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Functionalized triazolopeptoids – a novel class for mitochondrial targeted delivery†

Daniela Althuon,‡^a Franziska Rönicke,^b Daniel Fürniss,‡^a Jasmin Quan,^a Isabelle Wellhöfer,^b Nicole Jung,^{a,b} Ute Schepers*^b and Stefan Bräse*^{a,b}

Here we introduce linear 1,4-triazolopeptoids as a novel class of cell penetrating peptidomimetics suitable as organ targeting molecular transporters of bioactive cargo. Repetitive triazole moieties with up to three residues were assembled on solid supports using copper-catalyzed alkyne–azide cycloadditions (CuAAC) in a submonomer approach. Depending on the lipophilicity of their side chain appendages the 1,4-triazo-lopeptoids showed either endosomal localization or a strong colocalization with the mitochondria of HeLa cells with moderate toxicity. While the basic triazolopeptoids mainly target the neuromast cells in zebrafish embryos, the lipophilic ones colocalize with either cartilage in the jaws and the blood vessel system.

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Introduction

To date functional peptidomimetics often replace peptides with similar biological activities while displaying an enhanced stability towards enzymatic degradation. In addition, they also allow for additional spatial arrangements of hydrogen-donor and acceptor moieties. The key feature of most peptidomimetics is the arrangement of moieties that mimic the amido group. Peptide bond bioisosters, such as hydroxyethylamine (Hea), aromatic amides, and tetrazole rings have been used to stabilize metabolically labile peptide bonds in peptidomimetic drug design.^{1,2} In addition, foldamers containing a triazole ring peptide bond isoster have recently emerged as a novel class of peptidomimetics with properties and structural diversity similar to that of natural peptides.^{3,4-12} As such, they not only have a planar geometry like amides but also are enzymatically stable.13 Poly-1,4-triazoles with three and more triazoles in a linear backbone containing different spacers (C2,14,15 $C_{31}^{16,17}$ C_{4} -sugars¹⁸⁻²⁰ and likewise, non-racemic chiral poly-1,4-triazoles have been synthesized.^{14,16-20} They have been shown to play a pivotal role in materials science,²¹ drug development and medicinal chemistry.

†Electronic supplementary information (ESI) available: Characterization, toxicity, spectra for compounds **14** to **18**. See DOI: 10.1039/c5ob00250h ‡These authors contributed equally. Based on our interest in the use of aliphatic foldamers especially the cell penetrating peptoids (CPPos) as molecular transporters for a variety of cargos,^{5,13,22,34,35} we were interested to explore linear 1,4-triazolopeptoids as a novel class of cell penetrating foldamers. Inspired by the recent publication of Ke *et al.*,³⁵ we designed a set of novel triazolopeptoids (Fig. 1). In contrast to the structure **2** of Ke *et al.*, they are supposed to be less prone to dimerization and aggregation due to the lack of the amide hydrogen.

The herein presented synthesis of this rather unexplored class of 1,4-triazolopeptoid foldamers,^{36–38} is based on a modular approach for a straightforward assembly on solid supports which allows (a) for the incorporation of a huge variety of functionalized side chains, and (b) for the use of chiral building blocks. Besides a solid phase synthesis described by Fujino *et al.*, most of the linear compounds have been obtained by solution phase syntheses so far.^{16,17,25,39}

^aInstitute of Organic Chemistry, Karlsruhe Institute of Technology, Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany. E-mail: braese@kit.edu

^bInstitute of Toxicology and Genetics, Karlsruhe Institute of Technology, Campus North, Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany. E-mail: ute.schepers@kit.edu

Among the foldamers, aliphatic β -peptides and cell penetrating peptoids (CPPos)^{5,13,22,34,35} as well as aromatic amide foldamers² have already been shown to exhibit cell penetrating properties and can be used as transport moieties for targeted drug delivery.²³⁻³⁴ However, triazole foldamers, which are accessible by subsequent click reactions were not tested as mimetics of CPPs to facilitate drug uptake *in vivo* and the impact of the triazole bioisosters on the cell permeation characteristics is not well understood.¹ In contrast to the aliphatic foldamers, which mostly recapitulate the same structural features as their peptide counterpart, structural studies of foldamers with triazole containing backbones showed considerable differences in the overall structure compared to the corresponding peptide, which might have an impact on their cellular uptake capabilities.²



Fig. 1 Generic structure of 1,4-triazolopeptoids synthesized in this report 1 and by Ke *et al.*³⁵ 2.



