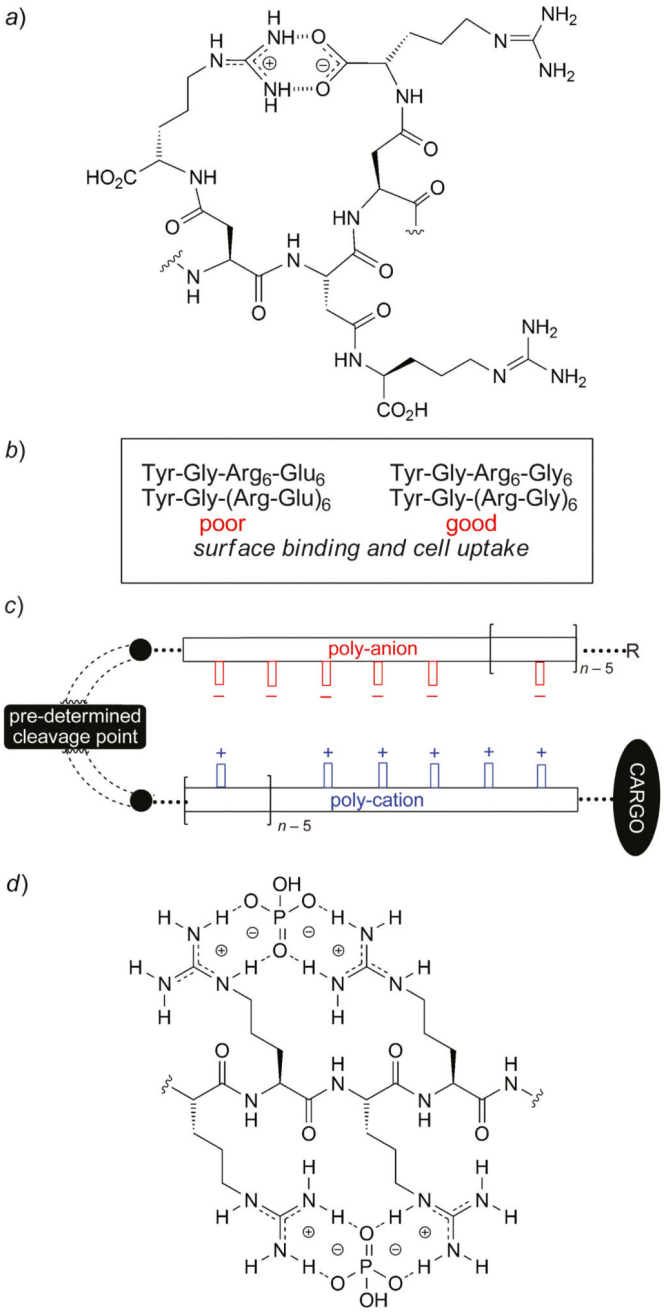

Figure 7.

Cation-anion interactions in oligoarginine derivatives. *a*) Possible salt-type structures in oligo-Adp chains derived from cyanophycin. *b*) Poor surface binding and cell uptake of neutral mixed Arg-Glu tetradeca-peptides as compared to poly-cationic analogs. *c*) 'Internally neutralized' polyelectrolytes, in which the anionic and cationic charges may be represented by the carboxylate and guanidinium groups in the side chains of Glu and Arg, respectively. *d*) Guanidinium neutralization by phosphate groups proposed in a discussion of the influence of counter-anions on cell and membrane permeability of oligo-arginines.[74]

