Brief Articles

Synthesis by Chemoselective Ligation and Biological Evaluation of Novel Cell-Permeable PKC-ζ Pseudosubstrate Lipopeptides

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The ability of lipopeptides to passively cross the cell membrane opens new opportunities for the intracellular delivery of bioactive peptides. However, the production of large series of cell-permeable lipopeptides is not trivial due to their generally low solubility. We have evaluated the possibility of associating the fatty acid to the functional cargo using generally applicable ligation chemistries. To this end, we have designed an amphiphilic shuttle in which arginine residues served to solubilize the lipid part in aqueous media, during both the assembly of the lipopeptide and the cellular assays. Our model peptide, the pseudosubstrate sequence of protein kinase C-ζ (PKC-ζ), was associated to the pentapeptide Gly-Arg-Gly-Arg-Lys(Pam)-NH_2 through thiazolidine, thioether, disulfide, or hydrazone linkages. The cytoplasm import of the resulting constructs was monitored through the quantification of the apoptosis specifically induced by PKC-ζ inhibition. Our observations suggested the interest of this noninvasive cellular import method to modulate the activity of an intracytoplasmic pharmacological target and showed the influence of a non-amide link created between the functional peptide and the lipidic vector: optimal results, in terms of both specific activity and low basal cytotoxicity, were obtained with the thiazolidine ligation product.

Introduction

The ongoing human genome sequencing effort continues to yield new structural information about proteins that participate in cellular activities. Their structure−function analysis however is hampered by the inability of most synthetic probes and, in particular, peptides to cross the cell membrane. While transfection experiments or invasive techniques provide valuable information, their application is relatively limited. Therefore, several approaches have been undertaken to permit noninvasive cellular import of synthetic peptides, through their covalent association with synthetic vectors. So far, the largest family of such carriers, reviewed by Derossi et al.[1] and Hawiger,[2] is represented by cell-permeable peptides. In contrast, little attention has been directed to the intracellular delivery of peptides modified by a simple lipid chain, first described by Eichholtz et al.[3] Recently however, we have observed that various 9−38 residues peptides modified by a simple N^{ϵ}-palmitoylated lysine could be delivered at 4 °C into the cytoplasm of various nonphagocytic cellular types, suggesting an energy-independent mechanism.[4−7]

The assembly of cell-permeable peptides through site-specific ligation of fully deprotected segments has been proposed to circumvent the need for the complete stepwise synthesis and purification of molecules which are prone to aggregation: typical methods of non-amide ligation, such as disulfide,[8] oxime,[9] thiazolidine,[10] or hydrazone[11] ligations, have been used to associate hydrophobic moieties to peptides or proteins.

The aim of this study was to develop a modular approach which could be adapted to the production of large series of cell-permeable lipopeptides. Starting from the hypothesis that cell permeability is associated with the presence of a long alkyl chain, but not necessarily dependent upon the overall hydrophobicity of the shuttle system, our approach was to design a generic amphiphilic vector containing both a palmitoyl chain and arginine residues. Generally applicable chemoselective ligation protocols were tested for its association to a cargo sequence: the interest of thioether, hydrazone, disulfide, or thiazolidine linkages was evaluated. The pharmacological importance of protein kinase C-ζ (PKC-ζ) and its subcellular localization[12] led us to select this tubulin-associated enzyme as a model target and its pseudosubstrate sequence as a functional cargo. The biological activity of the different constructs was assessed by investigating their effect upon specific induction of apoptosis in nonphagocytic human cells with reference to the previously described palmitoylated PKC-ζ pseudosubstrate lipopeptides **1a**.[7]

Results and Discussion

We have designed a shuttle system, Gly-Arg-Gly-Arg-Lys(Pam)-NH_2 herein referred as 'vector' (Scheme 1), that could be easily associated with bioactive peptides with minimal synthetic and purification efforts using known functionalities[13,14] and ligation protocols[15−17] as described in Scheme 2.

