PEGylated Prodrugs

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Controlled Systemic Release of Therapeutic Peptides from PEGylated Prodrugs by Serum Proteases**

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Bacterial infections are still the leading cause of death worldwide, with antibiotic resistance being one of the greatest pharmaceutical challenges. Media reports highlighting the threats from methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *S. aureus* infections (MDR-SA) have stimulated the search for new antibiotics. Thus novel drugs against Gram-positive pathogens have entered the market during the past decade.[1] Much less research, however, has been devoted to finding novel compounds against Gram-negative bacteria, which cause considerable concern due to the rapid spread of multidrug-resistant or extremely resistant strains.[2] One promising class of molecules are antimicrobial peptides (AMPs), which have proven their efficacy as part of innate immunity for millions of years.[3] As pharmaceuticals, however, AMPs have several disadvantages: a) toxic side effects observed at higher doses especially for lytic AMPs targeting the membrane, and b) the circulation time in blood, as linear peptides are degraded by blood-borne proteases and cleared rapidly by the kidneys.

A common approach to overcome such limitations relies on the covalent ligation of polyethylene glycol (PEG), which reduces renal clearance, enzymatic degradation, immunogenicity, and toxicity. PEG also improves the solubility and thus minimizes the injection volume.[4] As early concerns about PEG toxicity were not confirmed, PEG was approved for many drugs and cosmetics.[5]

Besides these obvious advantages, PEGylation often abolishes the activity of a drug, especially for small molecules. In these cases, PEG can be coupled by a hydrolysable or enzymatically cleavable linker (e.g. ester, carbonate, carbamate, hydrazone, or aromatic linker) to release the parent drug from the prodrug at a suitably designed position.[6] Here we present a novel concept to systemically release a peptide or small protein from PEGs by the proteolytic activity of blood using custom-tailored linkers, which makes it possible to tune the release kinetics to meet the pharmacokinetic requirements of the parent drug.

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We examined this concept in our efforts to optimize insect-derived proline-rich AMPs (PrAMPs) to systemically treat Gram-negative bacteria. The lead compounds Api88 and Onc72 are highly efficient in vivo and provide high margins of safety (Table 1).[7]

Recently, we improved both peptides to obtain half-life times of more than 4 h in mouse serum.[8] When these compounds were coupled to PEG to reduce the renal clearance, already the small PEG^{750}, an oligomer with an average mass of 750 Da, reduced the antibacterial activity dramatically. Longer PEGs abolished the activity completely. This was expected, as PrAMPs have to enter the bacteria to block intracellular target proteins, such as chaperone DnaK.[9] This uptake is probably prevented by the covalently coupled polar PEG. Thus, we elongated Onc72 (or Onc110) at the N-terminus by three residues (Gly-Ala-Arg, GAR) and coupled PEGs of different lengths, assuming that the active peptide would be released by trypsin-like proteases. Whereas PEG^{750} was coupled as a carboxylic acid to the unprotected N-termini of both peptides in reasonable yields, it was difficult to purify the products by RP-HPLC due to the broad peaks caused by the polydispersity of PEG. Thus, we altered the synthesis strategy and coupled iodoacetic acid to the free N-termini and coupled the peptides after purification to PEG^{750}- or PEG^{5000}-thiols. It was even possible to separate the final prodrug from the substrates by RP-HPLC. In serum both Onc72 (black) and Onc110 (blue) were slowly released from the prodrugs containing PEG^{750} (■) or PEG^{5000} (◆) at different kinetics (Figure 1).

Interestingly, both peptides were released from the PEG^{750} construct at almost the same speed as from the non-PEGylated GAR-Onc72/110 sequence (Figure S1 in the Supporting Information). This indicates that at least for short PEGs the release kinetics depends mostly on the recognition sequence and is not hampered by the polymer.

Table 1: Sequences of oncocin and apidaecin derivatives.

Peptide	Sequence[a]	PEG spacer (#)
Onc72	(#)-VDKPPYLPRPRPPROIYNO-NH_2	PEG-GAR
Onc110	(#)-VDKPPYLPRPRPZRZXYNO-NH_2	PEG-GAR
Api88	gu-ONNRPVYIPRPRPPHPRL-NH_2	
Api137	gu-O(#)NNRPVYIPRPRPPHPRL-OH	PEG-GARSG
Api300	gu-OO(#)NRPVYIPRPRPPHPRL-OH	PEG-GARSG
Api301	gu-ONO(#)RPVYIPRPRPPHPRL-OH	PEG-GARSG

[a] Abbreviations: O = L-ornithine (Orn), Z = *trans*-4-hydroxy-L-proline, X = *tert*-L-leucine, and gu = *N*,*N*,*N*′*N*′-tetramethylguanidino. (#) Hydrogen atom for the free peptide (e.g. Onc72) or PEG spacer in the PEGylated sequence (e.g. PEG-GAR-Onc72).

