Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 247–251

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

β-Homolysine Oligomers: A New Class of Trojan Carriers

C. García-Echeverría* and Stephan Ruetz

Oncology Research, Novartis Pharma Inc., CH-4002 Basel, Switzerland

Received 13 June 2002; revised 20 September 2002; accepted 9 October 2002

Abstract—We describe the design, synthesis and cell-membrane translocation properties of a series of β-peptides with the general sequence fluorescein-Adoa-(β-homolysine)$_n$-NH_2, $n = 5$–8 and Adoa = 8-amino-3,6-dioxaoctanoic acid. These β-peptides are able to cross the cytoplasmic membrane and accumulate in the nucleus of mammalian cells.

Introduction

Peptides, homopolymers and peptoid analogues containing cationic motifs have been shown to translocate across cell membranes and deliver a covalently linked 'molecular cargo' to cytoplasmic and/or nuclear targets.[1] This non-invasive method can be a powerful approach to study intracellular events, validate drug discovery targets in cellular settings and enhance delivery of drugs across tissue barriers.[2] As part of our efforts in this area of research,[3] we have used β-amino acids to develop polymers that can translocate across cell membranes. Based on previous studies with synthetic peptides,[4] we have designed and synthesised β-homolysine oligomers with the general sequence fluorescein-Adoa-(β-homolysine)$_n$-NH_2, $n = 5$–8 and Adoa = 8-amino-3,6-dioxaoctanoic acid. The ability of these oligomers to enter cells was assayed by laser scanning cytometry. Our data suggest the possible existence of an optimal number of β-homolysine residues for the efficient translocation of β-oligopeptides. These results support and expand recent contributions by different groups.[5]

Design, Synthesis and Biological Assays

β-Homolysine oligomer design and synthesis

Rothbard et al.[4] used short synthetic peptides containing cationic amino acids to determine the ability of these homopolymers to translocate across cell membranes. Based on this approach, we decided to synthesise oligomers containing β-homolysine residues to assess the cellular uptake of this new class of polymers. The minimal length required for an efficient translocation was explored by synthesising a set of polymers of five to eight residues. 8-Amino-3,6-dioxaoctanoic acid (Adoa) was selected to act as a flexible spacer between the cell-membrane translocating sequence and the detection probe in order to minimise undesired interactions between them. The β-homolysine oligomers were fluorescein-labeled on the N-terminal residue to examine their cell translocation properties by laser scanning cytometry.

The β-peptides **1**–**4** (Table 1) were prepared on solid-phase following protocols previously reported from our laboratory.[3] The general route for the synthesis of these oligomers is illustrated for compound **3**. β-Peptide **3** was synthesised manually on a 4-(2′,4′-dimethoxyl-phenyl-aminomethyl)-phenoxy resin ($f = 0.53$ mmol/g),[6] employing the Fmoc strategy.[7] The required N^{β}-Fmoc-N^{ω}-Boc-L-β-homolysine [2 equiv; (*S*)-7-(Boc-amino)-3-(Fmoc-amino)heptanoic acid, Fluka] was coupled with *O*-(1,2-dihydro-2-oxo-1-pyridyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (2.0 equiv)[8] in the presence of diisopropylethylamine (2.2 equiv). Coupling was achieved by first dissolving the Fmoc-β-homolysine derivative, the base, and the coupling agent in *N*-methyl-2-pyrrolidone, then waiting 3 min for pre-activation, adding the mixture to the resin, and finally shaking at room temperature for at least 90 min. The incorporation of the building block was monitored using the ninhydrin test and, in the event of being positive, a second coupling was performed using *N*-[(dimethyl-amino)1H-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]

*Corresponding author. Tel.: +41-61-696-1094; fax: +41-61-696-6246; e-mail: carlos.garcia-echeverria@pharma.novartis.com

0960-894X/03/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0960-894X(02)00885-5

