Bioorganic & Medicinal Chemistry Letters 20 (2010) 2238–2241

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

MR contrast agent composed of cholesterol and peptide nucleic acids: Design, synthesis and cellular uptake

Rajendra Joshi, Ritu Mishra †, Rolf Pohmann, Jörn Engelmann *

High-Field Magnetic Resonance Center, Max Planck Institute for Biological Cybernetics, Tübingen, Germany

ARTICLE INFO

Article history:
Received 8 December 2009
Revised 2 February 2010
Accepted 3 February 2010
Available online 8 February 2010

Keywords:
Peptide nucleic acid
Magnetic resonance imaging
Targeted contrast agent
Antisense targeting

ABSTRACT

A new mRNA targeting contrast agent consisting of three main functional domains, (i) gadolinium based magnetic resonance reporter part, (ii) antisense peptide nucleic acids targeted to mRNA, and (iii) cholesterol as the delivery vector, was developed and synthesized. The new contrast agent showed efficient cellular uptake and significant contrast enhancement at very low labeling concentrations (0.5 μM). However, after uptake into cells the agent was located predominantly in endosomes like a similar cell penetrating peptide conjugated probe. Our results indicate that this newly developed contrast agent could be used for the labeling of cells for optical as well as magnetic resonance imaging.

Magnetic resonance imaging (MRI) is currently one of the most important diagnostic tools available in medicine.[1] The specificity and sensitivity of MRI can be further enhanced by the application of contrast agents (CAs).[2] Since many clinically important targets reside inside the cell, the development of efficient intracellular targeted MR contrast agents might enlarge the applicability of MRI. Potential intracellular targets are, for example, DNA, mRNA or proteins/enzymes. We have chosen the mRNA of a red fluorescent protein (DsRed)[3] as model target and a 12mer antisense peptide nucleic acid (PNA) (agcgcctgtacc), specifically targeted to a complementary region of the DsRed mRNA, was used as the targeting sensor. PNAs are peptidase and nuclease resistant DNA mimics. Using PNAs allows versatile, stable and highly specific targeting.[4] In addition, the number of copies of mRNA per cell is higher than for DNA which makes mRNA targeting easier than DNA targeting. Antisense PNAs can hybridize uniquely to the complementary mRNA in the cytosol and by this could provide cell specific targeting for cells expressing the respective mRNA. Thus, the expression of the corresponding gene might be visualized non-invasively by MR imaging.[5] Unfortunately, the main obstacle to the effective use of PNAs is their relatively poor uptake by cells.[6] Therefore, enhancement of cellular uptake of PNAs was our main goal in this work.

In 2003, Heckl et al.[7] reported for the first time the potential of an intracellular CA which was composed of a gadolinium (Gd) complex (as reporter), a PNA sequence (as sensor) and a carrier peptide (as vector). A similar approach with PNA, Gd-DOTA and a cell penetrating peptide was reported by our group in 2007.[8] This CA suffered limitation in regards to endosomal entrapment and a complex synthetic scheme. The prerequisites for intracellular targeting are the efficient intracellular delivery of the CA as well as colocalization with the target. Thus, the design and synthesis of novel targeted intracellular CA remain a significant challenge. In order to circumvent the drawbacks of previous contrast agents, we herein report the synthesis and evaluation of a new efficient intracellular MR contrast agent which is based on a non-peptidic, lipid mediated delivery system. This CA includes three functional domains: (i) Gd based MR reporter part, (ii) antisense PNA targeted to mRNA, and (iii) cholesterol as the delivery vector. We have evaluated uptake efficiency and contrast enhancement ability for MRI and compared these to the CA previously reported by our group.

The new CA was synthesized in analogy to our previous publication[8] by Fmoc chemistry in solid phase peptide synthesis (Fig. 1, for a detailed description refer to the electronic Supplementary data). In brief, resin was downloaded (0.10–0.20 mmol/g) to avoid aggregation. D-Amino acids, aminoethoxyethoxyacetic acid (AEEA) and then PNA building blocks were added. The main purpose of adding basic amino acids like Arg and Lys was to enhance aqueous solubility of the construct and to initiate cell penetration by electrostatic interaction between basic residues and negatively charged phospholipids in the plasma membrane.[9,10] The use of D-isomer increases the stability against proteases while AEEA helps to increase aqueous solubility. Fmoc-Lys(Dde)-OH residue was incorporated as a spacer at the N-terminus of the PNA sequence. Afterwards the Fmoc group was deprotected selectively and cholesterol was coupled. The coupling reaction of cholesterol chloro-

* Corresponding author. Tel.: +49 7071601704; fax: +49 7071601702.
E-mail address: joern.engelmann@tuebingen.mpg.de (J. Engelmann).
† Present address: Department of Biological and Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, England.