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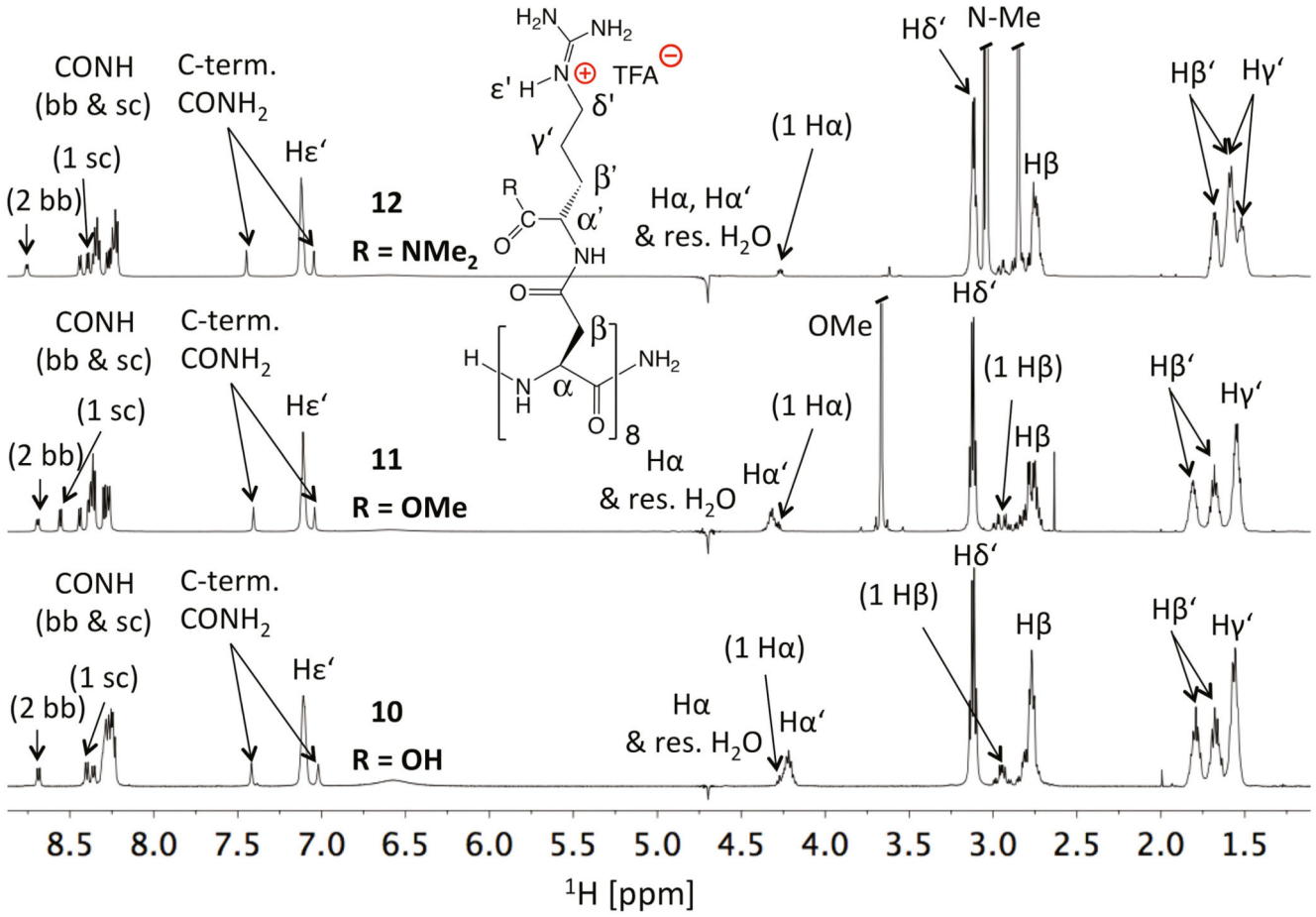

Figure 8.

^{1}H-NMR Spectra of the TFA salts of octa-Adp (**10**, a section of cyanophycin, recorded at 500 MHz) and of its methylester and dimethyl amide derivatives (**11**,**12**, recorded at 600 MHz) in H_2O/D_2O 95:5 at 25 °C. NMR Samples were prepared by dissolving 10.5 mg of **10**, 8.7 mg of **11**, and 7 mg of **12**, respectively, in 600 µl of the solvent mixture. Water suppression was achieved using excitation sculpting. Assignments indicated in the figure are based on DQF-COSY, TOCSY, ^{13}C-HSQC, ^{15}N-HSQC and ROESY, all recorded at 600 MHz. Tentative residue specific assignments of selected protons are indicated in parentheses. Proton resonances close to the position of the water signal are also suppressed or strongly attenuated.

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Table 1

Screen of herbicidal activity of glufosinate ammonium salt and of the octaarginine salts **1a**-4**Glufos** and **1a**-4**Fos** on whole plants

Plant Species[a]	[g/ha]	Glufosinate NH_4 Salt [%]	1a-4Glufos [%]	1a-4Fos [%]
AMAPA	500	100	50	40
	125	100	20	10
	60	100	0	0
CHEAL	500	100	10	80
	125	70	10	70
	60	40	10	0
EPHHL	500	100	30	60
	125	70	10	30
	60	30	0	0
IPOHE	500	100	10	60
	125	50	10	20
	60	30	0	0
SETFA	500	100	0	50
	125	100	0	10
	60	100	0	0
ECHCG	500	100	0	30
	125	90	0	10
	60	80	0	0
ELEIN	500	100	20	40
	125	100	0	20
	60	80	0	0
DIGSA	500	100	60	80
	125	90	10	3
	60	80	0	0
LOLPE	500	70	0	10
	125	30	0	10
	60	20	0	0