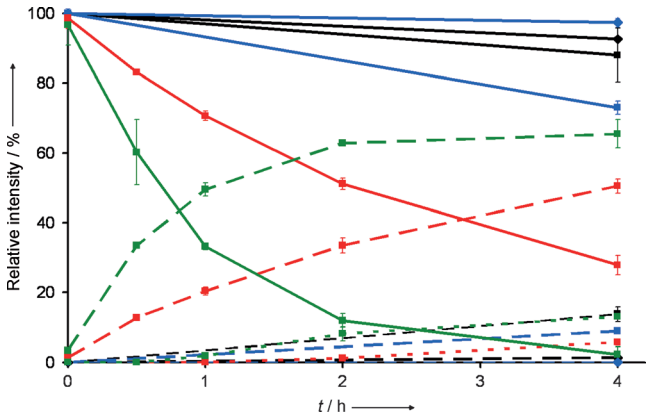

Figure 1. Degradation of PEG-GAR-Onc72 (black), PEG-GAR-Onc110 (blue), and Api137 containing PEG-GRSG (red) or PEG-GARSG (green) at the δ-amino group of Orn-1 in mouse serum (37 °C). The peptide amounts were calculated by the peak areas (RP-HPLC, absorbance at 214 nm) relative to the peak areas obtained for the original peptide construct dissolved in serum (set to 100%). Studied were PEG^{750} (■) and PEG^{5000} (◆) Solid lines represent the PEGylated prodrugs, dashed lines the released peptides Onc72, Onc110, and Api137(SG), and dotted lines the degradation product $^{1-16}$Api137(SG).

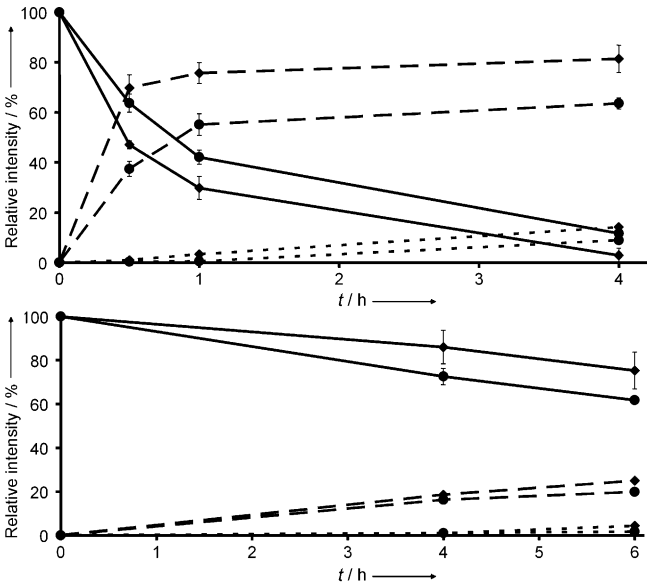

Figure 2. Degradation of Api301 PEGylated with PEG^{750} (top) and PEG^{5000} (bottom) at the N-terminus of GRSG (◆) and GARSG (●) in mouse serum at 37 °C. Solid lines represent the PEGylated prodrugs, dashed lines the released therapeutic peptide Api301(SG), and dotted lines the degradation product $^{1-16}$Api301(SG).

Before studying this in more detail, we probed the concept also for N-terminally modified peptides. In the case of Api137,[9] which bears a guanidated N-terminus (Table 1), linking PEG^{750} to the δ-amino group of ornithine in position 1 (Orn-1) reduced the antibacterial activity more than 200-fold. As proteases would not cleave off a peptide spacer directly linked to the side chain of Orn-1 by a C-terminal arginine, we tested the tetrapeptide Gly-Arg-Ser-Gly (GRSG) as a linker with the cleavage site being two residues away from the branched Orn. Indeed PEG^{750}-GRSG was cleaved with a half-life time of roughly 2 h (Figure 1, red, solid line).

When the linker was elongated by alanine (i.e. GARSG) the half-life time in serum was reduced to only 40 min (Figure 1, green, solid line). The quantities of the released Api137(SG) increased as the two prodrugs degraded (Figure 1, dashed lines). The late appearance of the metabolite $^{1-16}$Api137(SG) indicated that Api137 was not degraded as long it was coupled to the PEG. This was also confirmed by mass spectrometry, as the signal pattern of the prodrug was stable over time and did not shift to lower masses.