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.02.019

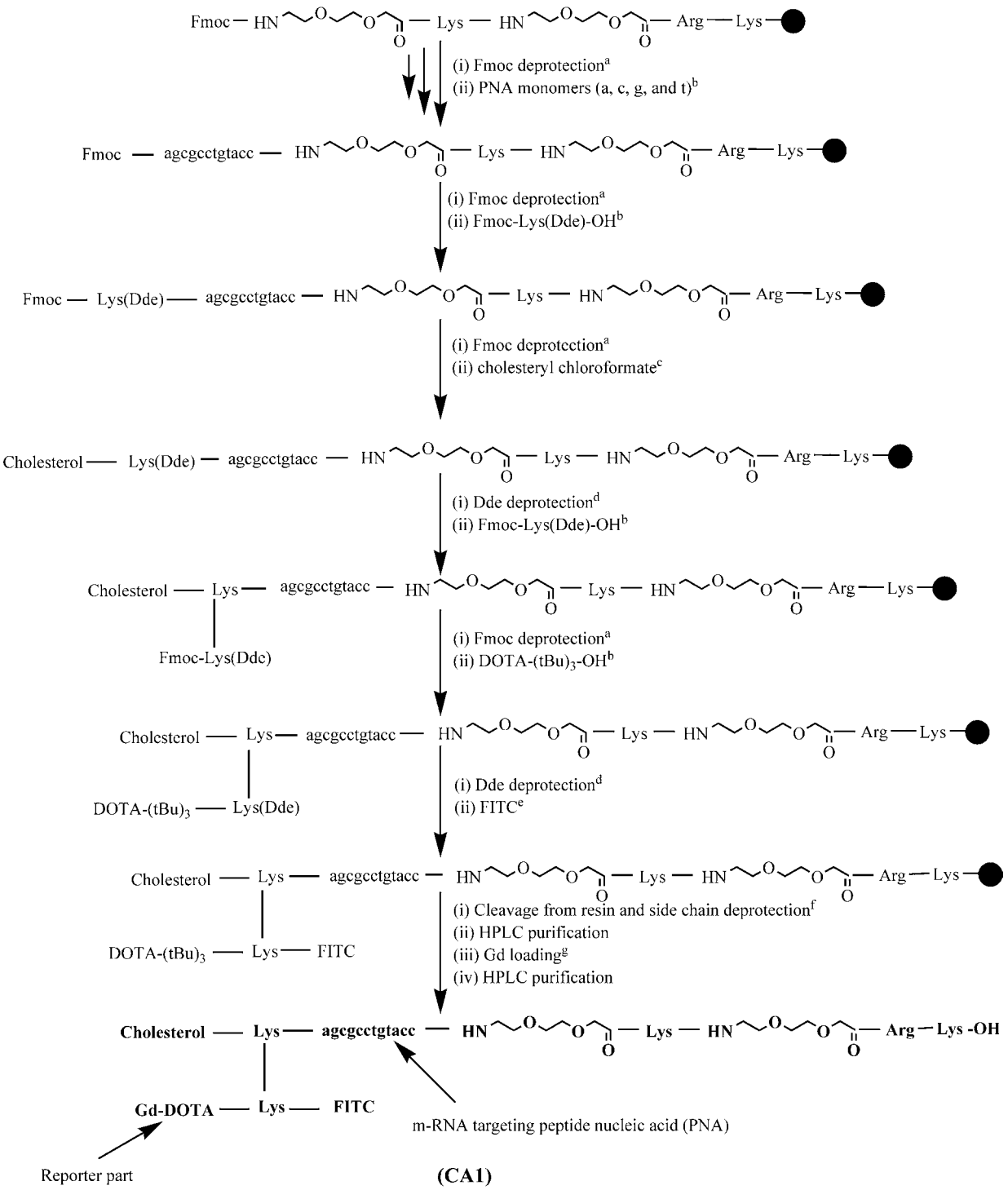

Figure 1. Synthetic scheme and chemical structure of **CA1**. Lys, Arg, AEEA, Lys, AEEA were appended by Fmoc chemistry in continuous scheme on Wang resin. (a) 20% piperidine in DMF, 2×, 5 min each; (b) HATU, DIPEA, DMF, 1 h; (c) DIPEA, DCM/DMF, 4 h; (d) 2% hydrazine in DMF, 2×, 2.5 min; (e) DIPEA, DMF, 12 h; (f) TFA, *m*-cresol, TIPS, H_2O (90:5:2.5:2.5); (g) $GdCl_3 \times 6H_2O$, MeOH, H_2O, 40 °C, 55 h.

formate was carried out in presence of DIPEA in DCM/DMF solution. One more spacer (Fmoc-Lys(Dde)-OH) was added to the ε-amino group of Lys and then tris (*tert*-butyl) DOTA and fluorescein isothiocyanate (FITC) were coupled to the α- and ε-position of Lys, respectively. After chelation of Gd, the final product (**CA1**) was purified by HPLC, lyophilized and finally characterized by ESI-MS. Labeling with FITC allowed to verify cellular uptake, which was estimated by fluorescence microscopy and spectroscopy in a mouse fibrosarcoma cell line expressing DsRed protein (DsRed).[11] The parent cell line (CCL-11), deficient of the target sequence was used as negative control.

Previously, our group demonstrated that a CA based on cell penetrating peptides (CPP) was efficiently taken up by cells.[8] The main drawback was an endosomal entrapment of the compound. The specific interaction between the sensor (antisense PNA) and the targeted mRNA (localized in the cytosol) as well as an effective

efflux from non-targeted cells were hindered although a specific binding of the CA to a synthetic target oligonucleotide sequence could be observed in a cell free in vitro assay.[12] Thus, modification of the CA was required to achieve the release from endosomes or a direct uptake into the cytosol of the cell. We tried to circumvent endosomal entrapment by the conjugation of cholesterol to the building blocks of the CA as the delivery vector. There are many reports about cholesterol in gene transfection and delivery[13,14] and a recently published report of Wolfrum et al. also highlights the use of cholesterol to facilitate cellular import of siRNAs for effective silencing of protein expression.