Table 1. Sequence and analytical characterization of the β-homolysine oligomers, fluorescein-Adoa-(β-homolysine)$_n$-NH_2

Entry	Sequence	MALDI-TOF (amu)	t_R (min)
1	FITC-Adoa-(β-homolysine)$_5$-NH_2	1261.7 (calcd 1261.6)	4.96
2	FITC-Adoa-(β-homolysine)$_6$-NH_2	1403.9 (calcd 1403.8)	5.16
3	FITC-Adoa-(β-homolysine)$_7$-NH_2	1546.9 (calcd 1546.0)	5.13
4	FITC-Adoa-(β-homolysine)$_8$-NH_2	1687.3 (calcd 1688.2)	5.13

N-methylmethanaminium hexafluorophosphate *N*-oxide (2.0 equiv)[9] or tetramethylfluoroformamidinium hexafluorophosphate (2.0 equiv)[10] as coupling agent in the presence of diisopropylethylamine (6 equiv). Fmoc-8-amino-3,6-dioxaoctanoic acid (Neosystem) was coupled as described for the Fmoc-β-homolysine derivative. Fluoresceinisothiocyanat isomer I (3 equiv) was incorporated to the N-terminal amino group in the presence of diisopropylethylamine (6 equiv). Coupling was achieved by dissolving the building block and the base in *N*-methyl-2-pyrrolidone, adding the mixture to the resin, and shaking at room temperature for 21 h. The complete β-peptide resin was simultaneously deprotected and cleaved by treatment with trifluoroacetic acid/water (95:5, v/v) for 2 h at room temperature. The filtrate from the cleavage reaction was precipitated in diisopropyl ether–petroleum ether (1:1, v/v, 0 °C), and the precipitate was collected by filtration. The crude compound was purified by reversed-phase medium-pressure liquid chromatography using a C_{18}-column (Merck, LICHROPREP RP-18, 15–25 μm; 46×3.6 cm; flow rate ≈53 mL/min; detection at 215 nm) eluted with an acetonitrile-water gradient. The purity of the final compound was verified by reversed-phase analytical HPLC (see, Table 1): linear gradient over 7 min of MeCN/0.09% TFA and H_2O/0.1% TFA from 1:49 to 1:0 and 3 min at 1:0; flow rate 2.0 mL/min, detection at 215 nm; SMT analytical C_{18} column (250×4.6 mm; 5 μm, 100 Å). The identity of the purified compound was assessed by correct mass spectral (matrix-assisted laser desorption ionisation time-of-flight mass spectrometry, MALDI-TOF) analysis (see, Table 1).

Cell permeability assay

Exponentially growing DU145 (p5+6) and HCT15 (p12+5) cells were trypsinized and diluted to 1×10^6 cells/mL. Aliquots (corresponding to a total amount of 1×10^6 cells per plate) were seeded into 100 mm dishes and incubated for 24 h at 37 °C in a 5% CO_2 incubator. Medium was removed and cells were incubated for 10 min ($T=4$ °C and $T=37$ °C) or 18 h ($T=37$ °C) with the compounds **1**–**4** ($c=0.1$, 1 and 10 μM) prediluted individually in fresh medium (10 mL aliquots per plate). As controls, additional aliquots of cells were incubated with fluorescein ($c=0.1$, 1 and 10 μM; British Drug Houses Ltd.). After 10 min or 18 h treatment, the medium was removed and cells were washed twice with PBS/O (phosphate buffered saline without calcium), trypsinized and collected by centrifugation. Cell pellets were then resuspended in 9 mL PBS/O, followed by the addition of 1 mL of a 10% paraformaldehyde stock solution to obtain a final concentration of 1%. After incubating for 5 min, cells were collected, washed once with PBS/O, finally fixed for 30 min in PreserveCyt fixative solution and kept overnight at 4 °C. Microscope slides were prepared following the standard procedure. Prior to analysis, cells were treated for 1 h with 100 μg/mL RNAse at 37 °C for 1 h following which the slides were coated with a 50% glycerol/PBS solution containing 0.2 μg/mL propidium iodide (PI) and protected with a coverslip. The cellular uptake experiments were repeated at least two times.