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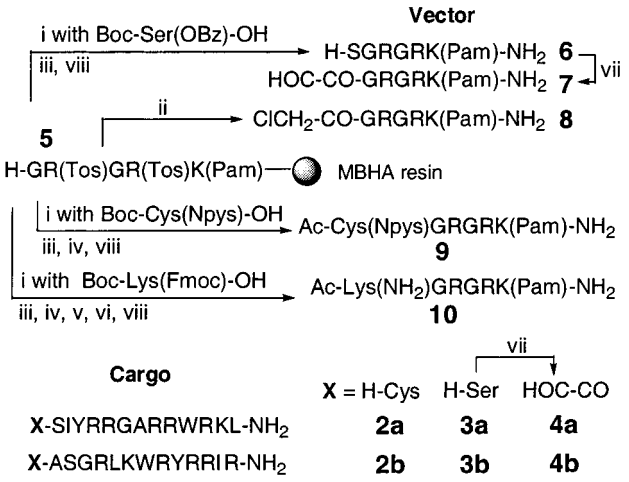
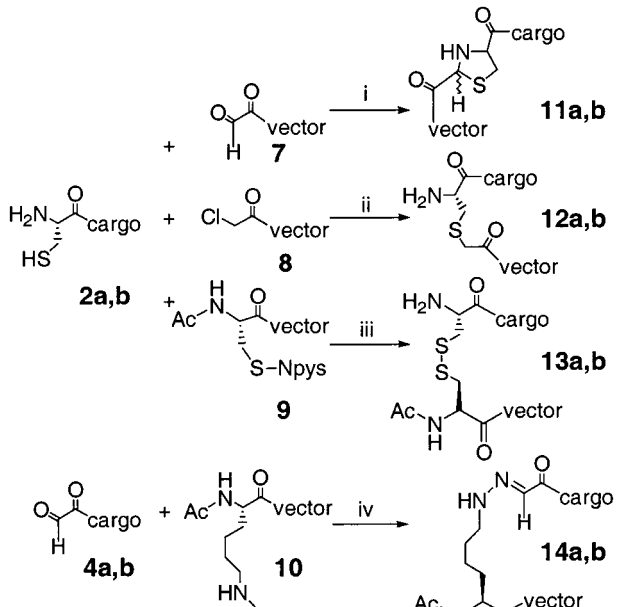
Scheme 1. Structures of the Functionalized Segments[a]



[a] Reagents: (i) amino acid, HBTU, HOBt, DIEA in DMF; (ii) chloroacetic acid, DIC in DMF; (iii) TFA/DCM (1/1); (iv) Ac_2O, DIEA in DCM; (v) piperidine (20%) in DMF; (vi) *N*-Boc-3-(4-cyanophenyl)oxaziridine in DCM; (vii) $NaIO_4$, buffer pH 7.0; (viii) HF, anisole.

Scheme 2. Structures of Non-Amide Ligation Products[a]



[a] Reagents and conditions: (i) NMP/pH 5.1 phosphate buffer (1/1), TCEP, 37 °C, 5 h; (ii) DMF/pH 7.95 phosphate buffer (95/5), TCEP, rt, 1 h; (iii) DMF/pH 7.95 phosphate buffer (95/5), rt, 5 min; (iv) DMSO/pH 6.0 phosphate buffer (1/4), rt, 20 h.

The important differences in retention times between the functionalized segments **2a,b**−**4a,b** and **6**−**9** and the ligation products **11a,b**−**14a,b** (Table 1) facilitated their separation by reversed-phase chromatography (e.g. Figure 1). The chemoselectivity and mildness of the experimental conditions produced unambiguous reaction products which were easily separated from the parent reagents.

Our series of PKC-ζ pseudosubstrate constructs differing in the nature of linker was compared to a second, scrambled-derived series. The biological activities of all constructs including the reference palmitoyl peptides **1a,b** were evaluated in parallel during the same experiments in Jurkat cells, a human leukemia T-cell line devoid of endocytic activity (Figure 2).

Table 1. Physicochemical Characterization of Segments and Ligation Products

		$[M + H]^+$ [a]			
	compd	calcd	found	k' [b]	isolated yield (%)
2a	cysteyl	1821.2	1821.2	8.5	45
2b	cysteyl	1821.2	1821.8	8.1	33
3a	seryl	1805.1	1805.0	nd	80[c]
3b	seryl	1805.1	1807.2	nd	96[c]
4a	glyoxylyl	1774.1	1776.2	6.3	38
4b	glyoxylyl	1774.1	1775.2	6.1	42
6	seryl	898.2	899.1	nd	39[c]
7	glyoxylyl	867.2	867.9	18.2	40
8	chloroacetyl	887.6	887.4	18.7	66
9	S-Npys	1110.4	1109.8	19.8	49
10	hydrazino	996.3	997.3	17.6	12
11a	thiazolidine	2669.3	2668.6	16.4	52
11b	thiazolidine	2669.3	2668.0	16.0	49
12a	thioether	2671.3	2670.0	16.1	60
12b	thioether	2671.3	2670.3	15.9	53
13a	disulfide	2774.4	2773.0	15.9	65
13b	disulfide	2774.4	2773.0	15.7	55
14a	hydrazone	2751.4	2750.2	16.4	54
14b	hydrazone	2751.4	2750.0	16.6	45

