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FULL PAPER

Photoregulation of Gene Expression with Amantadine-modified Caged siRNAs Through Host/Guest Interaction

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Abstract: RNA interference (RNAi) is an essential and powerful tool for targeting and verifying specific gene functions. Conditional control of siRNA activity, especially using light activation, is a potential method to regulate target gene expression and functions. Here, a series of photolabile siRNAs with amantadine modification were rationally designed and developed through host/guest interaction of amantadine and β-cyclodextrin derivatives to enhance the blocking effect of siRNA binding and/or RISC processing. These caged siRNAs with amantadine modification at the 5' end of antisense-strand RNA were efficiently inactivated due to the host/guest interaction of amantadine and β-cyclodextrin. Photomodulation of gene silencing activity of these amantadine-modified caged siRNAs targeting both exogenous and endogenous genes was successfully achieved, which indicated that host/guest interaction could be a new strategy for developing new caged siRNAs for gene photoregulation with low leaking activity.

Introduction

Small interfering RNAs (siRNAs), double-stranded RNA duplexes consisting of 20-25 base pairs, play a crucial role in RNA interference (RNAi)-induced gene silencing[1]. Considering the high failure rates of traditional drug discovery[2], RNAi-based nucleic acid drugs demonstrate significant potential for precise therapeutics, especially for currently undruggable targets[3]. Although patisiran, the first siRNA drug, was approved by FDA in 2018[4], there still exist some challenges associated with siRNA drugs[5], such as off-target effects[6], low stability[7], and difficult cellular delivery[8]. To solve these problems, many researchers have focused on the chemical modification of siRNAs. A variety of chemical modifications have been applied in recent decades to optimize the gene silencing effect of siRNA, including siRNA backbone modification and terminal conjugation of specific moieties[9]. These modifications achieved great enhancement of siRNA delivery efficiency and therapeutic effect. In particular, terminal conjugation of N-acetyl galactosamine (GalNAc) clusters showed great improvement in targeting hepatocytes due to the highly specific and efficient interaction of the moiety with its corresponding receptor through clathrin-dependent endocytosis[10]. This finding indicates that siRNAs conjugated with receptors or targeting ligands have considerable potentials for cellular uptake and distribution.

However, most covalent conjugation of siRNAs may lead to a partial decrease in siRNA activity if the conjugated moieties were not removed. To address this problem, conditional regulation of siRNA activity has received much attention, and many stimulus-responsive moieties have been introduced[11]. Light, as a noninvasively and easily operated stimulus, shows great potential for highly efficient conditional regulation with spatiotemporal resolution[12b]. With the introduction of caging technology, caged siRNAs are expected to precisely regulate gene functions. Many different caged siRNAs have been developed through nucleobase caging, phosphate caging, caging hairpins and siRNA cyclization[12]. Among them, caged siRNAs involving the attachment of caging groups to the phosphate backbone were the first type of photolabile siRNAs developed through a statistical caging strategy. However, the gene silencing effect of these caged siRNAs was not effectively abrogated due to their leakage activity[13]. Previously, we developed caged siRNAs with site-specific attachment of 2-(2-nitrophenyl) propyl (NPP) to each phosphate backbone of siRNA and found that the modification at 5' terminus of the antisense RNA strand of siRNA duplex had a more efficient inhibitory effect on siRNA activity, while modification at the 3' terminus of antisense RNA strand was partially tolerated[14]. Different modifications have been further applied to 5′ terminus of siRNA antisense strand through a photolinker, and these modifications showed effective photomodulation of siRNA activities and gene silencing.

According to previous reports, large blocking groups on the 5' end of antisense RNA strands could greatly enhance the photomodulation of caged siRNA activity[15] [16]. Host/guest interaction may be a new strategy for developing caged siRNAs only with easy modification of a small guest molecule. β-Cyclodextrin belongs to the cyclodextrin family of polysaccharides, containing an appropriate hydrophobic cavity that can host various compounds with high size selectivity and binding affinity through *host/guest* interaction, especially amantadine[17]. Both β-cyclodextrin and amantadine show good biocompatibility and have been used in clinical applications[18]. Herein, we rationally designed and developed a new *host/guest* siRNA caging strategy involving interaction of a β-cyclodextrin moiety and an amantadine-modified photolabile antisense RNA strand at 5′ terminus. The binding of amantadine with β-cyclodextrin through *host/guest* interaction greatly enhances the blocking effect of RISC formation and/or RNAi processing until light activation removes amantadine and the β-cyclodextrin complex.. Based on this new strategy both exogenous gene (firefly luciferase) and endogenous gene (Eg5) were targeted to demonstrate photomodulation of siRNA gene silencing ability using these caged siRNAs through *host/guest* interaction.

Results and Discussion

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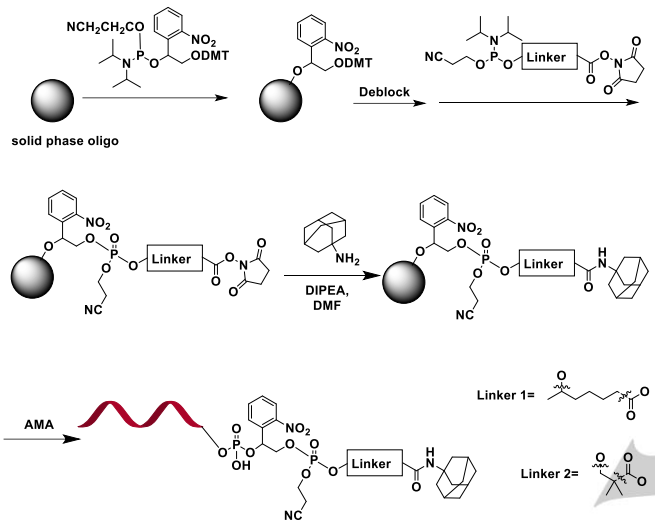
To synthesize amantadine modified caged siRNA, we first coupled a photolinker phosphoramidite (Scheme S1) and aliphatic N-hydroxysuccinimide (NHS) esters of alkyl linkers (Scheme S2 and Scheme S3) to 5' terminus of the antisense or sense RNA strands of anti-firefly luciferase siRNA (APAL and APSL) during RNA solid phase synthesis, as shown in Scheme 1. Further amantadine was conjugated to RNA on solid phase through amide bond formation. Two amantadine-modified caged sense or antisense RNAs with different lengths of aliphatic linker were then synthesized. After deprotection and HPLC purification, all obtained RNAs were characterized by ESI-MS analysis (see Supporting Information).