Cellular uptake of β-peptide 3 after treatment with sodium azide

The assays were performed as previously described with the exception that HCT15 cells were preincubated for 30 min at 37 °C with 0.5% sodium azide and 0.5% albumin in RPMI medium before the addition of β-peptide **3** ($c=0.1$ μM). After 10 min at 37 °C, the medium was removed and the cells were washed with the azide buffer followed by a second washing step with PBS/O. The cellular uptake experiments were performed twice.

Fluorescene measurements and quantification

The fluorescence emission of the stained cells was measured using a Laser Scanning Cytometer (CompuCyte). The slides were scanned using a 20×objective and an argon-ion laser operating at 5 mW at the 488 nm line. A minimum of 5000 cells were examined. The contouring parameter was the long red fluorescent signal of PI and a minimum pixel area threshold of 100 was used. Red and green fluorescence were collected by separate photomultipliers. The data are expressed in a scattergram showing the bivariant distribution of DNA content versus relative green fluorescent signal. To estimate the relative intracellular accumulation of FITC-labeled compounds, a background gate was defined using the values of the green fluorescence intensity within the contouring area of control cells treated with fluorescein alone.

Results and Discussion

Peptides, homopolymers and peptoid analogues containing a high number of cationic residues have been shown to be able to translocate across cell membranes and deliver functionally active molecules to cytoplasmic and/or nuclear targets.[1] On the basis of this information, we decided to explore the possibility of developing oligomers containing cationic β-amino acids as cellular transporters. Due to the unique properties of β-peptides,[11] these new carriers would have an advantageous toxicity, immunogenenicity, solubility and clearance

