siRNA Delivery

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Cell-Penetrating and Neurotargeting Dendritic siRNA Nanostructures**

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Abstract: *We report the development of dendritic siRNA nanostructures that are able to penetrate even difficult to transfect cells such as neurons with the help of a special receptor ligand. The nanoparticles elicit strong siRNA responses, despite the dendritic structure. An siRNA dendrimer directed against the crucial rabies virus (RABV) nucleoprotein (N protein) and phosphoprotein (P protein) allowed the suppression of the virus titer in neurons below the detection limit. The cell-penetrating siRNA dendrimers, which were assembled using click chemistry, open up new avenues toward finding novel molecules able to cure this deadly disease.*

RNA interference is a powerful tool that allows the sequence-specific suppression of gene expression by small interfering RNAs (siRNAs).[1] The major obstacle preventing the widespread use of siRNA-based therapeutics is the lack of efficient, specific, and nontoxic delivery systems to transport siRNA duplexes into cells and tissue.[2] Commonly used siRNA-delivery vehicles, such as liposomes or cationic polymeric systems, are heterogeneous in size and composition, and severe cytotoxic effects are observed in sensitive cell types such as neurons.[3] Recently we reported the ability of all-*cis*-configured anandamide (arachidonoylethanolamin, AEA) to target siRNA to cannabinoid receptors, thus leading to the efficient receptor-mediated internalization of the siRNA–AEA adducts.[4] As cannabinoid receptors are expressed on immune and neural cells, this approach enables the efficient delivery of siRNAs into these sensitive cell types.[5]

One important problem associated with receptor-mediated siRNA uptake is the limited expression of some receptors on cell surfaces. This leads to the rapid saturation of receptors, which limits the amount of siRNA that can be delivered. Here, we show that one AEA ligand is able to induce the receptor-mediated uptake of dendritic siRNA nanostructures with up to nine siRNA duplexes per ligand. Efficient RNA silencing is thus observed despite the complex structure (Figure 1). In principle this allows us to circumvent the saturation effect and to extend the uptake of siRNA.

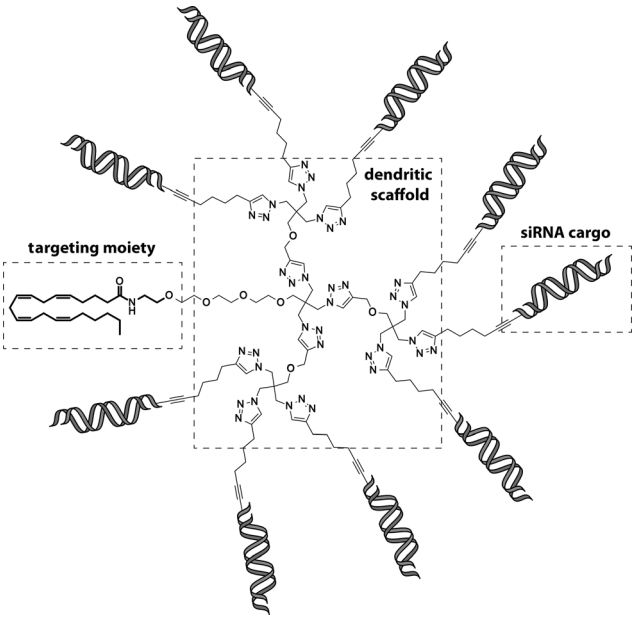

Figure 1. A dendritic siRNA nanostructure with an anandamide targeting unit. The siRNA passenger strands (red) are covalently connected to a dendritic framework, while the siRNA guide strands (blue) are hybridized to the nanostructure.

A click-chemistry-based approach enabled the efficient synthesis of the required monodisperse siRNA nanostructures (see Figure 1).[6] The synthetic strategy that enabled the assembly of the siRNA structures is depicted in Scheme 1. The starting material pentaerythritole was converted to the

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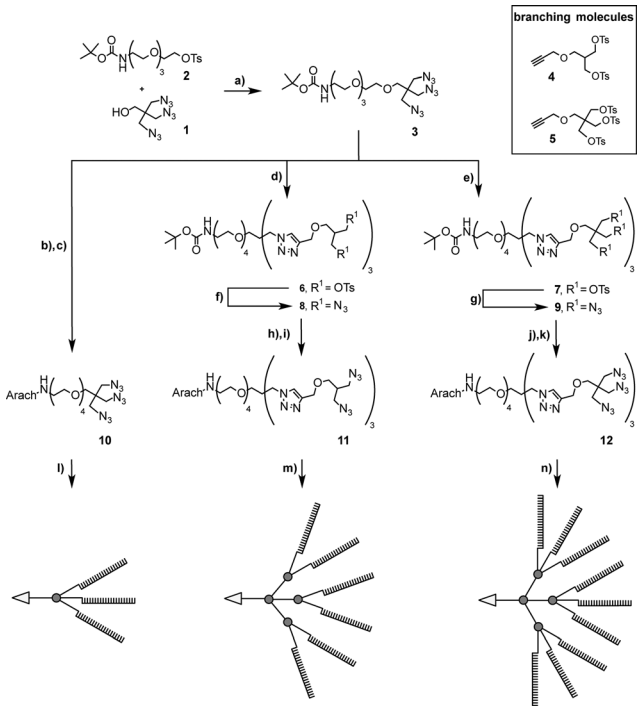