Scheme 1. Solid-phase synthesis of amantadine-modified caged oligonucleotides

To validate the photocleavage of amantadine-modified caged siRNA, different combination of amantadine-modified single-stranded sense and antisense RNAs (APAL1/SL, AL/APSL1, APAL1/APSL1, APAL2/SL, AL/APSL2, and APAL2/APSL2) were irradiated and analysed with 20% PAGE gels (Fig.S1 and Fig.S2). For double-stranded duplexes of caged oligonucleotides (APSL1/SL in lane 2-5, AL/APSL1 in lane 6-9 and ALAP1/APSL1 in lane 10-13), amantadine modification led to reduced mobility in comparison to the native RNA duplex (AL/SL in lane 1). Upon light activation (1-5 min irradiation), a new band of uncaged siRNA was observed (lanes 3~5, 7~9, 11~13 in Fig S1 and Fig S2), which showed similar mobility to the native control RNA duplex. This gel shift assay indicated that the cleavage of the photolinker and amantadine moiety led to the recovery of native RNA. Further ESI-MS data analysis of these uncaged RNAs confirmed the formation of native RNA with a terminal phosphate group. In addition, light irradiation for 3 min under our current conditions was sufficient for the full recovery of native siRNA, which was applied to the subsequent experiments.

To evaluate the photomodulation of target gene silencing using amantadine-modified caged siRNA, amantadine-modified caged antisense-strand RNA was subsequently hybridized with its complementary sense-strand RNA to generate siRNA duplexes. The β-cyclodextrin derivative was then incubated with amantadine-modified siRNAs for *host/guest* interaction to enlarge the blocking group of terminally modified siRNA. The reporter vectors (pGL-3 and pRL-TK) together with the native or caged siRNA duplex were cotransfected into HEK293T cells for photoregulation of target gene silencing.

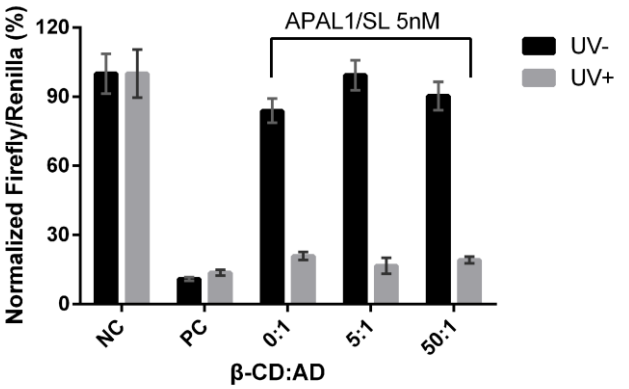


Figure 1. Photomodulation of firefly luciferase expression in AD-modified siRNA duplexes with natural sense strands and modified antisense strands (APAL1/SL) with different concentrations of β-cyclodextrin (β-CD). The concentration of the siRNA duplex was 5 nM, and the concentrations of β-cyclodextrin were 0 nM, 25 nM and 250 nM (β-CD:AD ratios are 0:1,5:1 and 50:1, respectively). NC, negative control; PC, positive control.

To demonstrate the effect of the host/guest interaction of amantadine/β-cyclodextrin on siRNA gene silencing, the complexes of amantadine-modified caged siRNA duplexes (APAL1/SL, 5 nM) with β-cyclodextrin at different concentrations (25 nM and 250 nM, ratios of 1:5 and 1:50) were first applied. The HEK293T cells transfected with the above control or caged siRNAs were placed in the dark for 4 h and were then divided into two groups. One group was kept in the dark, while the other was irradiated for 3 min. The luciferase activities were measured 44 h after co-transfection. As shown in Fig.1, cells from both negative and positive control groups showed almost no difference in firefly luciferase gene expression with or without light irradiation, which indicated that 3 min of light irradiation did not have an obvious toxic effect on cells. As expected, the positive control groups with native siRNA targeting firefly luciferase exhibited approximately only 10% gene expression with or without UV irradiation. Only 5′-end amantadine-modified caged siRNA without β-cyclodextrin incubation resulted in nearly 80% firefly luciferase activity before UV irradiation, which suggested that this modification at 5′ phosphate moiety partially disturbed RISC formation or processing similar to the previous observation with the caging group[12g]. The siRNA activity was recovered after the removal of the photolabile group with 3 min light irradiation. For cells treated with the complexes of amantadine-modified caged siRNAs and 5- or 50-fold concentration of β-cyclodextrin, approximately 96% and 90% luciferase expression remained before light activation, respectively, which exhibited greater enhanced inhibition than amantadine-modified siRNA alone. This result revealed that the amantadine-modified caged siRNAs were almost fully inactivated and that target firefly luciferase gene expression was scarcely silenced in the presence of β-cyclodextrin. The recovery of firefly luciferase gene silencing further confirmed the light-activated gene silencing ability of amantadine-caged siRNAs. Compared with the β-cyclodextrin-free group, the higher inhibition for gene silencing activity of caged siRNAs modified with amantadine was

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probably due to higher binding affinity of amantadine and β-cyclodextrin before illumination, which indicated that the host/guest interaction of amantadine/β-cyclodextrin might be useful for improving the photomodulation efficiency of caged siRNA.