Scheme 1 Strategies to form polymer-bound triazolopeptoids 6.

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As known from peptoid chemistry, the synthesis on solid supports not only allows for the coupling of fully functionalized monomers but also for the assembly of submonomers.²²

We therefore devised two different strategies in order to synthesize the 1,4-triazolopeptoids 1 (Scheme 1).

In the first submonomer-based approach, a stepwise addition of azidoacetic acids 3 and functionalized propargylic amines 4 were used to synthesize the immobilized 1,4-triazolopeptoids on solid supports.

In the second approach, a so-called monomer method adapted from well-established peptide and peptoid syntheses with the incorporation of building blocks of type 5 was used. We came to the conclusion that the stepwise approach is more promising. For the CuAAC reaction, we used copper(1) iodide in N,N-diisopropylethylamine (DIPEA) in THF furnishing the product **14c** in overall 32% yield (Scheme 2).

In order to test different 1,4-triazolopeptoids for their ability to act as cell penetrating moieties for the delivery of bioactive molecules, we synthesized a small library containing fluorophore-labelled 1,4-triazolopeptoids with different side chains with (14c, 17a-c, 18a-c) and without (15a-c, 16a-c) an *N*-methoxyethyl glycine spacer (Table 1, Fig. 2). As the synthesis of 14c resulted in significantly higher yields compared

Table 1	Yields of purified triazolopeptoids, see ESI and Fig. 2		
Peptoid	Yield [%]	Peptoid	Yield [%]
14c	32	16b	6.3
15a	$7.2-29^{a}$	16c	$1.4-4.6^{a}$
15b	$1.6-9.5^{a}$	17b	1.6
15c	$9.4-1.9^{a}$	17c	1.8
16a	2.0	18c	7.0

^{*a*} Result of several runs and/or variants (see ESI).



Scheme 2 Solid-phase synthesis of 1,4-triazolopeptoid 14c.



Fig. 2 Small library of 1,4-triazolopeptoids bearing different side chains (see Table 1).

to the reactions without spacer-unit, the spacer was used with the intention to increase the yields of the other 1,4-triazolopeptoids bearing different side chains. However, following syntheses did not confirm that the presence of the spacer improves yields of all derivatives suggesting that the charges of the side chains have considerable effect on the synthesis.



Fig. 3 1×10^4 HeLa cells were incubated with a 10 μ M of 15c, 16c, 17c, and 18c in DMEM at 37 °C for 24 h and subsequently subjected to fluorescent confocal microscopy using a Leica SP5-TCS (DMI6000) inverse microscope. Objective: HCX PL APO CS 63.0 \times 1.20 WATER UV. 1st column: Hoechst 33342, emission bandwidth: 417–468 nm; 2nd column: MitoTracker® Green, emission bandwidth: 499–522 nm; 3rd column: RhodB-triazolopeptoid, emission bandwidth: 593–696 nm; 4th column: merge of columns 1 to 3. Scale bar = 20 μ m.

The solid phase synthesis was either conducted with protected propargyl amines (**4-Fmoc**) or free amines (**4-H**). All 1,4triazolopeptoids were labelled with rhodamine B as a fluorophore.

