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Red light activated "caged" reagents for microRNA research†

A. Meyer,[a] M. Schikora,[a] V. Starkuviene[b,c] and A. Mokhir*[a]

"Caged" reagents for miRNA research (siRNA targeting EGFR, involved in miRNA maturation, and mimics of miR-20a, playing a key role in tumor formation and metastasis) were prepared. It was demonstrated that these reagents can be activated by non-toxic to cells red light both in cells and in cell free settings.

MicroRNAs (miRNAs) are 21–25 nucleotide non-coding ribonucleic acids, which target specific mRNAs in cells thereby affecting their stability and translation. They regulate cell differentiation, membrane trafficking, apoptosis, proliferation and other biological processes.[1,2] Moreover, some of these RNAs are up- or down-regulated during diverse human diseases, *e.g.* obesity, diabetes mellitus[3,4] and cancer.[5,6]

MiRNAs bind several mRNA targets and are, therefore, involved in complex regulatory networks by affecting the expression of many genes simultaneously.[2] Studies of such networks would benefit from light-activated miRNA mimics or inhibitors (termed either antagomirs or antimiRs), which can allow for accurate spatial and temporal control of miRNAs directly in cells. Several "caged" inhibitors of miRNAs have been reported. For example, W. Li and co-workers have prepared 2′-OMe RNA-based, hairpin-shaped "caged" antagomirs of lsy-6 miRNA, which can release the active antagomir upon exposure to 365 nm light in *C. elegans*.[7] Deiters and co-workers have synthesized UV-light responsive antagomirs of miR-122 and miR-21 by "caging" several uracil nucleobases of 2′-OMe RNA strands.[8] Heckel and co-workers have prepared "caged" antimiRs of miR-92a by modification of nucleobases with a 1-(2-nitrophenyl)ethyl protecting group, and applied them to improve angiogenesis in HUVEC cells.[9] In contrast to numerous examples of "caged" miRNA inhibitors, a single report describing "caged" miRNA mimics is available: Dmochowski and co-workers have synthesized "caged" mimics of let7 miRNA by covalent linking its 3′- and 5′-termini *via* a photocleavable moiety. The resulting inactive circular RNAs could be activated in zebrafish embryos by their exposure to 365 nm light.[10] In all known systems uncaging of miRNA inhibitors or mimics has been conducted by using toxic to cells UV-light[11] that limits broader applications of these reagents. To date neither an inhibitor nor miRNA mimics activated by non-toxic visible light are known.

We have recently reported a novel strategy for caging antisense oligonucleotides and small interfering RNAs (siRNAs), which can be activated by non-toxic to cells red light.[12–15] In particular, "caging" of siRNAs relies on blocking 5′-termini of their guide strands with a 9-anthracenyl (AN) fragment that prevents their intracellular phosphorylation and completely abandons their activity.[15] 3′-Termini of their lagging strands are modified with a red-light absorbing photosensitizer (**PS**). Upon irradiation of such RNAs with red light singlet oxygen ($^1O_2$) is generated in proximity to the AN, which induces its cleavage and release of active siRNA.[15] Since intracellular phosphorylation is also crucial for the miRNA activity, we hypothesized that this approach could be suitable for "caging" miRNA mimics as well (Scheme 1A). Herein we report on "caged" mimics of a selected miRNA (hse-miR-20a, further called miR-20a) and "caged" siRNA targeting mRNA of a representative protein involved in miRNA maturation (epidermal growth factor receptor (EGFR)). MiR-20a is important for a number of biological processes, *e.g.* membrane trafficking,[16] formation of tumors,[17,18] metastasis,[19] and it is overexpressed in some cancers.[20] EGFR is an oncogene in human cancers. It is known to modulate maturation of miRNAs through phosphorylation of argonaute 2 (AGO2).[21] We expect that these reagents will be useful tools for miRNA research.

As a starting point we selected a known siRNA (Scheme 2A) as an inhibitor of EGFR.[16] The 5′-terminal nucleoside in its guide strand is adenosine. We confirmed that this moiety can be replaced for 2′-deoxyadenosine without activity loss. For example, RNA1/RNA3, containing a terminal 2′-deoxyadeno-

[a]*Friedrich-Alexander-University of Erlangen-Nürnberg, Department of Chemistry and Pharmacy, Organic Chemistry II, Henkestr. 42, 91054 Erlangen, Germany. E-mail: Andriy.Mokhir@fau.de*
[b]*Ruprecht-Karls-University of Heidelberg, BIOQUANT, Im Neuenheimer Feld 267, 69120 Heidelberg, Germany*
[c]*Faculty of Natural Sciences, University of Vilnius, Vilnius, Lithuania*