In order to study the length effect of linkers on gene silencing of amantadine-modified siRNAs, two amantadine modified caged antisense strand RNAs with different length alkyl linkage were subsequently hybridized with their complementary sense strand RNA for siRNA duplexes. Similar to above study, the reporter vectors (pGL-3 and pRL-TK) together with native or caged siRNA duplexes with different linkers were cotransfected into HEK293T cells for gene silencing evaluation. As expected, the positive control groups with native siRNA targeting firefly luciferase exhibited a 10% level of gene expression (Fig.S3). Two experimental groups with 5′-terminal caged modification of siRNA (APAL1/SL and APAL2/SL) resulted in little effect on firefly luciferase activity before UV irradiation, which suggested that the modification at this position efficiently blocked the 5′ phosphate moiety and then disturbed the formation or processing of RISC. The siRNA activity was reactivated after removing the photolabile group with light irradiation for 3 min. And 4.4- and 4.7-fold enhancement of photomodulating firefly luciferase gene silencing activity were achieved for APAL1/SL and APAL2/SL, respectively (Fig S3). Thus, further cell experiments were mainly carried out using caged siRNA with the shorter linker 2.

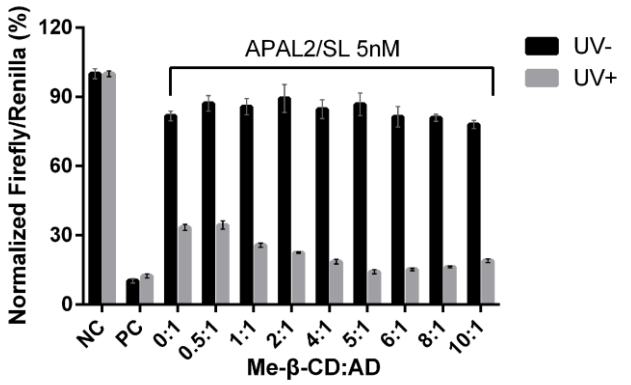

Figure 2. Photomodulation of firefly luciferase expression in siRNA duplexes mixed with nine different ratios （0:1 to 10:1） of methyl-β-cyclodextrin and amantadine-modified siRNA (5 nM) after 10 h of incubation before cotransfection

As we all know, methyl-β-cyclodextrin, a derivative of β-cyclodextrin, was previously confirmed to be an endocytosis inhibitor[19].We then investigated the real effect of β-cyclodextrin derivative on the entrance of amantadine modified caged siRNA. Methyl-β-cyclodextrin and other cellular entry inhibitors (chlorpromazine, genistein, and amiloride) were chosen for investing the uptake pathway of caged siRNA and gene silencing effect, respectively under inhibitory working concentration, as shown in Fig.S4 (Supporting Information). These inhibitors (chlorpromazine, genistein, or amiloride) had little effect on the cellular entry of amantadine-modified siRNA, whereas the internalization of the amantadine modified caged siRNA was effectively decreased by 18% in the presence of 4 μM methyl-β-cyclodextrin which was previous reported to specifically disrupt lipid rafts[20]. Methyl-β-cyclodextrin obviously decreased the uptake efficiency of amantadine-modified caged siRNA, suggesting that 4 μM methyl-β-cyclodextrin could actually bind the amantadine-modified siRNA and block lipid-raft-mediated endocytosis of amantadine-modified siRNA.

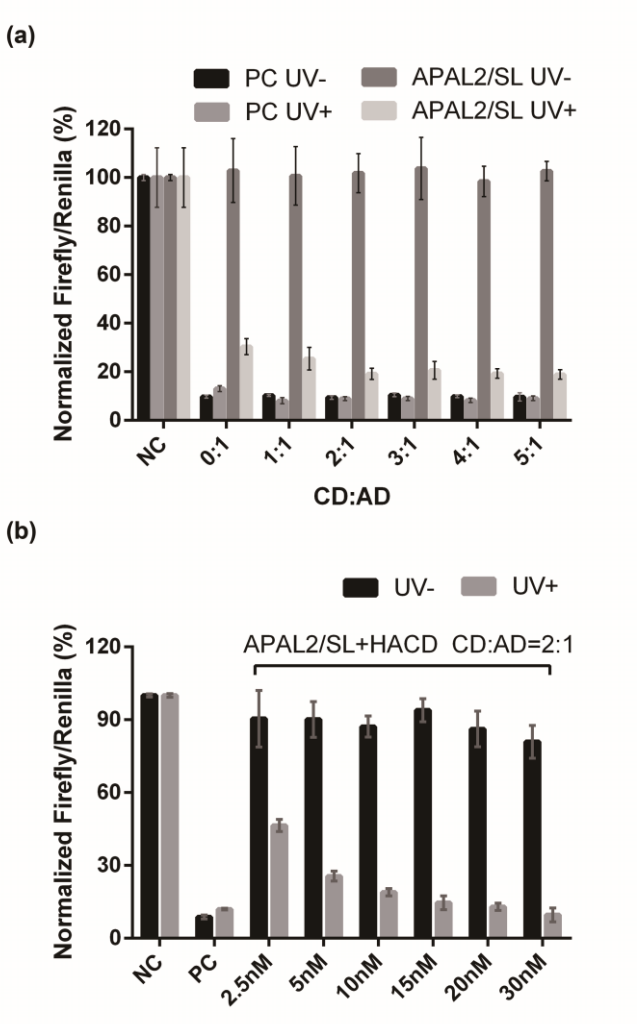

Figure 3. (a) Photomodulation of firefly luciferase activity (with Renilla luciferase as the internal control) using amantadine-modified caged siRNA (APAL2/SL) and HACD-17 at different concentrations (CD: AD from 0:1 to 5:1); AL/SL was used as a positive control (PC); the concentrations of all siRNAs were 5 nM. (b) Photomodulation of firefly luciferase activity (with Renilla luciferase as the internal control) using amantadine-modified caged siRNA (APAL2/SL) and HACD-17. The concentrations of the siRNAs were 2.5 nM, 5 nM, 10 nM, and 15 nM, and the CD to AD ratio was 2:1.