[15] Furthermore, it has been shown that a combined treatment of cholesterol conjugates with streptolysin-O (SL-O)[16] facilitated cellular uptake of these conjugates. SL-O is a bacterial protein which can reversibly permeabilize cell membranes and in turn enhance direct cytosolic uptake into these cells.[17] Therefore, keeping the role of cholesterol in mind, we first studied simple cholesterol constructs[18] and finally synthesized the complete cholesterol-based **CA1**.

All reactions described above, except Gd loading, could be successfully carried out on resin in an automated peptide synthesizer. The synthesis of the PNA sequence, often laborious and lengthy, was facilitated by developing and optimizing the scheme for an automated peptide synthesizer (see Supplementary data). All reactions were performed under mild conditions controlling the formation of side products. During the cleavage of the product from the resin, cholesterol was also partially cleaved off. This side reaction was dependent upon the incubation time of the resin in the cleavage cocktail and could be almost completely suppressed by choosing the proper reaction time. Gd loading after cleavage was achieved in the presence of water and methanol (1:1) at 40 °C for 55 h at pH 6.5. Methanol was used to increase the solubility. Excess Gd was removed using HPLC.

Results of fluorescence spectroscopy revealed the highly efficient intracellular uptake of **CA1** at very low labeling concentrations (Fig. 2). The internalization was concentration dependent and linearly increased up to 3 μM. However, at concentrations $\geqslant$3.0 μM precipitation was observed indicating a reduced solubility of these conjugates under physiological conditions.