Scheme 1. Synthesis of the siRNA dendrimers AEA-[3ORN], AEA-[6ORN], and AEA-[9ORN]. a) NaH, 15-crown-5, DMF, RT, 46%; b) TFA, $CHCl_3$, RT, 94%; c) arachidonic acid, TBTU, DIPEA, DMF, RT, 95%; d) **4**, $CuSO_4$, sodium ascorbate, H_2O/THF (1:1), RT, 80%; e) **5**, $CuSO_4$, sodium ascorbate, H_2O/THF (1:1), RT, 65%; f) NaN_3, DMF, 110°C, 85%; g) NaN_3, DMF, 110°C, 97%; h) TFA, $CHCl_3$, RT, 71%; i) arachidonic acid, HATU, DIPEA, DMF, RT, 81; j) TFA, $CHCl_3$, RT, 76%; k) TBTU, DIPEA, DMF, 40°C, 70%; l) alkyne-modified oligonucleotide (ORN), CuBr, TBTA, DMSO/H_2O, 89%; m) alkyne-modified oligonucleotide (ORN), CuBr, TBTA, DMSO/H_2O, 78%; n) alkyne-modified oligonucleotide (ORN), CuBr, TBTA, DMSO/H_2O, 65%.

corresponding triazide **1** in two steps. Compound **1** was next reacted with mono-Boc-mono-Ts-functionalized tetraethyleneglycole **2**, which provided the key branching molecule **3**. Using Cu^I-catalyzed click reactions, compound **3** was extended with the additional alkyne-containing branching molecules **4** and **5**, which were both obtained in two steps (Scheme S1). The tosylates **6** and **7** were next converted into the corresponding azides **8** and **9**. Subsequently, we cleaved the Boc groups in **3**, **8**, and **9** and reacted the amines with arachidonic acid. These reactions provided the dendritic anandamide azides **10**, **11**, and **12**. The products of this reaction were next connected to the alkine-modified siRNA passenger strands using a Cu^I-catalyzed click reaction to give the dendritic nucleic acid nanostructures.[6,7]

In all three cases we obtained one main product after the click reaction. These compounds were isolated using reverse-phase semipreparative HPLC. The correct structure and monodispersity of the dendritic siRNA assemblies was next confirmed by analytical HPLC and MALDI-TOF-analysis (for two examples, see Figure 2). In all cases, we obtained the dendritic structures in excellent purity. The correct molecular weights were obtained within the experimental limits of the MALDI-TOF measurements, proving the expected monodispersity of the nanoparticles. The final siRNA dendrimers

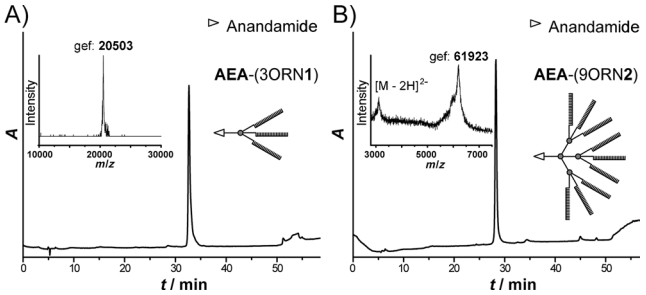

Figure 2. HPL chromatograms of the purified dendrimers obtained with **10** and **12**. The inserts show the MALDI-TOF spectra. MALDI-TOF data: A) calc. (AEA-[3ORN1]): *m/z* 20506, found: 20503. B) calc. (AEA-[9ORN2]): *m/z* 62124, found 61923 (broad signal).

ready for transfection were obtained by hybridizing the corresponding numbers of guide strands to the dendritic structures.

To investigate the biological activity of the siRNA dendrimers, we utilized a reporter assay in RBL-2H3 cells.[4,8] As depicted in Figure 3, all prepared siRNA dendrimers showed strong silencing effects. For the experiments, we kept the amount of siRNA duplexes constant by reducing the concentration of the dendritic structure to the reciprocal of the number of siRNA duplexes per nanoparticle. The dendrimer AEA-[3siRNA] with three siRNA strands attached showed by far the strongest silencing effect, even though the concentration of the dendrimer was reduced to one third compared to the anadamide-functionalized siRNA duplex. Surprisingly, even the dendrimer AEA–[9siRNA] showed a strong efficacy compared to the monomeric siRNA, despite its large size and the small total concentration (Figure 3).