To confirm our hypothesis that a suitable ratio of methyl-β-cyclodextrin and amantadine-modified caged siRNA is essentially important for the enhancement of their cellular uptake and subsequent gene silencing, we further investigated the detailed dose effect of methyl-β-cyclodextrin on photomodulation of the target gene silencing at relative low concentrations in comparison to that used in its inhibition assay. The same dual-luciferase evaluation system with siQuant vectors was applied with firefly luciferase (pGL-3) as the target gene and renilla luciferase (pRL-TK) as the internal control. Nine different ratios of methyl-β-cyclodextrin and amantadine-modified caged siRNA (from 0:1 up

to 10:1) were selected with the amantadine-modified caged siRNA APAL2/SL at a fixed concentration (5 nM). After methyl-β-cyclodextrin and the amantadine-modified siRNA were first pre-incubated for full non-covalent interaction to form the host/guest complex, the complex was then transfected into cells for the evaluation of gene silencing of firefly luciferase. As shown in Fig.2, when the concentration of methyl-β-cyclodextrin increased from 0 to 50 nM, the light activation of gene silencing efficiency induced an obvious enhancement from 33.5% (0:1) to 14.2% (5:1) of firefly luciferase activity with the increased ratios of Me-β-CD/AD, then photomodulation efficiency slowly decreased with higher ratios of Me-β-CD/AD, although little effect of Me-β-CD/AD modified caged siRNA on gene silencing was observed before light irradiation. These results demonstrated that β-cyclodextrin moiety could assist the entrance of amantadine-modified siRNA at a low concentration and large excess of β-cyclodextrin moiety actually saturated the binding site of cell surface which reversed to the inhibition of their cellular uptake.

Recently, a series of cyclodextrin-conjugated hyaluronic acids, namely, HACD-5.6~17 (which contains 5.6~17 β-cyclodextrin per chain), have been reported to exhibit excellent siRNA delivery activity and weak side effects[21]. Here, we chose HACD-17 for amantadine binding and delivery of amantadine-modified caged siRNA and further investigated siRNA gene silencing based on the *host/guest* interaction. HEK293T cells were co-transfected with two plasmids (pGL-3 and pRL-TK), as well as siRNA (APAL2/SL and AL/SL) and HACD-17. As shown in Fig.3a, the expression level of firefly luciferase for all the APAL2/SL groups without UV irradiation was similar to that of the negative control group, displaying almost no siRNA gene silencing activity. However, upon light activation of amantadine-modified caged APAL2/SL, firefly luciferase was efficiently down-regulated in cells treated with APAL2/SL. As the ratio of CD (in HACD) to AD increased from 0 to 5, the relative expression level (30.4%, 25.4%, 19.2%, 20.6%, 19.3%, and 18.9%) of firefly luciferase decreased (at ratios of 0:1, 1:1 and 2:1) and reached a minimum when the ratio of CD to AD was over 2. This observation demonstrated that the photomodulation of amantadine-modified siRNA was enhanced with HACD addition, and a CD to AD ratio of 2 was sufficient to achieve excellent photomodulation, which was consistent with the previous results shown in Fig.1.

According to the literature, the inhibitory effect of caged siRNAs was reduced when a high concentration of caged siRNAs was applied due to unsuccessful blocking of RISC formation and/or processing, which led to the “leakage” activity before light activation of caged siRNA. [13] To investigate the leakage activity of caged siRNA based on the new strategy of *host/guest* interaction of amantadine and β-cyclodextrin, we evaluated the dose dependency of amantadine-modified caged siRNA incubated with HACD-17 under the optimized CD to AD ratio (2 to 1). HEK293T cells were cotransfected with transfection vectors, HACD-17 and six gradient concentrations of amantadine modified caged siRNAs (2.5 nM, 5.0 nM, 10 nM, 15 nM, 20 nM, and 30 nM). As shown in Fig.3b, the control siRNA effectively down-regulated the firefly luciferase activity from 46.4% (2.5 nM) to 14.6% (15 nM) as expected. The cells treated with caged siRNA duplexes without irradiation maintained approximately 90% firefly luciferase activity compared to the negative control group with siRNA concentrations from 2.5 to 30 nM. The inhibition efficiency showed no obvious decrease as the concentrations of APAL2/SL siRNA increased, which demonstrated that the leakage activity of amantadine-modified siRNAs could be inhibited through the *host/guest* interactions between amantadine and HACD. In addition, the knockdown of firefly luciferase was also enhanced upon light activation with photomodulation ratios from 1.9-fold at 2.5 nM to 7.0-fold at 15 nM. The observed effective photomodulation of RNAi efficiency using amantadine-caged modified siRNA was consistent with the specific *host/guest* interactions with HACD, which could reduce the leakage activity efficiently.

For further verification of the photomodulation of endogenous gene silencing, amantadine-modified caged siRNAs targeting the endogenous Eg5 gene with or without the addition of HACD were also evaluated. As shown in Fig.4a, cells transfected with AE/SE (PC) exhibited obvious irregular phenotypes of cell nuclei due to siRNA-induced Eg5 silencing and cell mitosis suspension as well as arrested cells with two or multiple nuclei. As expected, the normal shape of cell nuclei was observed in cells transfected with caged siRNAs (both APAE2/SE and APAE2/SE/HACD) without light activation and was similar to that of the negative control, which indicated that amantadine modification of caged siRNA could efficiently block siRNA activity. However, upon light exposure for 3 min, irregular cellular phenotypes (bi- or multinuclear cells) were clearly observed, which were similar to the phenotypes of the positive control group (AE/SE) (Fig.4a). This indicated that the amantadine-caged APAE2/SE and APAE2/SE/HACD were optochemically activated to knockdown Eg5 gene expression. In addition to nuclear phenotypes, cell phase arrest was also analysed using flow cytometry analysis (Fig.S5) and high-content analysis (Fig.6b). For cells transfected with APAE2/SE, light activation led to over 1.8-fold increase in the percentage of abnormal cell nuclei compared to cells kept in the dark, which was similar to the enhancement of the positive control group (PC, AE/SE).