Fluorescence microscopy (Fig. 3) demonstrated that **CA1** entered DsRed-expressing cells and was mainly located in vesicles (green) around the nucleus (blue). A sufficient cytosolic distribution could not be observed. The localization in vesicles points toward a predominantly endosomal uptake mechanism for **CA1**. Because of vesicular entrapment, it can be expected that there would be still a lack of interaction between contrast agent and

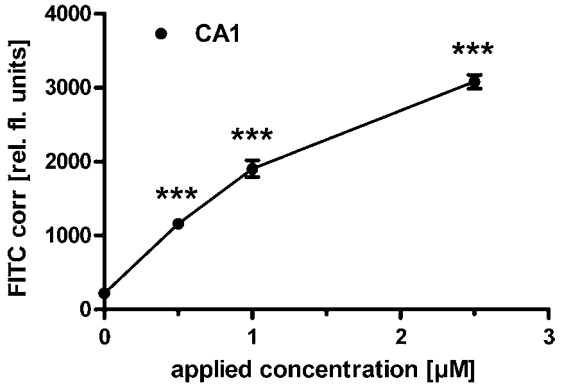

Figure 2. Cell internalization of **CA1** into DsRed cells measured by fluorescence spectroscopy. Cells were incubated with contrast agent at various concentrations in complete medium for 18 h. Values are presented as mean ± SEM (n = 11–12); ***p <0.001 significant difference to control.

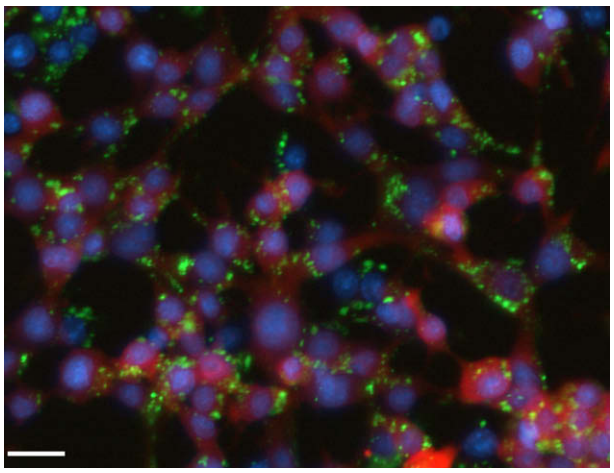

Figure 3. Cellular localization of **CA1** into DsRed cells. Cells were incubated with 0.5 μM concentration in complete medium for 18 h. Green: FITC fluorescence of **CA1**; blue: cell nuclei (Bisbenzimid H 33342); red: fluorescent protein DsRed; bar represents 20 μm.

mRNA in the cytosol. Although **CA1** was also able to bind specifically to a synthetic 63mer target oligonucleotide in cell free gel shift assay (data not shown), no significant inhibition of the DsRed gene expression could be detected. Therefore, SL-O was tested for its ability to improve the cytosolic uptake of **CA1**. In contrast to already published reports,[16,19] in our study SL-O did not increase the cytosolic distribution compared to a buffer control. The lack of inhibition of the DsRed expression might not only be due to a low number of molecules released to the cytosol but also to a non-optimized antisense sequence for blocking gene expression. Oligonucleotides (or PNAs) which are able to silence the DsRed gene have at least the double length or are even longer hairpin-type siRNAs compared to the 12mer sequence used in this study.[20] This short sequence was selected to fulfill the minimal requirements for a specific binding to the mRNA of DsRed, not for the efficient blocking of the synthesis of the fluorescent protein.

Our new cholesterol-based CA was able to enhance contrast in T_1-weighted MR images of labeled DsRed-expressing cells as well as in the non-target containing parent cells. An increase of the apparent cellular relaxation rate $R_{1,cell}$ of CA-loaded cells was al-

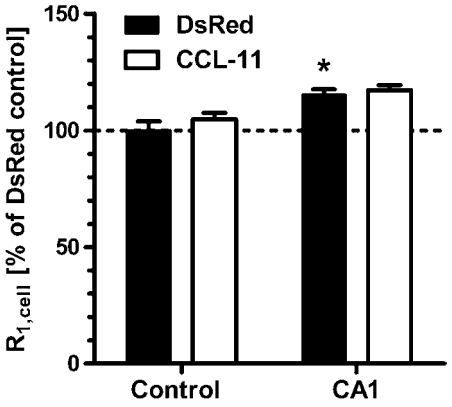

Figure 4. Cellular relaxation rate in DsRed and CCL-11 cells after labeling with **CA1**. Cells were incubated with **CA1** for 18 h, washed repeatedly and trypsinized. Cells were resuspended in culture medium without **CA1** at a cell density of 1.5×10^7 cells/500 μl and transferred into 0.65 ml tubes. The apparent cellular relaxation rate $R_{1,cell}$ ($1/T_{1,cell}$) was measured at 3T in an axial slice through the cell pellet and is plotted on the starting labeling concentration of **CA1**. Values are presented as mean ± SEM (n = 2); *p <0.05 significant difference to the corresponding control.