[a] AMAPA: *Amaranthus palmeri*, CHEAL: *Chenopodium album*, EPHHL: *Euphorbia heterophylla,* IPOHE: *Ipomoea hederacea*, SETFA: *Setaria faberi*, ECHCG: *Echinochloa crus-galli*, ELEIN: *Eleusine indica*, DIGSA: *Digitaria sanguinalis*, LOLPE: *Lolium perenne*. Three concentrations were used (500, 125, 60 [g/ha]); adjuvant 0.5% *Tween 20*; assessment 13 days after herbicide application, 0% meaning *no effect*, 100% meaning *complete kill*.

Table 2

In-vivo-Toxicity investigations with the octaarginine-fosmidomycin salts **1**- and **2**-4**Fos** by intravenous injection into the tail vein of mice non-infected or infected by *P. berghei*. For comparison, literature toxicity values of other oligo-arginines, **1** and **2**, are also shown (their corresponding concentrations may have to be corrected to lower values6). Parasitaemia reductions by Na-fosmidomycin are also presented. Parasitaemia reductions < 20% must be considered non-significant in this investigation: 'no parasitaemia reduction'. Administration cocktail 0.9% NaCl; volume administered: 0.01 ml/g mouse

Compound	*i.v.* dose [μmol/kg]	Observed effects
With *Plasmodium berghei*-infected mice		
Na-fosmidomycin	$1.5 \cdot 10^6$	95% Parasitaemia reduction
	$5 \cdot 10^5$	72% Parasitaemia reduction
	$1.5 \cdot 10^4$	No parasitaemia reduction
	$5 \cdot 10^3$	No parasitaemia reduction
1a-4**Fos**	50	Immediate exitus
	1.5	Exitus in 3 min
	0.5	No parasitaemia reduction, increased heart rate (HR), ataxia
ent-**1a**-4**Fos**	50	Immediate exitus
	1.5	Exitus in 3 min
	0.5	No parasitaemia reduction, increased HR, ataxia
2a-4**Fos** (*β*)	47	Immediate exitus
	1.4	Exitus in 3 min
	0.47	No parasitaemia reduction, no acute toxicity symptoms
With non-infected mice		
1a	79	Immediate exitus
	7.9	Increased HR, ataxia
1a-9**TFA**	43.6	Immediate exitus
	4.4	Immediate exitus
1b-8**TFA**	39.4	Immediate exitus
	4.0	Increased HR, ataxia
1a-4**Fos**	5	Exitus in 3 min
	0.5	Increased HR, ataxia
ent-**1a**-4**Fos**	5	Exitus in 3 min
	0.5	Increased HR, ataxia
Our previous work with rats		
2b (*β*)	0.7	No toxic effects[21][57]
Reports in the literature with mice		
ent-**1b**	5	Exitus within < 5 min[55]
	2.5	4/5 Survival[55]
mixed-(L/D)-**1a**	20	Exitus in < 5 min[56]
	10 and 5	Survival depends on ratio and position of L- and D-Arg[56]

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Table 3

In-vitro-Toxicity investigations with octa-Adp derivatives **10** – **12**. Intravenous injection into the tail vein of mice non-infected or infected by *P. berghei*. The parasitaemia reduction observed with **10-4Fos** was below 20%. For the experiment with octa-Adp **10** TFA-free peptide was used; peptides **11** and **12** were employed as TFA salts (*cf.* footnote 6 above). For comparison with octaarginine derivatives, for abbreviations, and for experimental details see Table 2

Compound	*i.v.* dose [μmol/kg]	Observed effects
With *Plasmodium berghei*-infected mice		
10-4**Fos**	1.10	No parasitaemia reduction
	0.34	No parasitaemia reduction
10	45	No parasitaemia reduction, no acute toxicity symptoms
	9	No parasitaemia reduction, no acute toxicity symptoms
	4.5	No parasitaemia reduction, no acute toxicity symptoms
With non-infected mice		
11-9**TFA**	30.1	Immediate exitus
	3.0	Increased HR, ataxia
12-9**TFA**	29.1	Immediate exitus
	2.9	Increased HR, ataxia