The influence of the branching site was further evaluated by substituting residues Asn-2 or Asn-3 of Api137, which can be replaced without major activity losses,[6] by Orn carrying either peptide linker at the δ-amino group. In this way, the positive charge at the N-terminal residue, which is important for the antibacterial activity especially against *P. aeruginosa*, was rebuilt. As the release kinetics of the Orn-2 and Orn-3 derivatives were identical for both linker sequences and both PEGs within the error range, we discuss here only the results of the Orn-3 peptides (Api301); the data of the Orn-2 derivatives (Api300) are given in the Supporting Information (Figure S2). The active peptide was released from Api301(PEG^{750}-GRSG) relatively fast ($t_{1/2} \approx 30$ min), reaching a level of approximately 70% relative to the prodrug within 1 h with a slight increase during the next 3 h (Figure 2, top). The corresponding PEG^{5000} derivative was more stable and Api301(SG) reached roughly 20% of the initial prodrug level after 4 h (Figure 2, bottom). The longer peptide linker sequence resulted in a slightly slower release of Api301(SG) and thus also lower quantities of Api301(SG) at the end of the incubation period (Figure 2). The influence of the prodrug design on the antibacterial activity was probed in TSB medium and a mixture of TSB medium and mouse serum (Table 2). Upon the addition of mouse serum to the TSB medium the antibacterial activity of Api137 increased slightly. In comparison to that, the activity of the more labile Api88 was decreased by fourfold under these conditions. In contrast to the inactive PEGylated compounds without a linker

Table 2: Antimicrobial activities of free and PEGylated Api and Onc derivatives as well as half-life times of the prodrugs in serum.

Peptide	MIC [μmol L^{-1}][a]		$t_{1/2}$ [min]
	TSB	Serum + TSB	
Api88	0.4	1.8	
Api137	0.5	0.2	
Api301 (SG)	0.8	0.2	
Api300 (PEG^{750}-GRSG)	2.9	0.4	50
Api300 (PEG^{750}-GARSG)	2.8	0.7	35
Api301 (PEG^{750}-GRSG)	2.8	0.4	45
Api301 (PEG^{5000}-GARSG)	9.3	0.6	> 360
Onc72	1.7	1.7	
Onc110	7.0	0.9	
PEG^{750}-GAR Onc72	12	6	> 360
PEG^{750}-GAR Onc110	24	12	> 360

[a] Minimal inhibitory concentrations (MICs) were determined in 33% TSB medium or 25% TSB-medium complemented with 25% mouse serum. Half-life times of the PEGylated prodrugs were determined in mouse serum (37 °C) using the peak areas obtained by RP-HPLC.

sequence, the PEG^{750}- and PEG^{5000}-G(A)RSG constructs were already slightly active in TSB medium. In the presence of mouse serum, however, the antibacterial activity increased further reaching the same MIC value as Api137. This high activity was surprising, as the active peptide is released slowly providing the bacteria an extended period of time to grow. Probably, the initial growth advantage is compensated by the constant release of Api301(SG) over the assay period, providing moderate peptide quantities for an extended period of time.

Finally, we tested the degradation of Api300(PEG-GRSG) in serum samples obtained from ten healthy male and female humans (56 ± 6 years) to evaluate individual release kinetics. The degradation was slower than in mouse serum with 60 ± 16% of the prodrug being present after 1 h (Figure S3 in the Supporting Information) and importantly with only small differences among the ten samples. It should be noted that most peptides including the Api and Onc peptides are mainly degraded in blood by trypsin-like proteases, which also release the peptides from the prodrugs. In persons with high protease activities in blood the drug should be released and degraded quickly, whereas in persons with low protease activities the drug should be released and degraded slowly. Thus, the drug concentration should be very similar among individuals and vary less than the release kinetics indicate.

In summary, we were able to establish a novel concept to protect peptidic drugs temporarily by a cleavable PEG-peptide linker, such that the active compound was released systemically by serum proteases. Based on the structural constraints of the peptidic drug, PEG can be linked either to the N-terminus or to a side chain that does not interfere with the function of the original drug. The concept could even be extended to the C-terminus of peptidic drugs containing a >C-terminal arginine or lysine residue. The presented strategy makes it possible to apply the advantages of PEGylation (e.g. reduced toxicity and immunogenicity, prolonged circulation times) to short peptides without diminishing their activities by permanent PEGylation, such as compounds like apidaecin and oncocin, which target intracellular targets. Importantly, the linker sequence can be optimized within a certain range for the desired release kinetic.

Experimental Section

Peptides were synthesized on solid phase on a 25 μmol scale on a multiple synthesizer (SYRO2000, MultiSynTech GmbH, Witten, Germany) using 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) chemistry and in situ activation with diisopropylcarbodiimide and 1-hydroxybenzotriazole. Ornithins to be branched contained the 4-methyltrityl (Mtt) protecting group. The N-termini of the apidaecin derivatives were guanidated before the Mtt group was cleaved with 2% trifluoroacetic acid (TFA).[7a,8a] The synthesized peptides were cleaved with TFA, precipitated with diethyl ether, purified by RP-HPLC (C_{18}-phase, aqueous acetonitrile gradient in the presence of 0.1% TFA) and confirmed by MALDI-TOF-MS. MIC values were determined in triplicate for *E. coli* BL 21 AI incubated in 33% TSB medium or in 25% TSB medium complemented with 25% mouse serum at 37°C overnight. Peptide degradation and peptide release from the PEGylated prodrugs were determined by incubating the compounds in mouse or human serum (37°C) and analyzing aliquots at different time points by RP-HPLC. The quantities of the studied compounds were estimated from the peak areas relative to its initial peak area ($t = 0$ min).

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