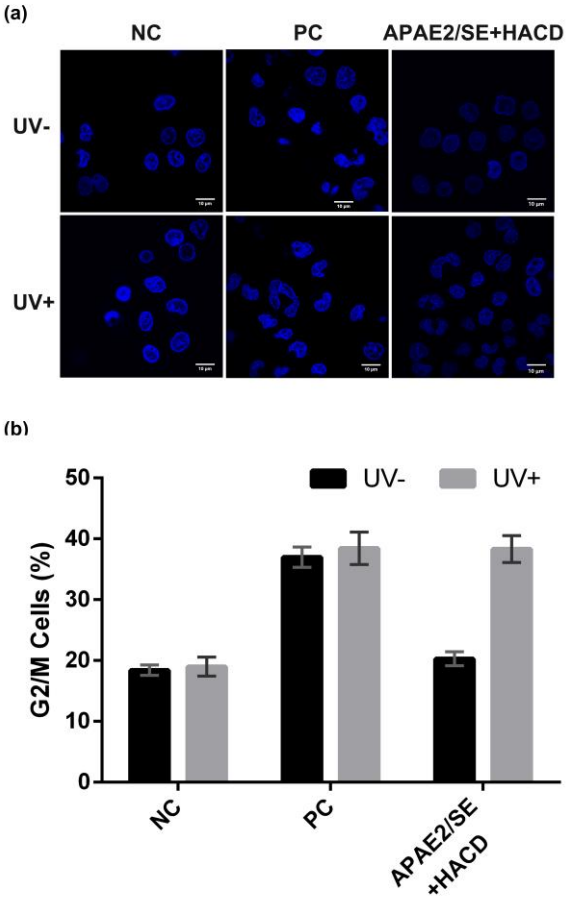

Figure 4. (a) Light activation of the Eg5 gene using NC, PC, and amantadine-modified caged siRNA (APAE2/SE, APAE2/SE+HACD) in HeLa cells. The cells were fixed and stained with Hoechst 33342 (blue). (b) DNA content of Hela cells at G2/M phase in the cell cycle after the cells were treated with AS/SE, APAE2/SE or APAE2/SE +HACD before or after light activation.

Eg5 mRNA levels under different treatments were also analysed to evaluate the photomodulation of amantadine-modified caged siRNA. The dose dependence of Eg5 siRNA activity before or after light activation was investigated. As shown in Fig.5, with the increase of siRNA concentration, caged siRNA (APAE2/SE) without HACD addition showed a decrease in Eg5 mRNA level down to 70% (at 50 nM) in comparison to the negative control even without light activation, which demonstrated the high leakage of siRNA activity with only the amantadine modification of siRNA. However, only a slight reduction in Eg5 mRNA levels was observed with the addition of HACD (the ratio of CD to AD was 2). Upon light irradiation, both Eg5 mRNA levels showed a similar concentration dependence with or without the addition of HACD, which indicated that the same active form of siRNA was recovered and showed very similar levels of gene silencing activity, from 40% at 5 nM caged APAE2/SE to 20% at 50 nM caged APAE2/SE, and photomodulation ratios was enhanced from 2.4 fold to 5.2 fold at 50 nM with the addition of HACD. These results confirmed that the *host/guest* interaction of CD/AD greatly decreased the leakage of target gene silencing activity and enhanced the photomodulation of target gene knockdown using amantadine-modified caged siRNA with the addition of HACD.

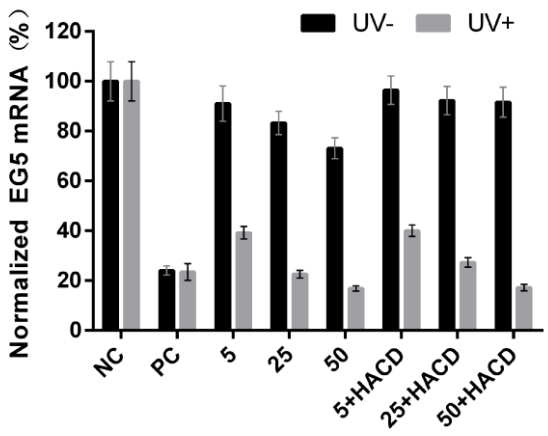

Figure 5. Relative amounts of Eg5 mRNA in HeLa cells treated with 5, 25, or 50 nM APAE2/SE with or without HACD incubation before or after light activation. GAPDH was used as an internal control.

To understand the HACD-17 enhancement of photomodulation of RNAi silencing of endogenous genes, we quantified the uptake of amantadine-modified caged siRNA hybridized with Cy3-labelled sense strand RNA. After siRNA transfection, the uptake efficiency of siRNA was quantified through analysing Cy3 fluorescence using confocal laser scanning microscopy and a cytofluorometer. The experimental groups with CD/AD ratios of 1, 2 and 5 (CD from HACD-17 and AD from APAE2/Cy3-SE) were investigated. As shown in Fig.6a, in comparison to the control group without amantadine modification, up to 50% improvement of cellular uptake was observed with CD:AD=2:1 for HACD and AD-modified cage Eg5 siRNA. These results indicated that the cellular uptake efficiency of amantadine-modified caged siRNA was enhanced based on the non-covalent *host/guest* interactions between amantadine and HACD, which suggested the great potential of *host/guest*-based delivery systems for siRNA delivery. And the delivery efficiency was not always positively correlated with the ratios of CD to AD. The transfection complex with a 2:1 ratio of CD (in HACD) to AD (in AD-modified caged siRNA) showed the highest efficiency of siRNA cellular uptake among the groups (1:1 and 5:1, CD to AD), which is consistent with the above studies. Thus, the correct ratio of CD/AD was very important for promoting the transfection efficiency of amantadine-modified siRNAs with the assistance of β-cyclodextrin derivatives. The fluorescence of NC (AE/Cy3-SE) and amantadine-modified siRNA (APAE2/Cy3-SE) with HACD-17 incubation (CD to AD 2:1) were analysed using confocal laser scanning microscopy. After 6 h of incubation, the cells were fixed and stained with Hoechst 33342 to label the cell nuclei and LysoTracker Red as a late endosome and lysosome marker. The Pearson's correlation coefficient between Cy3 fluorescence and LysoTracker Deep Red of negative control siRNA was 0.347, while the colocalization ability decreased with amantadine modified siRNA with the low Pearson's correlation coefficients (0.176 for CD to AD 2:1), which indicated the possible dissociation of amantadine and cyclodextrin after the complex was uptakenby cells.