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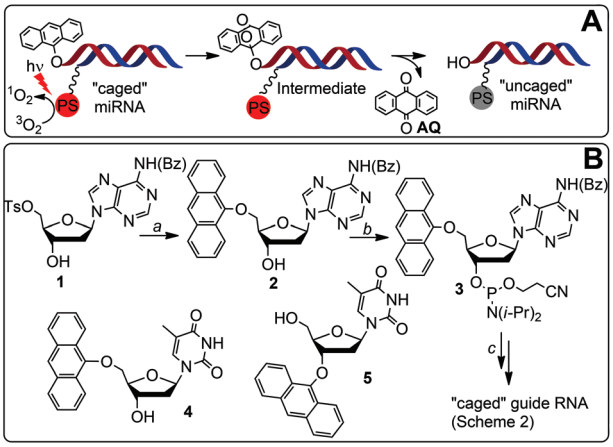
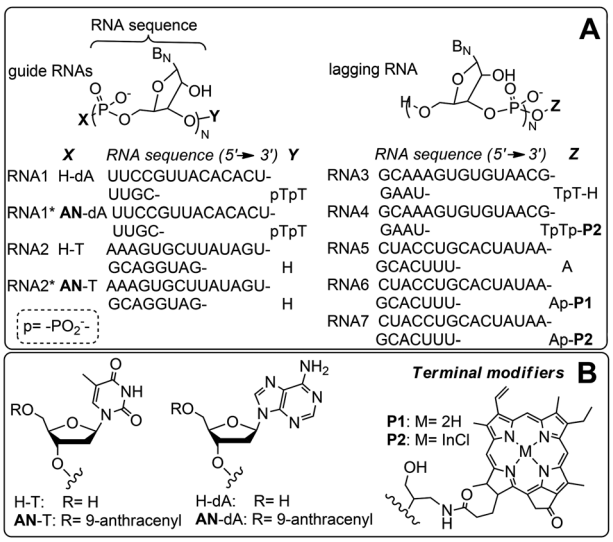


Scheme 1 A: "Caged" miRNAs activated by red light; **PS** = photosensitizer, *e.g.* InP; **PS** in the red circle is active and that in the grey circle – inactive (bleached). B: Synthesis of phosphoramidite **3** for preparation of "caged" RNAs and structures of previously reported 9-anthracenyl-substituted thymidine derivatives. (a) Anthrone, NaH, dimethylsulfoxide (DMSO), N_2, 50 °C; (b) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, *N*,*N*-diisopropylethylamine, CH_2Cl_2, 22 °C.



Scheme 2 A: Sequences of guide and lagging RNAs used for assembly of "caged" and control miRNAs and siRNAs. B: Structures and labelling of terminal modifiers of the "caged" and control RNAs.

sine (dA) residue, inhibited ~31% activity of EGFR, whereas its analogue, containing adenosine, inhibited ~38%.[16] Analogously, we confirmed that the replacement of 5′-terminal uridine for thymidine does not change substantially the activity of the selected miR-20a mimics (Scheme 2A).

Next, we prepared phosphoramidite **3** using **2** as a starting material (Scheme 1B, ESI†). Before synthesizing the corresponding RNAs, we tested whether **2** can be deprotected by $^1O_2$. Since in the latter reaction the AN chromophore is bleached, we followed it by using fluorescence and UV-visible spectroscopy (Fig. 1A and S6, (ESI†)). In particular, we observed

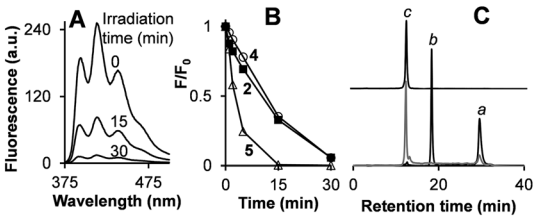


Fig. 1 A: Monitoring transformation of modified nucleoside **2** (1 mM) in CH_3OH containing In^{3+}(pyropheophorbide-a)chloride (100 μM), which was irradiated with red light (650 nm, 0.23 W) for the shown on the plot time periods: after the irradiation the mixtures were diluted 10 times with methanol and their fluorescence spectra (λ_{ex} = 368 nm) were acquired; a.u. – arbitrary units. B: Plots of decay of fluorescence intensities of nucleosides **2**, **4** and **5** upon their irradiation; all conditions are the same as described in A; *F* – fluorescence at 413 nm (for **2** and **4**) or 416 nm (for **5**) observed after the irradiation of the samples; F_0 – corresponding initial fluorescence. C: HPLC profiles of "caged" EGFR-targeting siRNA RNA1*/RNA4 (50 μM in HEPES buffer (6 mM) containing KCl (20 mM), $MgCl_2$ (0.4 mM) adjusted to pH 7.4 with KOH (5 mM)), which was either kept in the dark (lower, black dotted trace) or irradiated for 30 min (lower, grey trace) with an LED light source (650 nm, 0.23 W). An HPLC profile of pure RNA1 was used as a reference (upper, black trace). HPLC was conducted at denaturing conditions (60 °C): peak "a" corresponds to RNA4, peak "b" – to RNA1* ("caged" strand), peak "c" – to RNA1 ("uncaged" strand).