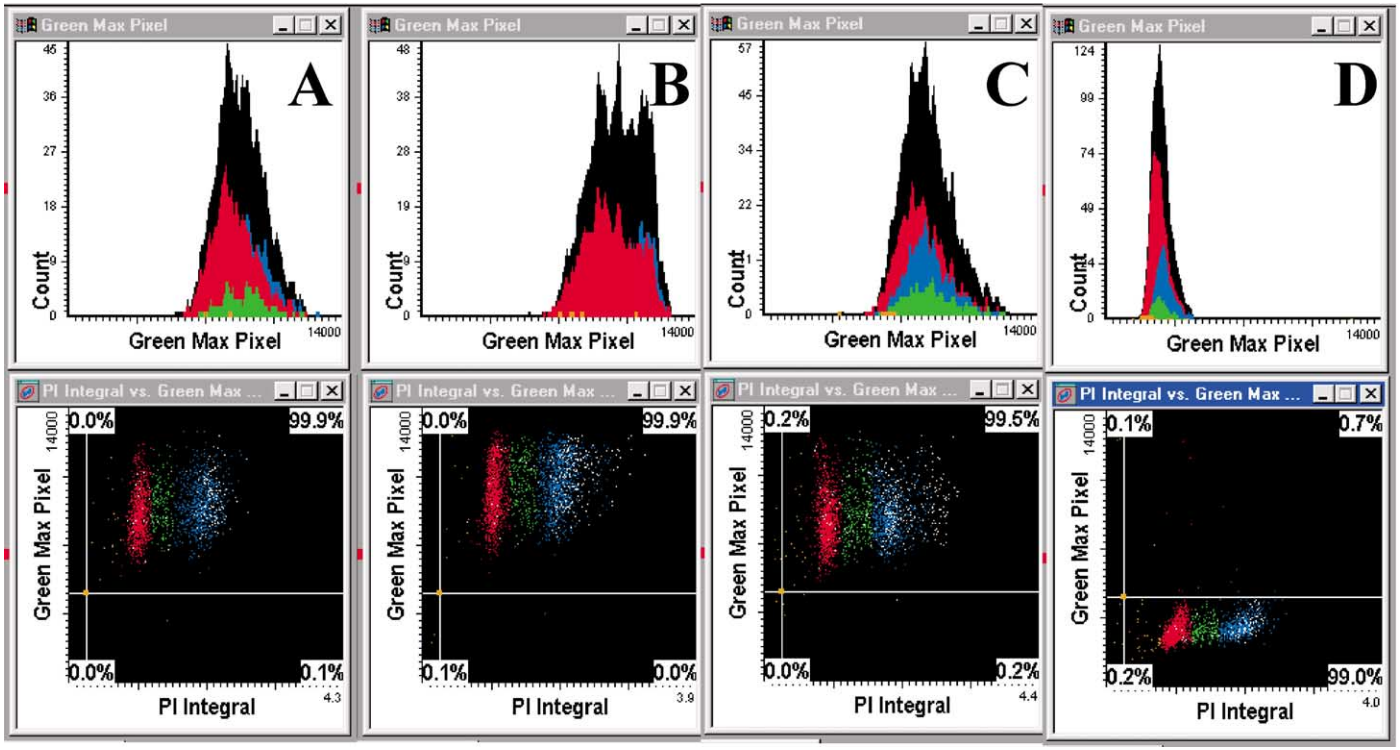

Figure 1. Cellular uptake of β-peptide **4** and fluorescein as determined by laser scanning cytometry (HCT15 tumour cells). Panel A, $c = 10$ μM (**4**); panel B, $c = 1$ μM (**4**); panel C, $c = 0.1$ μM (**4**); and panel D, $c = 10$ μM (fluorescein). Color codes: red, G1 cells; green, S cells; and blue, G2/M cells (DNA profile determined with propidium iodide).

Table 2. Uptake of fluorescein and β-peptides **1**–**4** into HCT15 and Du145 cells as determined by laser scanning cytometry

Compd	c (μM)	HCT15[a]	Du145[a]
Fluorescein	10	0.7	0.3
	1	1.1	0.8
	0.1	0.3	0.1
1	10	99.8	97.8
	1	99.6	76.5
	0.1	99.8	71.9
2	10	99.6	94.9
	1	99.7	69.5
	0.1	99.9	40.4
3	10	99.8	98.8
	1	99.8	85.8
	0.1	99.9	92.5
4	10	99.9	89.5
	1	99.9	98.9
	0.1	99.5	98.5

[a]% of compound uptaken at the indicated concentration and tumour cell line.

profile over some of the existing vectors. As a starting point in this work, we investigated the cellular uptake of β-homolysine oligomers using laser scanning cytometry. To determine whether there was an optimal length for cellular uptake, polymers of 5, 6, 7 and 8 β-homolysines were synthesised and labeled with fluorescein at the N-terminus using 8-amino-3,6-dioxaoctanoic acid as spacer (see, Table 1). The β-peptides **1**–**4** ($c = 10$, 1 and 0.1 μM) were incubated with the HCT15 colon tumour cells for 18 h at 37 °C and analyzed by laser scanning cytometry (for a representative example of the data, see Fig. 1A–C). The internalization of fluorescein was tested simultaneously under the same experimental conditions (Fig. 1D). As shown in Table 2, good cell penetration could be detected for the four oligomers at the three concentrations tested. The ability of β-peptides **1**–**4** to enter cells was further analysed using Du145, a prostate tumour cell line that, in our hands, has previously shown to be more resistant to the cellular uptake of exogenous biopolymers (e.g., oligonucleotides). An intermediate fluorescence intensity was observed in Du145 cells incubated with 0.1 or 1 μM of oligomers **1** and **2** (Table 2). However, incubation at a greater concentration ($c = 10$ μM) resulted in almost all cells incorporating these β-peptides. In contrast, oligomers **3** and **4** penetrated Du145 cells as efficiently as HCT15 cells (Table 2) suggesting that a minimal number of β-homolysine residues ($n = 7$) is critical for efficient translocation in different type of cells.