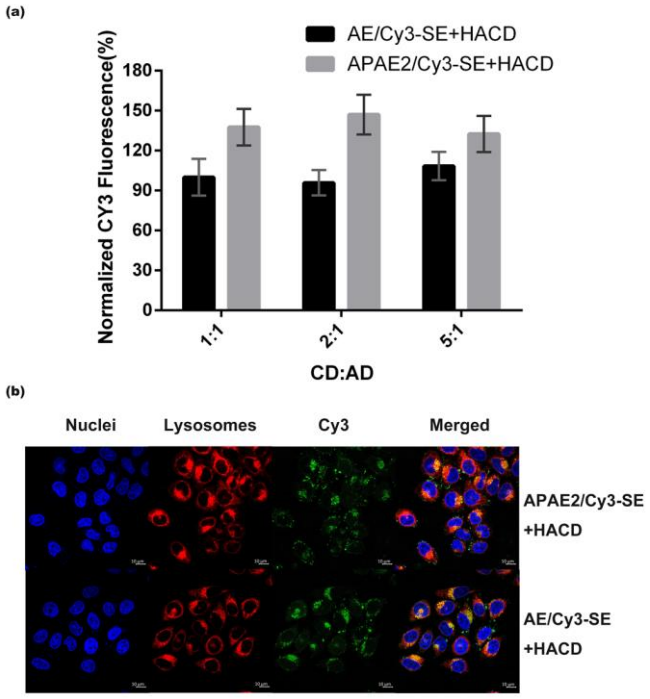

Figure 6. (a) The relative fluorescence of Cy3 labelled control siRNA (AE/Cy3-SE) and caged siRNA (APAE2/Cy3-SE) with different ratios of amantadine (AD) and HACD (CD: AD=1:1, 2:1, 5:1). (b) The fluorescence colocalization images of Cy3 labelled caged siRNA (APAE2/Cy3-SE) or control siRNA (AE/Cy3-SE) with HACD-17 (CD: AD=2:1).

Conclusion

In summary, we rationally designed and developed amantadine-modified caged siRNAs with single amantadine modification through a photolinker at the 5′ end of antisense RNA strand. The specific host/guest interaction of amantadine and β-cyclodextrin derivatives greatly enhanced the blocking effect on siRNA binding and/or processing. However, more excess of cyclodextrin (CD) actually exhibited a negative correlation with

siRNA cellular uptake and target gene silencing due to the inhibitory effect of excess amount of CD through lipid-raft-dependent endocytosis. Through optimization, the ratios of AD to CD moieties from 1:2 to 1:5 were favourable, and effective photomodulation of target gene silencing were successfully achieved under the optimal condition. With the assistance of HACD (CD conjugation on hyaluronic acid), the enhancement of cellular delivery of AD-modified caged siRNA was observed. In addition, the host/guest interaction of AD-modified siRNA with CD on hyaluronic acid further decreased the leakage activity of caged siRNA to target gene silencing before light activation. Upon light irradiation, siRNA activity was restored, and high photomodulation efficiency of both the exogenous firefly luciferase gene and endogenous Eg5 gene was achieved even at relatively high concentrations of AD-modified caged siRNA in the presence of HACD. Amantadine is not just a guest molecule but also an anti-viral and anti-Parkinson's agent[22], and the design also provided the possibility of a therapeutic combination of siRNA and small-molecule drugs. This new caging strategy for host/guest interaction provided another novel method for photoregulation of RNAi-induced gene silencing with the reduction of off-target effects due to the leakage of caged-siRNA activity.)

Experimental Section

Organic Synthesis and Characterization of photolabile linker and alkyl linkers

The photolabile phosphoramidite linker (PL) was synthesized under inert N_2 conditions with dry reagents and solvents according to our previous report[18b]. Solvents were distilled over CaH_2 before use. Silica gel column chromatography was performed on Merck silica gel. NMR spectra were recorded at 400 MHz (^{1}H) with a Bruker Avance III 400 MHz spectrometer. ^{31}P and ^{1}H NMR spectra were referenced using the external standard 85% H_3PO_4 and internal standard $(CH_3)_4Si$, respectively.

Synthesis of amantadine-modified photolabile RNA and native unmodified RNA was performed using an ABI 394 DNA/RNA synthesizer with 1 μmol scale based on standard phosphoramidite chemistry. PL and alkyl linker phosphoramidites were successively coupled at 5′ terminus of RNA strands. The terminal NHS carboxylic ester of RNA strands on the solid phase was further conjugated with 100 μmol amantadine and 50 μL of DIPEA in 1 mL DMF for 24 h at room temperature. The RNA CPG was then washed with DMF and ethanol three times to remove the residual amantadine. The modified RNAs on the solid CPG were then cleaved from CPG and deprotected in 400 μL of an AMA mixture solution containing 33% ammonium hydroxide solution and 40% methylamine ethanol solution (v/v=1) for 12 h at room temperature. After collection and concentration of RNA oligonucleotides, the obtained oligonucleotide residues were dissolved in 100 μL of anhydrous DMSO and 100 μL of triethylamine trihydrofluoride for 6 h at room temperature to remove t-butyldimethylsilyl group on 2′-hydroxyl group of each nucleotide. Then, 20 μL of 3 M sodium acetate and 300 μL of ethanol were added to the above solutions for RNA oligonucleotide precipitation. After cooling in a -80 °C freezer for 2 h, the precipitates were collected through centrifugation at 13000 rpm for 10 min at 4 °C.