Biological application

To investigate, whether the 1,4-triazolopeptoids are capable to act as peptidomimetics of CPPs, we tested the trimeric 1,4-triazolopeptoids **15c** (n = 3), **16c** (n = 3), **17c** (n = 3) and **18c** (n = 3) for their uptake in living cells and zebrafish embryos. 1×10^4 human cervix carcinoma cells (HeLa cells) were treated with 10 µM of the 1,4-triazolopeptoids, respectively.



Fig. 4 96 hpf zebrafish were incubated with a 5 μ M triazolopeptoids in E3-medium solution for 3 h at 28 °C in the dark. Control embryos were only treated with E3-medium. For subsequent live imaging, embryos were washed (3 × E3-medium), anesthetized (0.02% tricaine) and positioned in an 8 well μ -slide from IBIDI (lbitreat). Confocal fluorescence microscopy was performed on a Leica SP5-TCS (DMI6000) inverse microscope using a HC PL FLUOTAR 10.0 × 0.30 DRY objective. 1st column: RhodB-triazolopeptoid, emission bandwidth: 593–696 nm; 2nd column: brightfield; 3rd column: merge of column 1 and 2. Scale bar = 600 μ m.

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To determine their route of cellular uptake and their intracellular localization, the cells were counterstained with a mitochondrial marker (MitoTracker®Green) and the nuclear stain Hoechst 33342. While the 1,4-triazolopeptoids **16c** and **18c** comprising the lysine side chain accumulate in endosomal vesicles, 1,4-triazolopeptoids **15c** and **17c**, bearing the more lipophilic chlorobenzyl moiety show a strong accumulation in the mitochondria and a less pronounced colocalization with the endosomes (Fig. 3). This mitochondrial localization suggested a more amphiphilic nature of **15c** and **17c** and therefore a direct transport through the plasmamembrane *via* inverted micelles driven by the membrane potential, while **16c** and **18c** are taken up by classical endocytosis (Fig. 3).

All 1,4-triazolopeptoids showed moderate to low toxicity with LD₅₀ values between 10 μ M (**15c**) and 40 μ M (**17c**) for the mitochondrial derivatives, and even a lower toxicity with an LD₅₀ > 50 μ M for the endosomal derivatives **16c** and **18c** (see ESI, Fig. S2†).

To investigate the suitability of the 1,4-triazolopeptoids for drug transport and possibly organ specific localization *in vivo*, compounds **15c**, **16c**, **17c** and **18c** were analysed in naturally hatched zebrafish embryos (Casper strain, mitfa^{w2/w2}; roy^{a9/a9})⁴⁰ for their localization. Therefore 96 hpf (hours post fertilization) zebrafish embryos were treated with 5 μ M of the 1,4-triazolopeptoids in E3-medium solution (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) for 2 h and subjected to confocal fluorescence microscopy.

While the positively charged 1,4-triazolopeptoids and especially **18c** are specifically accumulating in the neuromast cells of the lateral line quite rapidly, the more lipophilic 1,4-triazolopeptoids especially **15c** are distributed to cartilage of the jaw and gills. In all cases a strong fluorescence is visible in the digestive tract, indicating uptake by ingestion. A strong staining of the blood vessel system and the gut by **15c** suggests a direct passage of **15c** through the colon endothelium to the blood stream (Fig. 4).

These results confirm that these 1,4-triazolopeptoids can function as molecular transporters, targeting even particular cell tissues or organs within a complex organism.

Conclusions

In this study, we present a new class of 1,4-triazolopeptoid foldamers synthesized by a submonomer approach on solid supports which are suitable as cell penetrating peptide (CPP) mimetics. They are highly selective for transport into endosomes and mitochondria. They also express certain organ targeting properties in living zebrafish embryos. While basic side chains seem to direct the 1,4-triazolopeptoids into neuromasts the more lipophilic side chains enrich in gills and the jaws.

In the future this submonomer solid phase approach can be exploited for the synthesis of larger diverse libraries for organ targeting transporters: currently, we are about to make medium-sized libraries with additional functionalities to unravel not only the global structures of these new foldamers in solution but also the structure–activity relationship for drug delivery purposes.

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