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Click-Modified Anandamide siRNA Enables Delivery and Gene Silencing in Neuronal and Immune Cells

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Supporting Information

ABSTRACT: Click chemistry of alkyne-modified RNA with different receptor ligand azides was used to prepare 3′-folate, 3′-cholesterol, and, as a new entity, 3′-anandamide-modified RNA in high yields and excellent purity. The anandamide-modified RNA shows surprisingly high transfection properties and enables the delivery of siRNA even into difficult-to-transfect RBL-2H3 cells which model neuronal uptake. Furthermore, the system was employed in human immune cells (BJAB), demonstrating silencing effects similar to those of a cationic, benchmark transfection reagent. In addition, the anandamide conjugates were found to be nontoxic. The reported chemistry and the described properties of the anandamide siRNA extend the possibilities of using siRNA-based gene silencing in neuronal and immune cells.

RNA interference (RNAi) is a powerful tool that utilizes 21mer RNA double strands to repress the formation of a particular protein in a cell.[1−3] In nature, the silencing RNA (siRNA) molecules are produced from larger transcripts that are cut by the Dicer complex.[4] For biotechnological application, however, the siRNA molecules are chemically prepared and administered. The idea to use siRNA as therapeutic agents[5] has been intensively pursued in the past decade, but the major obstacle, poor cellular uptake of RNA duplexes, could not be overcome.[6] Currently, RNA delivery systems as divergent as nanoparticles,[7,8] liposomes,[9,10] or polycation polymers[11] are under intensive investigation. Despite substantial progress in the field, however, the often still high toxicity[12−14] and low cellular specificity represent problems that have yet to be solved.[15]

Most recently, receptor-mediated endocytosis has evolved as an alternative delivery strategy[16−25] that allows targeting of the siRNA to special cell types. The method requires linking the siRNA to a ligand that binds to a cell-type-specific receptor. This initiates an internalization process leading to the uptake of the RNA−ligand conjugate. Currently, the strategy is most successfully implemented with cholesterol-modified RNA.[24] Here, we report that such a receptor-mediated strategy can be successfully used to solve the problem that sensitive neuronal[26,27] and immune cells[28] have been, up to now, difficult to transfect. We discovered that the cannabinoid receptor present on both cell types[29] can be efficiently targeted with arachidonoyl ethanol amide (anandamide)[30,31]-modified siRNA, despite the uncertainty that still exists regarding the cannabinoid-mediated uptake mechanism.[32,33] The synthesis of the anandamide-modified RNA strand was performed as depicted in Scheme 1. The central element of the synthesis is the Cu-catalyzed alkyne−azide click reaction[34−39] between an alkyne-modified RNA strand and the corresponding ligand azides **1**. To compare the anandamide-modified RNA strands to other systems, we utilized the click method also for the preparations of a folate−RNA[40] conjugate using folate azide **2** and of a cholesterol-modified RNA strand with the cholesterol azide **3**. We introduced in all cases a short tetraethyleneglycol spacer between the RNA strand and the respective ligand. The click technology enabled in all cases efficient ligation of the hydrophobic and often quite insoluble (folate) ligand molecules to RNA. In addition, the method enabled efficient conjugation at the more difficult to access 3′-terminus of the siRNA duplex. 3′-Modified siRNA strands are typically better tolerated by the RNAi machinery.[41] To achieve the 3′-end attachment, we used a deoxyuridine phosphoramidite with an octadiyne handle at C5 during RNA synthesis.

The anandamide azide ligand **1** was prepared in just one step from arachidonic acid **4** and the azido- and amino-functionalized oligoethyleneglycol **5**. The same strategy was employed for the synthesis of the cholesterol azide **3**. The folate derivative **2** was prepared via a slightly more elaborate synthesis starting with the protected glutamic acid derivative **6**, which was condensed with the amino−azide tetraethyleneglycol compound **5**. Cleavage of the Fmoc group and coupling with pteroic acid furnished, after deprotection, folate **2** (for more details see SI). Compound **2** contains in this way the ethyleneglycol spacer attached to the γ-carboxyl group, which provides a folate compound with superior receptor binding properties.[42] The three azides were subsequently clicked with excellent yields to the alkyne containing the RNA sense strand. After HPLC purification, the ligand-modified RNAs were hybridized to the antisense counterstrand to obtain the siRNA duplexes depicted in Chart 1.

