Green fluorescent protein specified small interfering RNA-cross-linked iron oxide nanoparticles-Cy5.5

siGFP-CLIO-Cy5.5

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Chemical name:	Green fluorescent protein specified small interfering RNA –crosslinked iron oxide nanoparticles-Cy5.5	
Abbreviated name:	siGFP-CLIO-Cy5.5	
Synonym:	MN-NIFR-siGFP	
Agent category:	Nucleic acid, small molecule (nanoparticle)	
Target:	RNAse III	
Target category:	Enzyme	
Method of detection:	Magnetic resonance imaging (MRI), near-infrared (NIFR) optical imaging	
Source of signal/contrast:	Iron oxides, Cy5.5	
Activation:	No	
Studies:	• *In vitro* • Rodents	No structure is Currently available in PubChem.

Background

[PubMed]

Ribonucleic acid interference (RNAi) modulates intracellular activation *via* the use of small interfering RNA (siRNA) (1). RNAi suppresses gene expression through degradation of a specific, targeted mRNA, which leads to gene silencing. siRNA is a 21-23 nucleotide (nt) double-stranded RNA (dsRNA) with symmetric 2-3nt 3' overhangs and 5'-

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phosphate and 3'-hydroxyl groups so that it is recognized by an RNAse III enzyme (2). In general, intracellular siRNA undergoes 5'-phosphorylation to unwind the RNA duplex, followed by association with RNA-induced silencing complex (RISC) (1). Then the activated RISC and the unwound anti-sense strand of the siRNA interact with the mRNA target to generate single site-specific cleavage at the mRNA target. The efficiency of gene silencing primarily relies on the optimal incorporation of siRNA into RISC and its stability in RISC, as well as a perfect complementary base pairing with the mRNA target (3). Mismatches can abolish the target degradation, the mRNA cleavage, and the RISC turnover. The high specificity in gene silencing makes siRNA a popular research tool for various gene-inactivation studies such as differentiation, apoptosis, and tumorigenesis (3). siRNA is also used in therapeutic applications to identify drug targets and to characterize gene functions *in vivo* without the use of gene knockout mice (4). Formulation of siRNAs with compounds to promote transit across cell membranes is being developed to address the major challenge of cellular delivery of siRNAs (5). Several imaging modalities have been used for localized *in vivo* delivery of siRNA (6, 7).

Green fluorescence protein (GFP) is a reporter protein of 238 amino acids, and it emits a bright green fluorescence (λ_{max} = 509 nm) when illuminated with a blue light (λ_{max} = 395 nm) (8). Red fluorescent protein (RFP; excitation = 558 nm, emission = 583 nm) is another reporter protein of 28 kDa that shares ~25% sequence identity with GFP (9). GFP has a cylinder-like structure composed of eleven β-sheets slightly twisted around the central axis and a tripeptide (serine^{65}-tyrosine^{66}-glycine^{67}) fluorophore attaching to the α-helix in the cylinder center (9, 10). As a tag or indicator, GFP is widely used to detect gene expression, protein trafficking, and cellular localization (11). Its fluorescence directly reflects the levels of gene expression or locations in subcellular compartments. Because GFP has no inherent localization of its own, fusion of GFP with functional proteins will typically result in the subcellular distribution pattern of the target protein (9). Thus, the fusion of GFP with host proteins is used to separate subcellular compartments in various cell organelles, including plasma membrane nucleus, endoplasmic reticulum, Golgi apparatus, secretary vesicles, mitochondria, peroxisomes, and phagosomes (12). The GFP/RFP reporter gene can be incorporated into recombinant plasmids, where it is directly linked to the transcriptional regulatory elements (11). Transfection of such recombinant plasmids into cells of interest allows for evaluation of promoter and enhancer activity. In combination with the RNAi technique, a GFP-specified siRNA (siGFP) can be used to generate animal models that are directly detectable with an optical microscope without the need for additional histological staining (13). For instance, 9L gliosarcoma cells can be transfected with the pcDNA3 (phGFP-S65T)-GFP/RFP plasmid to generate 9L-GFP/RFP glioma cells (13).