[a] ES−MS spectra were recorded on a Micromass Quatro II electrospray mass spectrometer. [b] Capacity factors $k' = (t_r - t_0)/t_0$ were determined with the following RP-HPLC conditions: Zorbax C3 column, eluted at 50 °C with a flow rate of 1 mL/min; solvent composition A = 0.05% TFA in H_2O, B = 0.05% TFA in H_2O/acetonitrile (20:80); linear gradient 0−100% B over 30 min, monitoring 215 nm. [c] Crude yield. nd, not determined.

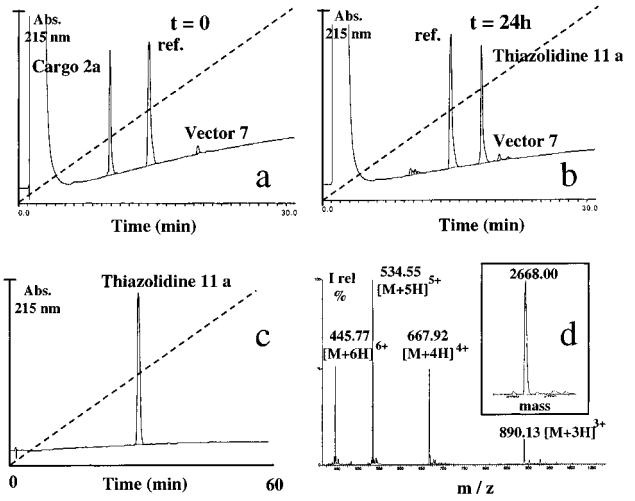


Figure 1. Monitoring the synthesis and characterization of the thiazolidine ligation product **11a** from the cysteinyl cargo sequence **2a** and the glyoxylyl vector **7**. RP-HPLC monitoring was performed as described in the legend to Table 1: (a) RP-HPLC profile of the initial reaction mixture; (b) after 5 h, reference Boc-L-Trp-OH; (c) RP-HPLC analysis, 0−100% B over 60 min linear gradient was performed, using the same solvent conditions; (d) ES−MS spectrum of the purified ligation product.

As previously described,[7] inhibition of the enzyme led to cell death as a result of apoptosis. Not surprisingly, some cytotoxicity was observed when testing high concentrations of the lipopeptides which could not be attributed to the specific inhibition of the target enzyme, as this was also observed with the scrambled analogues. Alteration of the membrane fluidity, interaction of peptide cargo with the membrane surface, or subtle configuration changes[18] could lead to membrane alteration as well as the cationic amphipathic properties of

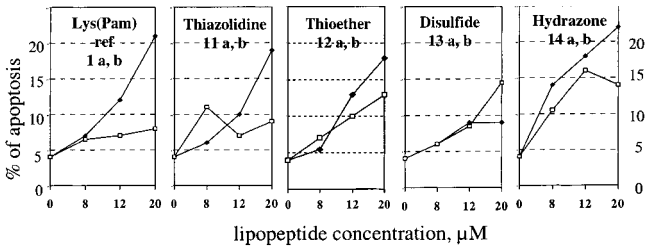


Figure 2. Quantification of apoptosis induced in Jurkat cells incubated with the PKC-ζ-derived lipopeptides (■) or their scrambled analogues (□). Apoptosis was quantified by flow cytometry as described in the Experimental Section. Results are representative of three independent experiments. Structure of the reference compounds: **1a**, Ac-K(Pam)SIYRRGARRWRKL-NH_2; **1b**, Ac-K(Pam)ASGRLKWRYRRIR-NH_2.

the constructs. However, the presence of additional arginine residues in the sequence of the vector did not result in additional cytotoxicity with reference to compound **1b**.