To visualize the delivery of the RNA duplexes into live cells, we initially hybridized the anandamide- and folate-modified RNA sense strands to antisense strands containing a fluorescein label. Figure 1 shows the result of confocal microscopy studies performed with two different cells lines. For the anandamide-modified RNA duplex we utilized RBL-2H3 cells, which serve

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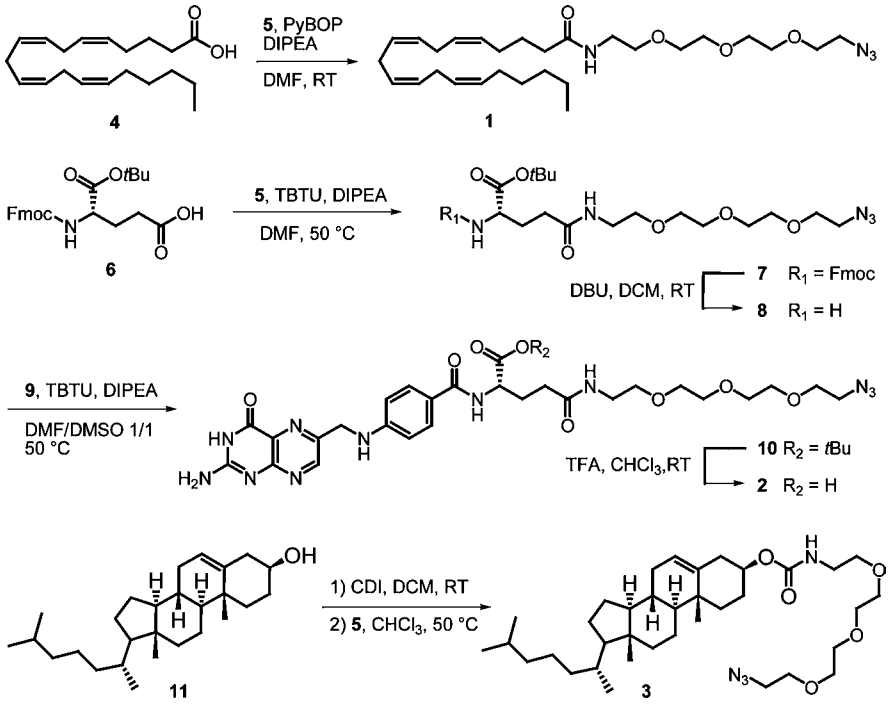
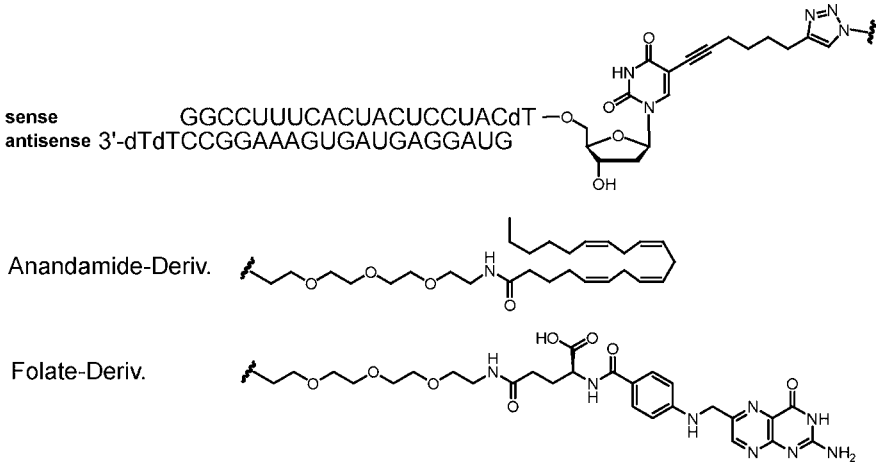
Scheme 1. Synthesis of Azide-Modified Anandamide 1 as Well as Folate (2) and Cholesterol (3) Derivatives[a]



[a]Reagents: **5** = 11-azido-3,6,9-trioxaundecan-1-amine, PyBOP = benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate, TBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, DIPEA = *N*,*N*-diisopropylethylamine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, **9** = pteroic acid, TFA = trifluoroacetic acid, CDI = carbonyldiimidazole.

Chart 1. Chemical Structure and Sequence of Anandamide- and Folate-Modified siRNA Targeting *Renilla* Luciferase



as a model for immune cell function.[43] Barker et al. were able to show that the uptake of anandamide by RBL-2H3 cells is functionally identical to uptake by neuronal cells and astrocytes.[44] Thus this cell line is an excellent model for anandamide uptake in immune cells and neurons.