RNA oligonucleotides were then purified using a Waters HPLC system (Alliance e2695, with a C18 reversed-phase HPLC column) with a flow rate of 1.0 mL/min. buffer A: 0.05 M triethylammonium bicarbonate buffer (TEAB), and buffer B: acetonitrile. Gradient B follows the procedure: 0–30% in 20 min, 30–100% in 5 min, 100% in 5 min, 100–0% in 5 min, and 0% in 5 min. Purified RNA oligonucleotides were then characterized using ESI mass analysis in negative mode (Fig S6).

PAGE gel shift assay

The mobility of amantadine-modified caged siRNAs was studied using 20% native PAGE gels to evaluate the photocleavage ability of caged siRNAs. Antisense RNA oligonucleotides (AL, APAL1) and 1.0 equivalent of the corresponding complementary sense strand (SL, APSL1) were mixed in PBS to form four siRNA duplexes (AL/SL, APAL1/SL, AL/APSL1, APAL1/APSL1). The mixtures were heated at 90 °C for 5 min and then cooled to room temperature for siRNA hybridization. Four microliters of 1 μM modified siRNA was loaded to 20% native PAGE gels to analyse the photorelease of caged siRNAs after light irradiation (365 nm, 1-5 min, 7 mW/cm2). Native siRNA and caged siRNA with no light irradiation were chosen as control. All PAGE gels were run at 100 V for 2 h and were then subjected to ChemiDoc XRS imaging after staining with SYBR Gold nucleic acid gel stain (Invitrogen) for 5 min. Gel shift assay of APAL2 and APSL2 and their duplex followed the same procedure.

Cell culture

HEK293T and HeLa cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Pan), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, Gibco). The cells were seeded into 24-well plates at a density of 1×10^5 cells/well and incubated for 24 h before transfection.

Dual-luciferase reporter assay for caged siRNA

A dual-luciferase reporter assay was chosen for evaluation of caged siRNA with firefly luciferase as the target gene and Renilla luciferase as the internal control. HEK293T cells were incubated in 24-well plates at a density of 5×10^4 cells/well and grown to approximately 80% confluence in 24 h. Complementary strands were paired to form a 10 μM corresponding siRNA duplex solution. The prepared siRNA solution was stored at -20 °C for next use. DMEM in the cell culture plate was replaced with 400 μL of OptiMEM was added per well. The transfection complex was added to the cell culture plate, and the final volume in each well was 500 μL. The concentration of the siRNA duplex (APAL1/SL) was 5 nM, and the concentration of β-cyclodextrin was 0 nM, 25 nM or 250 nM. Each set of samples was prepared in 6 multiple wells for a total of 24 wells and was then separated to 2 groups. The samples were placed in cell incubator, and after 4 hours, one group of samples were illuminated from the bottom of the plate with an LED ultraviolet lamp, while the other group of cells were

not treated. The OptiMEM medium was then replaced with DMEM containing FBS.

After being cultured in the cell incubator for another 20 hours, the cells were washed with 1×PBS once. The expression levels of firefly luciferase and renilla luciferase were examined by a Dual-Luciferase Reporter Assay System using Centro XS3 LB 960 fluorophotometer. The ratio of Firefly/Renilla luciferase was calculated, and the data were obtained. All experiments were performed in at least triplicate, and the error bars represent the standard deviations in three independent experiments.

The AD-modified siRNAs with different linkers was examined using the same above procedures. The concentration of the siRNA duplex (APAL1/SL and APAL2/SL) was 5 nM, the cells transfected with caged siRNAs were placed in the dark for 4 h, then UV illumination was introduced to irradiate cells for 3 min, the ratio of Firefly/Renilla luciferase data were examined using above method after another 20 h incubation.

Uptake inhibition analysis

The inhibitors used in these assays were genistein (37 μM, cavelin-mediated endocytosis), chlorpromazine (42 μM, clathrin-dependent endocytosis), amiloride (13 μM, mocropinocytosis) and methyl-beta-cyclodextrin (4 μM, lipid-raft-dependent endocytosis). The working dose of each inhibitor was chosen based on previous studies[18b]. Prior to incubation with the amantadine-modified caged siRNA APAL2/Cy3-SL, cells were pre-incubated with culture medium containing inhibitors to block various endocytic pathways. After 1 h of treatment with these inhibitors, the culture medium was removed and replaced with solutions containing transfection complex performed as described previously and fresh inhibitors at the same concentration for further 5-6 h incubation under a 5% CO_2 atmosphere at 37 °C. Then Cy3 fluorescence intensity was analysed with flow cytometry (BD FACSAria II).

The ratio effect on photoregulation of caged siRNA for firefly luciferase gene silencing with methyl-β-cyclodextrin derivatives

Methyl-β-cyclodextrin together with amantadine-modified cage siRNA (5 nM) with different ratios of CD to AD moieties (CD:AD=0:1, 0.5:1, 1:1, 2:1, 4:1, 5:1, 6:1, 8:1, 10:1) or control siRNA were first incubated for full complex formation before transfection. The firefly (200 ng, pGL-3) and Renilla (50 ng, pRL-TK) reporter plasmids together with the corresponding control siRNA or caged siRNAs were co-transfected to cells using Lipofectamine 2000 in OptiMEM medium, and the final concentrations of both the control siRNA and amantadine-modified caged siRNA were fixed at 5 nM. After 4 h of incubation, the wells in 24-well plates with transfected HEK293T cells were separated to two groups. One group was kept in the dark, while the other group was irradiated with UV light (365 nm, 7 mW/cm^2) for 3 min to remove the photolabile linkers for the recovery of gene silencing activity of caged siRNAs. The cells were washed twice with 1× PBS, and fresh DMEM was then added for cell culture. After 20 h of incubation, firefly luciferase activity was evaluated using a high-sensitivity microplate luminometer (Centro XS3 LB 960, Berthold Technologies) and the Dual-Luciferase Reporter Assay System (Promega) according to the standard protocol. Firefly luciferase activity was normalized to Renilla luciferase activity for each condition. All experiments were performed in at least triplicate, and the error bars represent the standard deviations in three independent experiments.