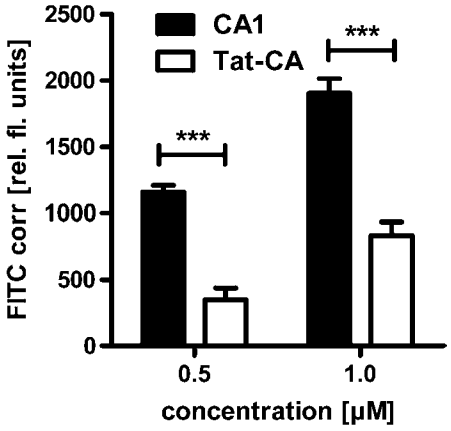

Figure 5. Comparison of cell internalization of cholesterol appended CA (**CA1**) and D-Tat$_{49-57}$ appended CA (Tat-CA).[8] Cells were incubated with both types of contrast agents at 0.5 and 1 μM concentrations in complete medium for 18 h. Values are presented as mean ± SEM (n = 3–12); ***p <0.001 significant difference between CAs.

ready observed at a labeling concentration of 0.5 μM (Fig. 4). The observed contrast MR enhancement and cellular internalization at low concentrations makes such conjugates useful for biomedical applications.

Various imaging probes have been developed so far[8,21,22] but the application of such probes in MR imaging is limited most likely due to their endosomal entrapment, less specific nature or insufficient accumulation at the target site. Heckl et al.[7] reported a CA that showed increase in MR signal and prolonged retention in tumor cells, but the applied concentration (0.5 mM) was 1000 times higher than the concentration that we used with our compound. Our new CA showed significant increases in the relaxation rate $R_{1,cell}$ after loading within cells. Cholesterol conjugation in the present study offered several advantages like (i) an efficient cellular delivery, (ii) contrast enhancement in low concentration, (iii) easy conjugation, and (iv) chemical stability. In addition, it was reported that cationic lipids containing cholesterol were biodegradable and showed low toxicity.[14] By coupling cholesterol in the present construct, less chemical steps and better cellular internalization was achieved as compared to previously reported Tat-CA based on a peptidic vector (Fig. 5). However, poor aqueous solubility and endosomal entrapment are disadvantages of using cholesterol.

In conclusion, our newly designed and synthesized targeted cholesterol-conjugated contrast agent was internalized efficiently into cells. A labeling concentration of 0.5 μM was sufficient to significantly enhance MR image contrast. However, modifications are still required to circumvent endosomal entrapment and to achieve a better cytosolic distribution of the CA and a specific accumulation in the target cells.

Acknowledgements

The authors thank Hildegard Schulz for technical assistance, Wu Su for identifying antisense PNA sequence. This work was supported by the German Ministry for Education and Research (BMBF), FKZ 01EZ0813, and the Max Planck Society and was performed in the frame of COST D38 action.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.02.019.