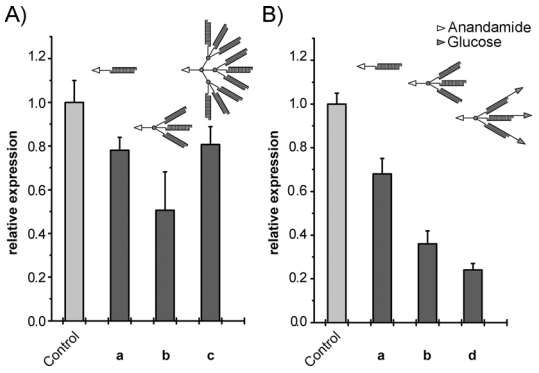

Figure 3. Relative silencing of the Renilla luciferase compared to the silencing of Firefly luciferase mediated by dendritic anandamide-siRNAs in RBL-2H3 cells. Investigated was: A) the influence of branching (1, 3, 9 siRNA duplexes per ligand) on silencing efficacy mediated by novel dendritic structures; B) the influence of additional glucose modifications introduced by modified siRNA guide strand. In all cases the total amount of siRNA duplexes was normalized to the monomeric structure. Quantification was achieved by luciferase activity. a: AEA-[1siRNA-Luc], b: AEA-[3siRNA-Luc], c: AEA-[9siRNA-Luc], d: AEA-[3siRNA-Luc]-glucose.

The 3′ end of the siRNA guide strand allows further functionalization.[9] This modification can stabilize the RNA

inside the cell, which should lead to a better silencing effect.[7e,10] In order to exploit this possibility, we next functionalized the siRNA duplexes of the dendrimers with a glucose molecule at the 3′ ends.[11] Glucose forms H-bonds with the RNA duplex and increases the water solubility. The introduction of the 3′ modification was achieved by hybridizing the passenger-strand-containing dendrimers with the corresponding glucose-modified guide strands. Indeed, the glucose-end-capped dendrimers showed a significantly enhanced efficacy. It is likely that the large siRNA dendrimes suffer from serious degradation in the endosomes after internalization.[10a,12] This may be retarded by glucose capping.[13]

The advantage of capping of the trimeric siRNA dendrimer is quite large, as shown in Figure 3B. The threefold glycosylated structure finally gave a silencing effect of approximately 80%. Thus, in comparison to the monomeric duplex, the silencing efficiency was increased by a factor of 2.5, despite the reduced total concentration.

We next investigated the possibility of utilizing the dendritic siRNA structure to induce silencing in difficult to transfect cells such as neurons (Figure 4). Consequently, we tested the uptake of the nanoparticles into neural stem cells. We prepared siRNA dendrimers in which we replaced the glucose units at the 3′ end of the guide strand by Alexa Flour 647. Indeed, when we added the fluorophore-modified siRNA trimer to the neural stem cells, efficient uptake was detected by confocal microscopy (Figure 4A).

In order to prove the ability of the siRNA dendrimers to silence an endogenous gene, we next transfected the neural stem cells with a trimeric siRNA dendrimer targeting Tet1. This enzyme was recently shown to oxidize 5-methylcytosine to 5-hydroxymethylcytosine and 5-carboxycytosine, which is essential for the differentiation process.[14] Using qPCR, we monitored the expression level of Tet1 (Figure 4B). Indeed, the siRNA dendrimer led to a significant reduction of the level of endogenous Tet1 expression, proving the effect of the siRNA despite it being incorporated into a dendritic structure. Again, we observed the strongest silencing effect with the trimeric siRNA structure.

Finally we were interested in the ability of our dendritic siRNA structures to exhibit a medicinally relevant function in primary neurons that are hard to transfect by normal methods.[3f,15] As a target, we chose the neurotropic rabies virus (RABV), which causes over 55000 deaths per year and is not treatable after the onset of clinical symptoms.[16] For this study, we infected mouse E14 cortical neurons with the RABV and subsequently treated the cells with two siRNA dendrimers (AEA-[3siRNA]-Glc) against the mRNAs of the viral nucleoprotein (N protein) and the phosphoprotein (P protein). The siRNA sequences were designed in silico using the program siDESIGN. Both proteins are essential for viral transcription and replication and are therefore considered promising targets to efficiently counteract RABV infection.[17] In addition, the P protein of RABV is an important antagonist of the innate immune system, completely suppressing both IFNβ induction and signaling pathways.[18] Down-regulation of P protein expression should consequently promote the innate immune response, thereby