HACD-17 was dissolved in RNase-free water to form a 1 μM HACD-17 solution, meanwhile the concentrations of CD residues (in HACD) were 17 μM. HACD-17 was then added to siRNAs (APAL2/SL, AL/SL) solutions to form transfection complexes, the final concentration of the siRNA duplexes were 5 nM, and the concentrations of CD residues (in HACD) were 0, 5, 10, 15, 20, or 25 nM, meanwhile the ratios of CD to AD were from 0:1 to 5:1. In the dose effect of HACD experiment, The concentration of siRNAs was 2.5 nM, 5 nM, 10 nM, 15 nM, 20 nM or 30 nM, and the CD to AD ratio was fixed to 2:1 during the dose-effect experiment. The ratio of Firefly/Renilla luciferase data with or without UV irradiation were obtained using similar procedures described previously.

Phenotype of Eg5 gene silencing

HeLa cells were incubated in 35-mm laser confocal plates at a density of 2× 10^5/well and grown to approximately 50% confluence in 24 h. Then, cells in each plate were transfected with natural siRNA or amantadine-modified caged siRNA with the assistance of Lipofectamine 2000. After 6 h of incubation at 37 °C, culture plates were divided into two groups. One group was kept in the dark, and the other was irradiated (3 min, 365 nm). After replacement of the medium, the cells were incubated for an additional 42 h at 37 °C. All the cells were fixed with 3.8% formaldehyde and stained with Hoechst 33342 (Sigma) for 30 min. Then, the medium was replaced with 1× PBS. Laser scanning confocal microscope (Nikon, A1R) was used to image cell nuclei at an excitation wavelength of 405 nm.

Real-time quantitative polymerase chain reaction (RT-qPCR) of Eg5

HeLa cells were incubated in 12-well plates at a density of 1×10^5 cells/well and grown to approximately 50% confluence within 24 h. After the similar experimental processing for cells treated with control siRNA and caged siRNA before and after light irradiation , all cells were then collected and their total RNAs were extracted using Trizol reagent (Invitrogen). Eg5 cDNAs were then reverse-transcribed with HiScript II Q RT SuperMix for PCR (Vazyme Biotech). GoTaq qPCR Master Mix (Promega) was used for real-time fluorescence quantitative PCR. The threshold cycles of each sample were normalized to the GAPDH housekeeping gene. The following primer sequences were used: Eg5 forward primer 5′-CAGCTGAAAAGGAAACAGCC, Eg5 reverse primer 5′-ATGAACAATCCACACCAGCA, GAPDH forward primer 5′-TGCACCACCAACTGCTTAGC, and GAPDH reverse primer 5′-GGCATGGACTGTGGTCATGAG.

Cell culture

HeLa cells were treated according to the same procedure as that used for phenotype experiment. After a total 48-h incubation at 37 °C, all cells were digested with trypsin-EDTA and collected

through centrifugation (2,000×g, 5 min). Then, the cells were washed once with 1× PBS and fixed in cold 70% ethanol at 4 °C for 24 h. The fixed cells were collected by centrifugation (2,000×g, 5 min), followed by resuspension and washing with cold 1× PBS buffer. Finally, the cells were incubated with propidium iodide and RNase A solution at 37 °C for 30 min in the dark and were analysed with flow cytometry (BD FACSAria II). The percentage of cells in the G2/M phase of the cell cycle was calculated using ModFitLT 5.0 software.

Subcellular localization analysis by confocal microscopy

A Zeiss LSM880 laser scanning confocal microscope was used to examine the intracellular distribution of amantadine-modified caged siRNA in living cells. All images were obtained using a 100× oil lens and a charge-coupled device (CCD) camera. Hoechst 33342, Cy3 and LysoTracker Deep Red were excited at 405 nm, 561 nm, and 633 nm, respectively. All images were analysed using ZEISS ZEN Microscope Software.

Native siRNA (AE/Cy3-SE) and amantadine-modified siRNA (APAE2/Cy3-SE) with Cy3-labelled sense-strand RNA were used for the localization study. These duplexes were transfected into the cells using Lipofectamine 2000 or HACD under the same conditions. After 4 h incubation, the medium was replaced with fresh DMEM. After an additional 2h of incubation, the transfected cells were stained with LysoTracker Deep Red and hoechst 33342 for further imaged. Colocalization of AE/Cy3-SE or APAE2/ Cy3-SE with LysoTracker Deep Red was investigated via fluorescence confocal microscopy.

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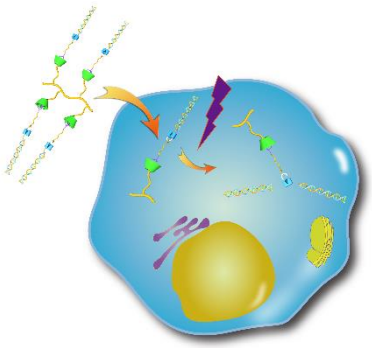

A new type of caging strategy with host/guest interaction was developed using amantadine modified caged siRNAs. The host/guest interaction of amantadine and β-cyclodextrin derivatives greatly enhanced the blocking effect of RISC formation and/or processing. Photoregulation of both exogenous and endogenous gene silencing was successfully achieved with low leaking activity