GFP-specified siRNA-crosslinked iron oxide nanoparticles (CLIO)-Cy5.5 (siGFP-CLIO-Cy5.5) is a magnetofluorescent nanoparticle used for multimodal imaging of the delivery and silencing of siGFP in tumors (6). This agent consists of four components: five siGFPs (22nt) to target GFP mRNA, three fluorescence probes (Cy5.5) for optical imaging, four myristoylated polyarginine peptides (MPAP) for mediating transportation to the cytoplasm, and an iron oxide nanoparticle core for enhancing magnetic resonance

imaging (MRI) contrast and delivering siGFP to tumors. The siGFP is linked to magnetic nanoparticles by a stable thioether bond without compromising silencing efficiency. Cy5.5 is a cyanine dye consisting of two quaternized heteroaromatic bases (A and A') joined by a polymethine chain with five carbons (14), which binds directly to the nanoparticles. This dye possesses high quantum yield, good chemical stability, easy conjugation, and high sensitivity (mole extinction coefficient, ~250,000 mol/cm) (15, 16). As a membrane translocation module, MPAP has a hydrophobic 14-carbon moiety of myristic acid (Myr, -C(=O)-$(CH_2)_{12}$-CH_3) linked to a polyarginine peptide to generate Myr-Ala-$(Arg)_7$-Cys-$CONH_2$ (17). MPAP can cross the cellular membrane of live cells efficiently and target the cytoplasm without registered toxicity (17). The nanoparticle contains an icosahedral core of superparamagnetic crystalline Fe_3O_4 (magnetite) that is caged by epichlorohydrin cross-linked dextran and functionalized with amine groups (CLIO-NH_2) (18). They have a high magnetic susceptibility to induce a significant magnetization inside a magnetic field. This creates microscopic field gradients that diphase nearby protons and causes a shortening of T_2 relaxation times (19). Enhanced permeability and retention effects in tumors and an increased fluid-phase endocytosis in tumor cells results in the accumulation of magnetic nanoparticles in the tumors (6). With the assistance of MPAP, sufficient siGFP can be delivered to the tumors. siGFP-CLIO-Cy5.5 allows for fine resolution (10–100 μm) and unlimited depth penetration of MRI with the high sensitivity (10^{-9}–10^{-17} mol/L) and the short acquisition times of optical imaging (6).

Synthesis

[PubMed]

The synthesis of siGFP-CLIO-Cy5.5 was conducted in multiple steps (6). Initially, monocrystalline iron oxide (MION) was synthesized by neutralization of ferrous salts, ferric salts, and dextran with ammonium hydroxide, followed by ultrafiltration (20). The obtained MION was cross-linked in strong base with epichlorohydrin and then reacted with ammonia to produce CLIO-NH_2. Next, *N*-hydroxysuccinimide ester of Cy5.5 was reacted with the CLIO-NH_2 (21). The produced CLIO-Cy5.5 was conjugated with a heterobifunctional cross-linker, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), *via N*-hydroxy succinimide ester. Then, Myr-Ala-$(ARg)_7$-Cys-$CONH_2$ MPAPs were attached to this linker *via* a sulfhydryl reactive pyridyl disulfide residue (pH 7). The produced CLIO(MPAP)-Cy5.5 was coupled with a m-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) cross-linker (pH 8.5). At the same time, commercial GFP siRNA duplex, a 22nt siRNA duplex directed against the sequence 5'-GCA AGC TGA CCC TGA AGT TC-3' at nucleotides 122–141 of phGFP-S65T, was modified with a thiol moiety *via* a hexyl spacer (5'-S-S-$(CH_2)_6$-) for bioconjugation. Finally, the free thiol single-stranded RNA was reacted with the CLIO(MPAP)-Cy5.5 *via* the MBS cross-linker to produce siGFP-CLIO-Cy5.5. For the biodistribution studies, the MPAP peptide on the siGFP-CLIO-Cy5.5 was modified to contain an additional tyrosine at its carboxyl terminus for exchange with ^{125}I (^{125}I-labeled siGFP-CLIO-Cy5.5). The analysis results

demonstrated that on average there were three Cy5.5, four MPAP, and five siGFP molecules per paramagnetic nanoparticles.