that irradiation of a mixture of **2** (1 mM) with In^{3+}(pyropheophorbide-a)chloride (InP, 0.1 eq.) in CH_3OH with red light (650 nm, 0.23 W) leads to decrease in the intensity of both fluorescence and absorbance bands characteristic for the AN, which indicates its bleaching. Additionally, ^1H NMR spectroscopy data confirm that under these conditions the AN fragment is cleaved with the formation of anthraquinone and *N*-benzoyl-2′-deoxyadenosine (Fig. S7 and S8†). However, unidentified side products were also formed under the chosen experimental conditions (Fig. S7 and S8†). We observed that rates of the reaction of $^1O_2$ with **2** and previously studied analogue **4**,[15] containing thymidine in place of 2′-deoxyadenosine, are practically identical. In contrast, 3′-*O*-substituted **5** [22] is cleaved quicker by $^1O_2$ (Fig. 1B).

Modified and unmodified RNAs were prepared by solid phase synthesis as described in the ESI† (Scheme 2A), identified by MALDI-TOF mass spectrometry and their purity (>90%) was confirmed by analytical HPLC (Fig. S10†). RNA duplexes were obtained by annealing of the corresponding single stranded RNAs in aqueous buffer (pH 7.4).

Uncaging of EGFR specific siRNA RNA1*/RNA4, containing **P2** as a **PS**, in cell-free settings was studied by using analytical HPLC (Fig. 1C). We observed that in the dark this compound is stable for at least 24 h. However, when it was irradiated with red light for 30 min, the conversion of RNA1*/RNA4 was quantitative, whereas the yield of the desired uncaged product RNA1 was found to be ~85% (peak c). Analogous uncaging efficiency has been observed for the photoactivation of the previously reported "caged" AcGFP–siRNA by its exposure to red light for 30 min: 100% conversion of "caged" siRNA, yield of the uncaged product – 83 ± 2%.[15]

Though "caged" miR-20a mimics RNA2*/RNA6 and RNA2*/RNA7, containing correspondingly **P1** and **P2**, are also converted to the uncaged products under these conditions, the efficiency of this reaction was found to be lower: RNA2*/RNA6, conversion of the starting material – 84%, yield of the uncaged product – 38%; RNA2*/RNA7, conversion of the starting material – 96%, yield of the uncaged product – 43%, Fig. S11.† In the latter reaction an identical side product is formed (labelled with * in Fig. S11†), which according to MALDI-TOF MS analysis has 232 Da higher mass than RNA2* (the accuracy of mass determination for RNAs with $m/z \sim 7000$ was 0.1% of the molecular mass of the analyte or ±7 Da), and does not contain the anthracene chromophore or the photosensitizer, but contains RNA nucleobases as indicated by it strong absorbance at 260 nm. Unfortunately, we could not identify this side product.

Similarly to previously reported "caged" siRNAs targeting AcGFP and KIF11[15] the uncaging process in this case is accompanied by bleaching of the photosensitizer (**P2**). This is indicated by the decrease in the intensity of the peak corresponding to RNA4 (peak a in Fig. 1C: compare lower black and grey traces).

Next, we investigated red-light induced inhibition of EGFR in HeLa cells. The corresponding "caged" siRNAs and controls were brought into the cells *via* transfection with Lipofectamine™ 3000. After 2 h of incubation with the cells, the RNAs were irradiated with red light (650 nm, 0.23 W) for 5 min and further incubated for 18 h 55 min at 37 °C. Then the cells were starved for 3 h as described in the ESI† and the amount of EGFR in the cellular membrane was probed by binding to biotinylated epidermal growth factor (EGF) pre-labelled with streptavidin–Alexa Fluor® 488. After 15 min of internalization and fixation of the cells, the fluorescence of endocytosed EGF was quantified by flow cytometry (Fig. 2A).