References and notes

1. (a) Weissleder, R.; Mahmood, U. *Radiology* **2001**, *219*, 316; (b) Jin, A. Y.; Tuor, U. I.; Rushforth, D.; Filfil, R.; Kaur, J.; Ni, F.; Tomanek, B.; Barber, P. A. *Contrast Media Mol. Imaging* **2009**, *4*, 305.
2. (a) Jacques, V.; Desreux, J. F. *Top. Curr. Chem.* **2002**, *221*, 123; (b) Ghaghada, K. B.; Ravoori, M.; Sabapathy, D.; Bankson, J.; Kundra, V.; Annapragada, A. *PLoS One* **2009**, *10*, e7628.
3. Bevis, B. J.; Glick, B. S. *Nat. Biotechnol.* **2002**, *20*, 83.
4. Hyrup, B.; Nielson, P. E. *Bioorg. Med. Chem.* **1996**, *4*, 5.
5. Liu, C. H.; Kim, Y. R.; Ren, Q. J.; Eichler, F.; Rosen, B. R.; Liu, P. K. *J. Neurosci.* **2007**, *27*, 713.
6. (a) Gait, M. J. *Cell. Mol. Life Sci.* **2003**, *60*, 844; (b) Kilk, K.; Langel, U. *Methods Mol. Biol.* **2005**, *298*, 131.
7. Heckl, S.; Pipkorn, R.; Waldeck, W.; Spring, H.; Jenne, J.; von der Lieth, C. W.; Corban-Wilhelm, H.; Debus, J.; Braun, K. *Cancer Res.* **2003**, *63*, 4766.
8. Su, W.; Mishra, R.; Pfeuffer, J.; Wiesmueller, K.-H.; Ugurbil, K.; Engelmann, J. *Contrast Media Mol. Imaging* **2007**, *2*, 42.
9. Fischer, R.; Fotin-Mleczek, M.; Hufnagel, H.; Brock, R. *ChemBioChem* **2005**, *6*, 2126.
10. Stewart, K. M.; Horton, K. L.; Kelley, S. O. *Org. Biomol. Chem.* **2008**, *6*, 2242.
11. Cellular uptake study was estimated by fluorescence microscopy and spectroscopy in DsRed as well as CCL-11 mouse fibrosarcoma cells plated in 96-well plates. Cells were treated with conjugates in complete medium for 18 h at 37 °C. After incubation, cell nuclei were counterstained with Bisbenzimid H 33342 and external fluorescence was quenched with trypan blue followed by repeated washes. Internalized fluorescence was measured in a multiplate reader (BMG, Labtech Germany) and microscopic images were made at an Axiovert 200M (Zeiss, Germany).
12. Mishra, R.; Su, W.; Pohmann, R.; Sauer, M. G.; Pfeuffer, J.; Ugurbil, K.; Engelmann, J. *Bioconjugate Chem.* **2009**, *20*, 1860.
13. Futaki, S.; Ohashi, W.; Suzuki, T.; Niwa, M.; Tanaka, S.; Ueda, K.; Harashima, H.; Sugiura, Y. *Bioconjugate Chem.* **2001**, *12*, 1005.
14. Aissaoui, A.; Oudrhiri, N.; Petit, L.; Hauchecorne, M.; Kan, E.; Sainlos, M.; Julia, S.; Navarro, J.; Vigneron, J.-P.; Lehn, J.-M.; Lehn, P. *Curr. Drug Targets* **2002**, *3*, 1.
15. Wolfrum, C.; Shi, S.; Jayaprakash, K. N.; Jayaraman, M.; Wang, G.; Pandey, R. K.; Rajeev, K. G.; Nakayama, T.; Charrise, K.; Ndungo, E. M.; Zimmermann, T.; Koteliansky, V.; Manohoran, M.; Stoffel, M. *Nat. Biotechnol.* **2007**, *25*, 1149.
16. Holasova, S.; Mojzisek, M.; Buncek, M.; Vokurkova, D.; Radilova, H.; Safarova, M.; Cervinka, M.; Haluza, R. *Mol. Cell. Biochem.* **2005**, *276*, 61.
17. Barry, E. L. R.; Gesek, F. A.; Friedman, P. A. *Biotechniques* **1993**, *15*, 1018.
18. Joshi, R.; Mishra, R.; Su, W.; Engelmann, J. Peptides 2008, Proceedings of the 30th European Peptide Symposium; Helsinki, H. Lankinen, Ed., Chemistry of Peptides in Life Science, Technology and Medicine, 2009.
19. Spiller, D. G.; Giles, R. V.; Grzybowski, J.; Tidd, D. M.; Clark, R. E. *Blood* **1998**, *91*, 4738.
20. Kasim, V.; Taira, K.; Miyagishi, M. *J. Gene Med.* **2006**, *8*, 782.
21. Tian, X.; Aruva, M. R.; Qin, W.; Zhu, W.; Sauter, E. R.; Thakur, M.; Wickstrom, E. L. *Bioconjugate Chem.* **2005**, *16*, 70.
22. Sun, X.; Fang, H.; Li, X.; Rossin, R.; Welch, M. J.; Taylor, J. S. *Bioconjugate Chem.* **2005**, *16*, 294.