Uptake of the folate-modified RNA duplex was studied with HeLa cancer cells known to overexpress the folate receptor. The microscopy studies showed that unmodified siRNA is, as expected, unable to enter both cell lines. Anandamide- and folate-modified siRNA, however, were readily detected inside the respective cells, proving uptake. The same result was also observed with modified dsDNA, which shows that the uptake is entirely ligand dependent (Figure 1).

To demonstrate that the delivered siRNA exhibits the desired RNAi effect, we utilized a commercially available dual-luciferase reporter assay. A plasmid containing two luciferases (Renilla and Firefly) was transfected into the cells. RNAi was evaluated by targeting the expression of the Renilla luciferase, whereas the Firefly luciferase served as an internal standard. For these studies we used the ligand-modified siRNA without transfection reagent. Initial control experiments with unmodified RNA duplexes (no ligand, no fluorescein) showed that the Renilla expression was not affected. In contrast, we observed a dose-dependent silencing of Renilla expression in presence of ligand-modified siRNA in both cell lines (Figure 2a). Most importantly, even a relatively low amount of ligand-modified siRNA showed a considerable effect that ultimately led to a relative silencing of about 60%. A final control experiment was performed with a scrambled siRNA. Here again we were unable to detect any luminescence reduction, showing that the

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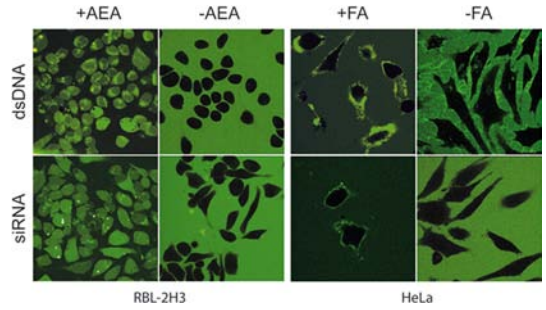

Figure 1. Delivery of fluorescein-labeled siRNA and dsDNA to RBL-2H3 and HeLa cells. Cannabinoid receptor expressing RBL-2H3 cells were incubated with anandamide (AEA)-modified dsDNA and siRNA. Folate receptor expressing HeLa cells were incubated with folate (FA)-modified dsDNA and siRNA. As a negative control, both cell lines were incubated with duplexes lacking a ligand modification.

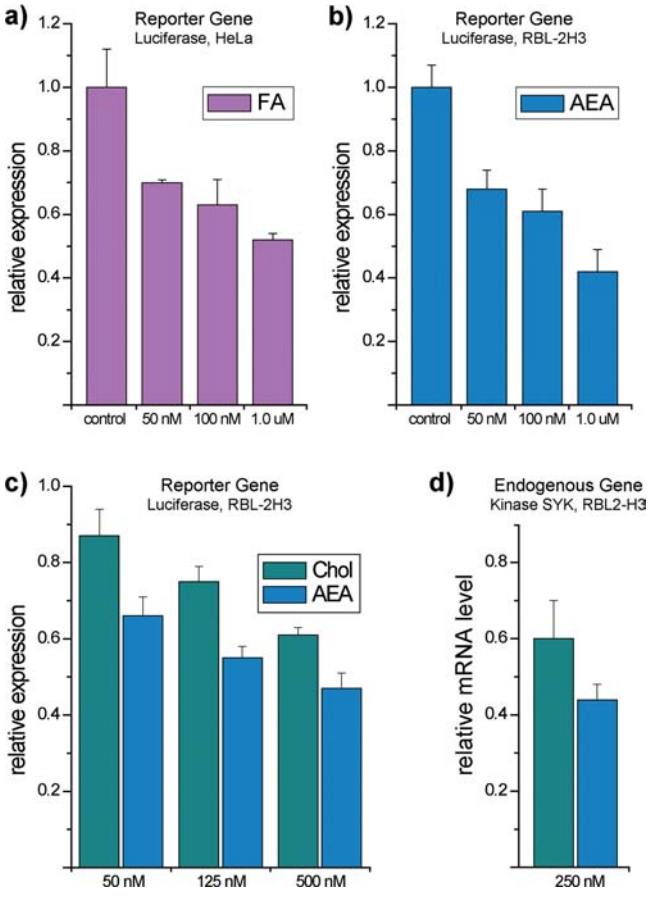

Figure 2. (Top) Relative silencing of Renilla luciferase mediated by (a) folate (FA)-modified siRNA in HeLa cells and (b) anandamide (AEA)-modified siRNA in RBL-2H3 cells. Quantification via luciferase activity. (Bottom) Relative silencing of (c) Renilla luciferase and (d) spleen tyrosine kinase mediated by cholesterol (Chol, green)- and anandamide (AEA, blue)-modified siRNA in RBL-2H3 cells. Quantification via mRNA level.