Additional studies were performed using β-peptides **2**–**4** to evaluate the effect of time, temperature and sodium azide on cellular translocation. Uptake of these oligomers seems to be a relatively fast process. Medium to high fluorescence intensity was observed when HCT15 cells were incubated with β-peptides **2**–**4** ($c = 0.1$ μM) for 10 min at 37 °C (Table 3). Oligomer **3** shows the highest fluorescence intensity indicating that this β-peptide probably internalizes at a higher rate than oligomers **2** and **4**. Incubation of cells with β-peptides **2**–**4** for 10 min at 4 °C exhibited fluorescence intensities similar to the ones obtained at 37 °C (Table 3). This finding is consistent with prior reports with regard to α- and β-peptides translocation at low temperatures.[4,5] Although this result has been considered as an indication that cell penetration does not depend on endocytosis, we cannot completely exclude it. Poly(L-lysine), which is a close analogue to our β-homolysine oligomers, has been shown to associate with the membrane and be internalized by a process termed non-specific adsorptive endocytosis.[12] Furthermore, low temperature can alter the structure and integrity of the cellular membrane and affect its permeability towards exogenous compounds. Sodium azide, which inhibits energy-dependent cellular uptake, did not affect substantially the translocation of β-peptide **3** ($c = 0.1$ μM; 81.0% vs 88.8%, + and − sodium azide, respectively).

Table 3. Uptake of fluorescein and β-peptides **2**–**4** ($c = 0.1$ μM) into HCT15 as determined by laser scanning cytometry

Compd	Fluorescein	**2**	**3**	**4**
$T = 37$ °C	5	75.5	88.8	68.1
$T = 4$ °C	2	70.6	85.4	60.3

In all experiments performed, no toxicity was observed and the β-homolysine oligomers tend to accumulate in the nucleus (data not shown). Nuclear localization is a common feature of previously described polycationic α- and β-peptide carriers.[4,5] Although this property has been ascribed to the potential recognition of the oligocationic motifs of these oligomers by the nuclear import machinery, it is also known that compounds of <60 kDa can passively diffuse through the nuclear membrane.[12e,13]

In summary, we have shown that short oligomers of β-homolysine are able to cross the cytoplasmic membrane and accumulate in the nucleus of mammalian cells.[14] Our findings are complementary to those of Gellman and Seebach[5] and point to the possibility of using β-amino acids in the design and development of intracellular delivery vectors. Additional work is required to elucidate the mechanism of transport of β-peptides and their assessment as carriers for intracellular delivery in vitro and in vivo.

Acknowledgements

We thank D. Arz, R. Wille and V. von Arx for technical assistance and Dr. M.A. Pearson for helpful suggestions and for kindly reviewing this letter.

References and Notes

1. (a) For representative examples, see: Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, *276*, 5836. (b) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinmann, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003. (c) Friedler, A.; Friedler, D.; Luedtke, N. W.; Tor, Y.; Loyter, A.; Gilon, Ch. *J. Biol. Chem.* **2000**, *275*, 23783. (d) Murphy, J. E.;

Uno, T.; Hamer, J. D.; Cohen, F. E.; Dwarki, V.; Zuckermann, R. N. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1517. (e) Derossi, D.; Chassaing, G.; Prochiantz, A. *Trends Cell Biol.* **1998**, *8*, 84. (f) Sheldon, K.; Liu, D.; Ferguson, J.; Gariépy, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, 2056.