The fluorescence intensity of the cells treated with Lipofectamine™ 3000 only and labelled with EGF–biotin/streptavidin–Alexa Fluor® 488 was used as a reference, which corresponds to non-inhibited levels of internalized EGF. We observed that uncaged siRNA RNA1/RNA3 reduced EGF internalization by a factor of 0.317 ± 0.026. The introduction of **P2** at the 3′-terminus of the guide strand as in RNA1/RNA4 slightly enhanced the inhibition: 0.431 ± 0.021. In contrast, "caged" siRNA RNA1*/RNA4 and RNA1*/RNA3 were found to be inactive (Fig. 2A). The irradiation of cells pre-incubated with RNA1*/RNA4 with red light restored the inhibitory activity of this reagent completely (0.415 ± 0.025), which was similar to that obtained for the uncaged analogue RNA1/RNA4 (0.457 ± 0.025). In contrast, RNA1*/RNA3 lacking a **PS** remained inactive (Fig. 2A).

Finally, we explored red light induced activation of "caged" miR-20a mimics RNA2*/RNA6 and RNA2*/RNA7 in HeLa cells. The former reagent contained **P1** as a **PS** and the latter – **P2** (Scheme 1B). Since similar effects were obtained with both **PS**'s, only **P2**-containing RNAs will be discussed further. The activity of miR-20a mimics was determined by using a dual-luciferase reporter assay, in which miR-20a expression correlates with decrease in the activity of *Renilla* luciferase (hRluc). The activity of *firefly* luciferase (Fluc) was used as a reference. Both enzymes

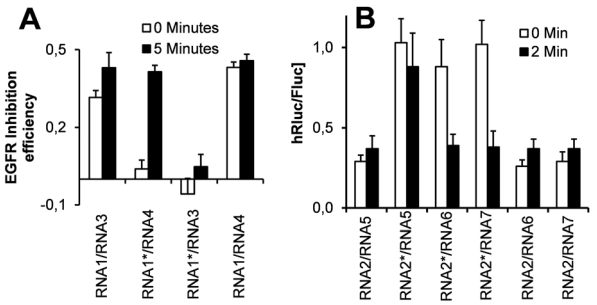


Fig. 2 A: Red light controlled EGFR expression in HeLa cells: amount of each RNA strand (in "caged" siRNA RNA1*/RNA4, unmodified siRNA RNA1/RNA3 and controls RNA1*/RNA3 and RNA1/RNA4) was 3 pmol per well in a 24-well plate. Samples were either non-irradiated (colourless bars) or irradiated with red light (LED, 650 nm, 0.23 W) for 5 min (black bars). B: Red light-controlled activity of "caged" miR-20a mimics (RNA2*/RNA6 and RNA2*/RNA7), unmodified miR-20a (RNA2/RNA5) and controls (RNA2*/RNA5, RNA2/RNA6 and RNA2/RNA7) in HeLa cells determined using dual luciferase reporter assay and expressed as a change of expression of hRluc relative to that of Fluc – hRluc/Fluc. Amount of each RNA strand was 1 pmol per well in a 24-well plate. Samples were either non-irradiated (colourless bars) or irradiated with red light (LED, 650 nm, 0.23 W) for 2 min (black bars). The data obtained in three independent experiments were averaged and their standard deviation was determined. Other experimental details are provided in the ESI.†

were brought into cells by the transfection of a psiCheck2 reporter plasmid containing a miR-20a binding site. We set a ratio of hRluc/Fluc to 1 for cells treated with the plasmid only. For uncaged miR-20a mimic RNA2/RNA5 we observed a hRluc/Fluc ratio of 0.29 ± 0.04. Labelling 3′-terminus of the lagging strand with **P2** as in RNA2/RNA7 did not affect the activity: hRluc/Fluc = 0.29 ± 0.06. In contrast, the "caged" mimic RNA2*/RNA7 was practically not active (hRluc/Fluc = 1.02 ± 0.15). As expected, its exposure to red light (650 nm, 0.23 W, 2 min) restored the activity completely: hRluc/Fluc = 0.38 ± 0.10, compared with hRluc/Fluc = 0.37 ± 0.06, observed for the irradiated uncaged RNA2/RNA7. Since the activity of a **PS**-free, "caged" control RNA2*/RNA5 was not restored upon irradiation, we concluded that the effect of light was mediated by the **PS**.

In summary, we prepared a "caged" siRNA, which targets a gene affecting miRNA maturation (epidermal growth factor receptor), and "caged" mimics of miR-20a, which is a representative miRNA participating in membrane trafficking, formation of tumors and metastasis. We demonstrated that these reagents can be efficiently activated by short (2–5 min) exposure to non-toxic red light (650 nm) of low power (0.23 W) both in cells and in cell-free settings. These and analogous reagents can potentially become useful tools for studying complex miRNA-mediated regulatory pathways.

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