The biological activity of the thiazolidine **11a** and the relative nontoxicity of its scrambled analogue **11b** in the same concentration range confirm the utility of this ligation chemistry, which has been recently described as an acceptable alternative of the amide bond for the association of a functional peptide to membrane-permeable sequences.[19] Conversely, the observation of the cytotoxicity associated with the hydrazone **14b** can be related to previous observations suggesting that this linker can be more than a simple bond between moieties responsible for the bioactivity and/or biodistribution of a drug as it can sometimes play a direct role in the bioactivity.[20]

The cytotoxicity of the thioether derivatives **12a,b** as well as the inactivity of the disulfides **13a,b** were unexpected. Disulfide ligation was successfully used for intracellular delivery of the PKC-α pseudosubstrate associated with the penetratin peptide.[21] However, the disulfide construct **13a** led to no appreciable specific effect when compared to the scrambled sequence **13b** or the reference palmitoyl compound. This observation may be due to a more difficult access to the target, as PKC-ζ is found associated with the tubulin while the PKC-α isoform translocates to the internal face of the plasma membrane upon cellular activation. Alternately, the lack of specific biological activity with reference to the other constructs of our series could be due to the cleavage of the disulfide linkage in the reductive cytoplasmic medium, as the lipophilic tail could contribute positively to the interaction with the target, as previously observed in vitro with palmitoylated derivatives of the PKC-α pseudosubstrate.[22]

An original noninvasive cellular import modular system was obtained by combining a short amphiphilic lipopeptide vector and adapted chemoselective ligation protocols for its association to a model peptide, the PKC-ζ pseudosubstrate sequence. Among the different possibilities, the thiazolidine ring represents the most attractive linker, due to its ease of formation, its chemical stability, the straightforward access to functionalized segments, and the low nonspecific cytotoxicity of the resulting hybrid compounds. This approach could potentially be applied to the rapid preparation of new cell-permeable lipopeptide or peptide mimetic libraries for validation of intracytoplasmic pharmacological targets.

Experimental Section

Synthesis of the Segments. The different segments (0.25-mmol scale) were elaborated on a methylbenzhydrilamine (MBHA) resin (Applied Biosystems, Foster City, CA), using Boc/benzyl chemistry and the *N*-[(1*H*-benzotriazolyl)(dimethylamino)methylene]-*N*-methylmethaminium hexafluorophosphate *N*-oxide (HBTU) in situ protocol[23] in an Applied Biosystem 430A peptide synthesizer (Foster City, CA). Boc-protected amino acids were purchased from Propeptide (Vert-Le-Petit, France).

Glyoxylyl Vector 7. Boc-L-Ser(OBzl)-OH was coupled to peptidyl-resin **5** using HBTU/HOBt/DIEA activation. The peptide was deprotected and cleaved from the solid support in anhydrous HF (dry resin/HF/anisole: 1 g/10 mL/1 g, 1 h 30 min at 0 °C). Then, HF was evaporated and the crude peptide was precipitated in cold diethyl ether (200 mL). The precipitate was centrifugated, dissolved in aqueous acetic acid and lyophilized.

The crude seryl vector **6** was purified by RP-HPLC on a C3 Zorbax column (15 × 500 mm) using a linear water−acetonitrile gradient containing 0.05% TFA, flow rate 3 mL/min, detection at 235 nm. Starting from 0.25 mmol of MBHA resin, 111.7 mg (overall yield 39%) of peptide **6** was obtained.

Purified seryl vector **6** (25 mg, 22.2 mmol) was treated for 10 min with sodium periodate (10 mg, 46.7 mmol) in 100 mM pH 7.0 sodium phosphate buffer (2 mL). The reaction was stopped with 20 mL of 1,2-ethanediol and the crude reaction mixture was purified on a Vydac C4 column (15 × 500 mm) using the above eluent system. 9.7 mg (overall yield 40%) of glyoxylyl vector **7** was obtained.

Chloroacetyl Vector 8. Peptidyl-resin **5** was treated for 30 min with pre-formed chloroacetic acid anhydride (chloroacetic acid/diisopropylcarbodiimide (DIC): 8 equiv/4 equiv in DMF for 15 min). Peptide **8** was deprotected, separated from the solid support and purified as described above. Starting from 0.25 mmol of resin **5**, 185.0 mg (overall yield 66%) of chloroacetyl vector **8** was obtained.

Ac-Cys(Npys) Vector 9. Boc-L-Cys(*S*-nitro-2-pyridylsulfenyl)-cysteine (Boc-L-Cys(Npys)-OH) was coupled to resin **5** using HBTU/HOBt/DIEA activation. The α-amino group was deprotected with TFA/DCM: 1/1 (by vol) for 20 min and acylated with acetic anhydride (Ac_2O)/DIEA/DCM: 10/5/85 (by vol) during 15 min. Peptide **8** was deprotected, cleaved from the solid support and purified as described above. 164.0 mg (overall yield 49%) of Ac-Cys(Npys) vector **9** was obtained.