observed silencing is caused by specific binding of anandamide-modified siRNA to the mRNA target. The silencing efficacy of anandamide-modified siRNA was next evaluated in comparison to that of the cholesterol–siRNA conjugate.[24] The result of this comparison is depicted in Figure 2. To our surprise, we noted that the new anandamide **1**-modified siRNA is constantly significantly more potent than the broadly exploited cholesterol system, which establishes the anandamide ligand as a powerful new delivery tool.

To investigate if the anandamide–siRNA conjugate is able to down-regulate a therapeutically important endogenous gene, we next attempted to suppress the expression of the spleen tyrosine kinase (SYK), which is a key protein involved in the IgE-dependent inflammatory signaling cascades. As such, the protein is a prospective target for the treatment of allergic and inflammatory disorders.[45,46] For the experiment we prepared an ananamide-modified siRNA having the sequence described by Sanderson et al.[47] After addition of the siRNA conjugate to RBL-2H3 cells, we monitored the expression level using real-time PCR. Indeed, the expression of the SYK protein was successfully reduced by about 55%, and again the anandamide conjugate was found to be substantially more active than cholesterol-modified siRNA (Figure 2).

To highlight the advantages associated with anandamide conjugation, we compared the siRNA effect of anandamide-modified siRNA with and without an additional transfection agent (*jetPRIME*). The comparison was performed directly in human immune B cells (BJAB) known to express the cannabinoid receptors.[48] The data depicted in Figure 3a show

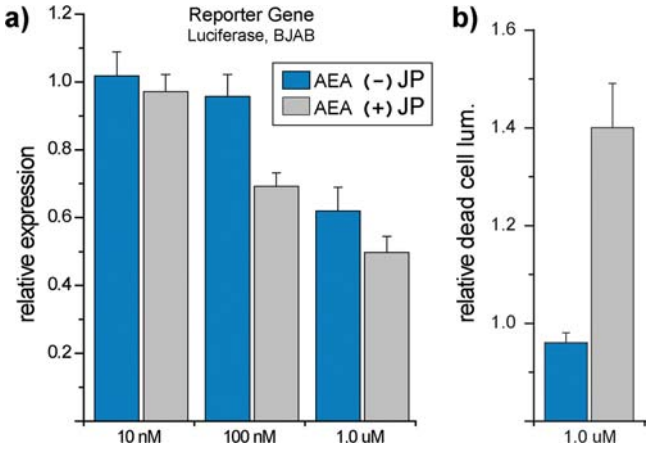

Figure 3. (a) Relative silencing of *Renilla* luciferase mediated by anandamide-modified siRNA and (b) relative toxicity of anandamide-modified siRNA in the absence of *jetPRIME* (blue) and in the presence of *jetPRIME* (gray) in human immune B cells (BJAB). Quantification via luciferase luminescence.

that the silencing effect is in the same range, proving that anandamide conjugation can replace the transfection agent. We furthermore observed that anandamide conjugation increases the activity of the *jetPRIME*-complexed siRNA by a factor of almost 2 (Figure S3g). Most important, however, are the toxicity data reported in Figure 3b, which show that the anandamide conjugates are not toxic, in comparison to mock-treated cells, which allows repeated treatment of the cells with the conjugates (see also Figure S4).

In summary, we report here the use of the Cu-catalyzed alkyne–azide chemistry for the construction of novel anandamide–siRNA conjugates. The chemistry enables the efficient construction of RNAs modified with different ligands at the 3′-end in excellent yield and purity. This is particularly noteworthy for folate-modified oligonucleotides, which are notoriously difficult to access. The anandamide conjugation allows transfection of immune cells and provides excellent

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silencing data comparable to cationic transfection reagents without showing any cytotoxicity.

■ ASSOCIATED CONTENT

Supporting Information

Preparation of **1**−**3**, oligonucleotide synthesis, purification and click modification, convocal microscopy studies, dual luciferase assay, and real-time PCR. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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