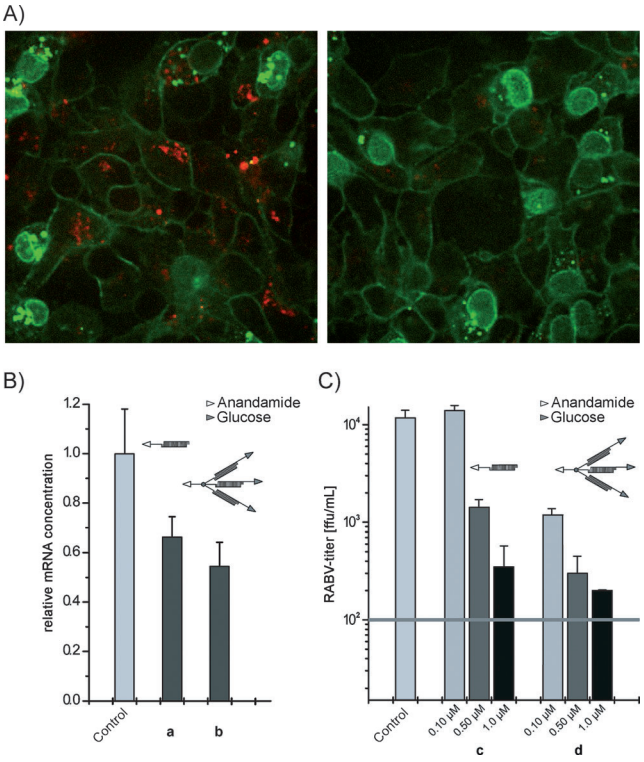

Figure 4. A) Anandamide-mediated delivery of dendritic siRNA nanostructures to neural stem cells. Left: trimeric siRNA modified with Alexa Fluor 647 was incubated with neural stem cells. Right: As a negative control the stem cells were incubated with siRNA lacking the ligand modification (red: siRNA, green: cell membranes. The contrast settings for siRNA signals were equal for both images). B) Successful delivery of nanostructures to stem cells was additionally demonstrated by regulation of Tet1 monitored by real-time PCR. **a**: AEA-[1siRNA-Tet1], **b**: AEA-[3siRNA-Tet1]-Glc. C) Down-regulation of RABV titers in E14 cortical neurons by treatment with different AEA-modified siRNA structures targeting the P protein of rabies virus. **c**: AEA-[1siRNA-P-protein], **d**: AEA-[3siRNA-P-protein]-Glc.

further restricting RABV infection. The data of the experiment clearly show that treatment of the infected neurons with either one of the dendrimers AEA-[3siRNA]-Glc (anti-P and anti-N) lead to a strongly reduced viral titer (Figure 4C; for data of targeting N protein Figures S2 and S3). The titer was reduced by two orders of magnitude in relation to the control experiment performed with a nontargeting anandamide-modified siRNA. The trimeric dendrimer was able to reduce the viral titer to a level close to the detection limit of the experiment. Important are also the observations that the reduction of the viral titer is dose-dependent and that with the standard lipofectamin 2000-based transfection, only a tenfold reduction of the viral titer was observed. These results show the improved efficacy of the siRNA dendrimers.

In summary, we have presented novel dendritic siRNA nanostructures that allow the transfection of difficult-to-transfect cells, such as stem cells and primary neurons.[19] A click-chemistry-based approach, in which the passenger strands of the siRNAs are covalently bound to the dendritic structure, allowed us to exactly control the monodispersity of the particles, which enabled the control of the number of

siRNA duplexes bound to one AEA ligand for receptor-mediated uptake. The dendritic siRNA nanostructures allow the effective control of gene expression in neural stem cells and in primary neurons. We were also able to use the system to reduce the viral titer of the rabies virus in primary neurons to close to the detection limit, thus illustrating a possible way to a novel siRNA-based therapy of this deadly disease.

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Communications

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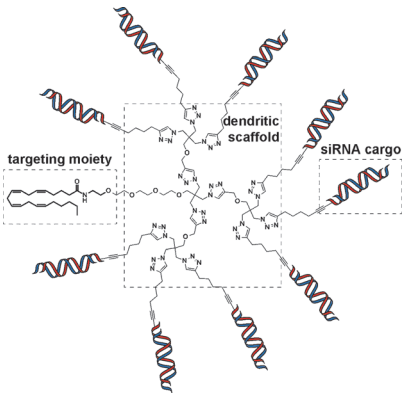
Cell-Penetrating and Neurotargeting Dendritic siRNA Nanostructures



siRNA dendrimers with an anandamide receptor ligand are accessible through a click-chemistry approach, and are taken up even by sensitive neural cells. Silencing of two key proteins of the rabies virus was achieved, allowing the suppression of the viral titer in infected neurons below the detection limit.