In Vitro Studies: Testing in Cells and Tissues

[PubMed]

The uptake and silencing efficiency of siGFP-CLIO-Cy5.5 were measured in 9L-GFP (GFP-S65T) gliosarcoma cells with 9L-RFP (DsRed2) gliosarcoma cells as the control (6). The cells were incubated with siGFP-CLIO-Cy5.5 at different concentrations. The Cy5.5 fluorescence measured at 48 h after injection exhibited a linear dependence on the concentration of siGFP-CLIO-Cy5.5, which reflected increased uptake of siGFP-CLIO-Cy5.5 by the cells. The presence of MPAP membrane translocation peptides appeared to enhance cellular uptake of siGFP-CLIO-Cy5.5 substantially. The silencing efficiency in 9L-GFP cells was concentration-dependent, and the decrease in GFP fluorescence became significant at 48 h. In comparison, the fluorescence in 9L-RFP cells remained nearly the same as preincubation levels. This result was further confirmed with confocal microscopy, in which extensive cytoplasmic Cy5.5 fluorescence was found in both 9L-GFP cells and 9L-RFP cells after 48 h of incubation. siGFP-CLIO-Cy5.5 was found to be located in the perinuclear region as a result of sequestration by the RNA-induced silencing complex (RISC).

Animal Studies

Rodents

[PubMed]

Biodistribution studies of siGFP-CLIO-Cy5.5 were conducted in mice (6). ^{125}I-Labeled siGFP-CLIO-Cy5.5 at a dose of 10 mg Fe/kg (440 nmol siRNA/kg) was intravenously injected into mice (n = 3) that were bilaterally implanted with 9L-GFP and 9L-RFP tumors. Mice were euthanized 24 h after injection, and tissues were harvested for gamma counting. The distribution (percent injected dose (% ID)/g tissue) of ^{125}I was 4.5 in the liver, 0.45 in the spleen, 0.22 in blood, 0.2 in 9L-GFP tumors, and 0.21 in 9L-RFP tumors. This was consistent with the accumulation and metabolic process found for iron oxide nanoparticles.

Medarova et al. studied the *in vivo* effect of siGFP-CLIO-Cy5.5 in tumors (6). Mice (n = 5) were bilaterally implanted with 9L-GFP and 9L-RFP tumors. The delivery of siGFP-CLIO-Cy5.5 was examined with MRI at 4.7 T; T_2-weighted imaging was performed before and 24 h after injection of siGFP-CLIO-Cy5.5 at a dose of 10 mg Fe/kg (440 nmol siRNA/kg). The tumor appeared bright before injection and darkened significantly after injection. The delivery of siGFP-CLIO-Cy5.5 to the tumors was further confirmed *ex vivo* with MRI at 14 T and with near-infrared optical imaging measurement of Cy5.5 signal. The silencing efficiency of siGFP-CLIO-Cy5.5 was measured with *in vivo* optical imaging in the GFP and RFP channels before injection and 48 h after injection. GFP fluorescent signal in the

9L-GFP tumors was decreased 48 h after injection. The silencing effect was further confirmed with *ex vivo* optical imaging and confocal microscopy in the excised tumors. The confocal microscopic images of frozen tumor sections demonstrated that siGFP-CLIO-Cy5.5 accumulated in tumor-recruited macrophages. Quantitative reverse-transcription polymerase chain reaction was used to analyze the levels of *Gfp* mRNA in tumors. The level for mice treated with siGFP-CLIO-Cy5.5 was found to be 85 ± 2% lower than for mice treated with saline; these levels were 97 ± 1% lower than mice treated with mismatch siRNA.

Medarova et al. also examined the immunostimulatory and cytotoxic effects of siGFP-CLIO-Cy5.5 (6). No significant increase in the levels of serum interferon-α or inflammatory cytokines (interleukin-6) was found in mice treated with siGFP-CLIO-Cy5.5 compared with the levels of the parental unmodified magnetic nanoparticle (CLIO) or to non-treated controls. No elevated toxicity of siGFP-CLIO-Cy5.5 was found in treated mice compared to the controls by evaluation of serum aspartate aminotransferase and alanine aminotransferase levels. The apoptosis in tumors treated with siGFP-CLIO-Cy5.5 remained at the same levels as in tumors treated with CLIO.

Other Non-Primate Mammals

[PubMed]

No publication is currently available.

Non-Human Primates

[PubMed]

No publication is currently available.

Human Studies

[PubMed]

No publication is currently available.

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