Hydrazino Vector 10. The hydrazino vector **10** was obtained from a selectively deprotected ϵ-amino group by solid-phase N-electrophilic amination as described.[14] Briefly, the peptidyl resin was reacted with 60 mg (1 equiv) of *N*-Boc-3-(4-cyanophenyl)oxaziridine (BCPO; Acros Organics, Noisy le Grand, France) in 6 mL of DCM for 3 h. The resin was treated with 50 mg of *N*-benzylhydrazine dihydrochloride in 5 mL of DMF/acetic acid/H_2O (3 × 10 min) to hydrolyze the imine formed between the ϵ-amino group and the 4-cyanobenzaldehyde generated during the reaction. The resin was neutralized with DIEA 5% in DCM for 2 min, and the N-electrophilic amination/hydrolysis/neutralization procedure was repeated until the obtention of a negative Kaiser test (10 cycles). Peptide **10** was deprotected, cleaved from the support and purified as described above. 40.0 mg (overall yield 12%) of Ac-Lys(NH_2) vector **10** was obtained.

Chemical Ligation Studies. The progress of the chemical ligation was followed by analytical RP-HPLC as indicated in the legend to Figure 1. At the end of the reaction, the reaction medium was purified by RP-HPLC on a C3 Zorbax column using a linear water−acetonitrile gradient containing 0.05% TFA; 0−60% acetonitrile in 80 min, flow rate 3 mL/min, detection at 215 nm.

Thiazolidine Peptides 11a,b. 6.0 mg (2.1 μmol) of peptide **2a**, 5.0 mg (4.6 μmol) of peptide **7** and 48 μL (1.1 μmol) of an

aqueous solution of tris(2-carboxyethyl)phosphine (TCEP) at 6.8 mg/mL were dissolved in 1 mL of 25 mM pH 5.1 citrate/phosphate buffer and 1 mL of *N*-methylpyrrolidinone (NMP) for 12 h at 37 °C: **11a**, 4.2 mg (overall yield 52%); **11b**, 4.0 mg (overall yield 49%).

Thioether Peptides 12a,b. 8.0 mg (3.0 μmol) of peptide **2a**, 5.7 mg (5.1 μmol) of peptide **8** and 23 μL (1.5 mmol) of an aqueous solution of TCEP at 19.2 mg/mL were dissolved in 200 μL of 0.1 M pH 8.0 phosphate buffer and 3.8 mL of DMF for 5 h at room temperature: **12a**, 6.9 mg (overall yield 60%); **12b**, 5.5 mg (overall yield 53%).

Disulfide Peptides 13a,b. 8.0 mg (3.0 μmol) of peptide **2a** and 5.3 mg (3.9 mmol) of peptide **9** were dissolved in 100 μL of 100 mM pH 8.0 phosphate buffer and 1.9 mL of DMF for 10 min at room temperature: **13a**, 7.2 mg (overall yield 65%); **13b**, 6.3 mg (overall yield 55%).

Hydrazone Peptides 14a,b. 6.7 mg (2.7 μmol) of peptide **4a** were dissolved in 2.4 mL of a 250 mM pH 6.0 citrate/phosphate buffer. The pH of the solution was adjusted to 6.1 with a 100 mM aqueous Na_2HPO_4. Peptide **10** (7.3 mg, 5.4 μmol) was dissolved in 600 μL of dimethyl sulfoxide (DMSO) and added to the peptide solution for 20 h at room temperature: **14a**, 5.3 mg (overall yield 54%); **14b**, 4.45 mg (overall yield 45%).

Determination of Apoptosis. Jurkat cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS; GIBCO), 2 mM glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 100 U/mL penicillin and 100 μg/mL streptomycin under 5% CO_2 at 37 °C. Jurkat cells (2×10^6 cells) were resuspended in FCS-free medium and then incubated for 6 h with different concentrations of various PKC-ζ pseudosubstrate lipopeptides. After 3 washes with PBS−BSA (1%), cells were fixed in PBS−paraformaldehyde (4%) for 1 h on ice. Apoptotic cells were detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), with the Apoptosis Detection System (Promega, Madison, WI). Briefly, cells were resuspended in 70% ice-cold ethanol solution and stored overnight at −20 °C. Samples were labeled with TdT buffer according to manufacturer's instructions. Reactions were terminated by adding EDTA. Cells were analyzed by flow cytometry (Elite Epics II, Coulter, Hialeah, FL) for the amount of fluorescein-12-dUTP. To identify both apoptotic and non-apoptotic events, cells were resuspended in propidium iodide solution (diluted to 5 μg/mL in PBS) and analyzed by flow cytometry.

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