2. (a) Dunican, D. J.; Doherty, P. *Biopolymers* **2001**, *60*, 45. (b) Schwarze, S. R.; Hruska, K. A.; Dowdy, S. F. *Trends Cell Biol.* **2000**, *10*, 290. (c) Rothbard, J.; Garlington, S.; Lin, Q.; Kirschberg, T.; Kreider, E.; McGrane, P. L.; Wender, P. A.; Khavari, P. A. *Nature Medicine* **2000**, *6*, 1253. (d) Hawiger, J. *Curr. Opin. Chem. Biol.* **1999**, *3*, 89. (e) Schwarze, S. R.; Ho, A.; Vocero-Akbani, A.; Dowdy, S. F. *Science* **1999**, *285*, 1569. (f) Huang, Ch.-Y; Uno, T.; Murphy, J. E.; Lee, S.; Hamer, J. D.; Escobedo, J. A.; Cohen, F. E.; Radhakrishnan, R.; Dwarki, V.; Zuckermann, R. N. *Chem. Biol.* **1998**, *5*, 345.

3. (a) García-Echeverría, C.; Furet, P.; Chène, P. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2161. (b) García-Echeverría, C.; Jiang, L.; Ramsey, T. M.; Sharma, S. K.; Chen, Y.-N. P. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1363.

4. Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. *B. J. Peptide Res.* **2000**, *56*, 318.

5. During the preparation of this letter, we learned about the contributions by Gellman et al. (Umezawa, N.; Gelman, M. A.; Haigis, M. C.; Raines, R. T.; Gellman, S. H. *J. Am. Chem. Soc.* **2002**, 124, 368) and Seebach et al. (Rueping, M.; Mahajan, Y.; Sauer, M.; Seebach D. *ChemBioChem* **2002**, *2–3*, 257) on the cellular uptake of β-peptides. The cellular translocation of β-peptides was also reported by Prof. D. Seebach in a lecture at the 17th American Peptide Symposium (San Diego, 9–14 June 2001) and at the Department Forschung Kantonsspital Basel and Zentrum für Pharmazeutische Wissenschaften (Basel, 29 November 2001).

6. Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787.

7. For information on Fmoc peptide chemistry, see: (a) Atherton, E., Sheppard, R. C. In *Solid-Phase Peptide Synthesis — A Practical Approach,* Rickwood, D.; Hames, B. D., Eds.; IRL Press at Oxford University Press: Oxford, 1989. (b) Atherton, E.; Sheppard, R.C. In *The Peptides*; Gross, E.; Meinhofer, J., Eds.; Academic Press: New York, 1987; Vol. 9, pp 1–38.

8. Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*, 1927.

9. (a) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397. (b) Carpino, L. A.; El-Faham, A.; Albericio, F. *Tetrahedron Lett.* **1994**, *35*, 2279.

10. Carpino, L. A.; Triolo, S. A.; Griffin, G. W.; Herman, L. W.; Tarr, G.; Sole, N. A.; Diekmann, E.; El-Faham, A.; Ionescu, D.; Albericio, F. *Innovation Perspect. Solid Phase Synth. Comb. Libr., Collect. Pap., Int. Symp., 4th* 1996; pp 41–50.

11. Frackenpohl, J.; Arvidsson, P. I.; Schreiber, J. V.; Seebach, D. *ChemBioChem.* **2001**, *2*, 445.

12. (a) Leonetti, J. P.; Degols, G.; Lebleu, B. *Bioconj. Chem.* **1990**, *1*, 149. (b) Shen, W. C.; Ryser, H. J.-P. *Mol. Phrmacol* **1979**, *16*, 614. (c) Ryser, H. J.-P.; Shen, W. C. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3867. (d) For additional studies on branched peptides containing lysine residues, see: Singh, D.; Kiarash, R.; Kawamura, K.; LaCasse, E. C.; Gariépy, J. *Biochemistry* **1998**, *37*, 5798. (e) Sheldon, K.; Liu, D.; Ferguson, J.; Gariépy, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2056.

13. Breewer, M.; Goldfarb, D. S. *Cell* **1990**, *60*, 999.

14. Interestingly, these features were not observed previously for an heptamer of L-lysine, see ref 4. The change in the backbone seems to have in this case a positive effect on the translocation activity of the oligomer.