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Impact of delivery systems on siRNA immune activation and RNA interference

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ABSTRACT

Small interfering RNAs (siRNAs) induce robust degradation of homologous mRNAs. Highly specific silencing of target genes makes siRNA an interesting tool in drug development. However, several non-specific effects complicate the use of RNA interference (RNAi). One of the most prevalent unspecific effects is triggering the innate immune system in mammals. In parallel, activating the immune system may open the possibility to develop dual siRNAs for treatment of a variety of diseases including cancer. Here, we show that the best use of unmodified siRNAs for RNAi and immune activation depends on the delivery system, formulation condition, sequence and siRNA design concerning ORN motifs. Testing several commercial delivery systems identified that the optimal siRNAs for dual functions should contain TLR7/8 ORN motifs at least in the antisense strand and be delivered by either Dharmafect or HiPerfect. Superfect delivery system only activates TLR7 and opens new capabilities in RNAi and immune activation.

1. Introduction

RNA interference (RNAi) is a conserved mechanism in plants and animals to block gene expression by sequence-specific degradation of mRNA. RNAi results in the formation of small interfering RNA (siRNA) duplexes. They consist of 21–28 nucleotides in length with 3′-overhangs of two nucleotides at either terminus or are blunt ended [1–5]. The siRNA is cleaved by Dicer into 21–25 nucleotides long molecules and processed into the RNA-induced silencing complex (RISC) [6,7]. This protein helicases cleave the target mRNA complementary to the antisense strand of the siRNA. In the literature different synthetic siRNA designs were described: beside the classical siRNA design with blunt ended 21 nucleotides or with two UU or dTdT nucleotides as 3′-overhangs, siRNA duplexes with a length of 25–30 bases were described (so called Dicer design). These longer Dicer siRNAs duplexes resulted in a 100-fold increase in potency [1,2,4,8].

Several non-specific effects may complicate the use of RNAi. One of the most prevalent unspecific effects is the activation of the innate immune system. Beside endosomal pathways for immune activation such as with toll-like receptors 3, 7 or 8 (TLR3, TLR7, TLR8), also cytoplasmatic pathways via RIG-I (retinoic acid-inducible gene-I), Mda-5 (melanoma-differentiation-associated gene 5) or PKR (protein kinase R) are discussed in the literature to play a key role for siRNA-mediated immune activation. Studies demonstrated that synthetic unmodified siRNAs activate the mammalian immune response. The presence of specific TLR7 and TLR8 RNA motifs in chemically synthesized siRNAs has been shown to induce cytokine and chemokine immune responses. The siRNA-induced immune activation was proposed to depend on TLR7 and 8, which are previously defined as receptors for single-stranded RNA (ssRNA) [9–16]. Here, the ssRNA and siRNA mediated TLR7 and TLR8 immune activation was shown to be sequence-dependent and to require endosomal localization [17]. Beside TLR7 and TLR8 immune activation, binding of double stranded RNA (dsRNA) >25 bp to RIG-I was demonstrated by biochemical and cell based studies [18,19]. In addition, delivery reagents can alter the stimulatory potential of ssRNAs or siRNAs: cytosolic delivery by Lipofectamine, for example, is required to activate RIG-I, whereas endosomal delivery leads to TLR7- and/or TLR8-dependent immune responses [20–25].

In this study we demonstrate that the specific commercial available delivery systems and especially the complexation conditions such as the N/P ratio influence the potency (EC50) and maximal cytokine release of siRNA-mediated immune effects, whereas incomplete double-strand formation does not affect the overall immune response. Using optimal formulation conditions, the immune response induced by unmodified siRNAs is sequence-dependent but not siRNA design- or length-dependent. The target cell selectivity and diversity of TLR7 and TLR8 is a useful tool to differentiate *in vitro* on primary immune cells between potential TLR7, TLR7/8 and TLR8 effects [11,23,25–27]. We show that TLR7/8 and TLR8 RNA motifs described for ssRNAs [11,23–25] present both or alone in siRNA duplexes induce TLR7 and/or TLR8 motif-dependent type I interferon and TNF-α immune effects. In addition, we could

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identify three commercially available delivery systems inducing both: RNAi and immune response via TLR7 and/or TLR8. Although such immune stimulatory siRNAs reveal "unwanted" side effects, a recent study could show dual anti-tumor effects in cancer therapy by combining RNAi and TLR7/8 activation [28].

2. Material and methods

2.1. Reagents

ORNs and siRNAs were provided by Coley Pharmaceutical GmbH (Düsseldorf, Germany). All oligonucleotides were controlled for identity and purity by Coley Pharmaceutical GmbH and had undetectable endotoxin levels (<0.1 EU/ml) measured by the Limulus assay (BioWhittaker, Verviers, Belgium). Compounds were suspended in sterile DNAse- and RNAse-free water (Life Technologies, Eggenstein, Germany) and were stored and handled under aseptic conditions to prevent contamination. Sequences are listed in Table 1. DOTAP (Roche Mannheim, Germany), ESCORT (Sigma–Aldrich, Munich, Germany), Lipofectamine (Invitrogen GmbH, Darmstadt, Germany), Dharmafect (Thermo Fisher Scientific Epsom, United Kingdom), HiPerfect (Qiagen Hilden, Germany), JetPEI (PolyPlus Transfection Illkirch, France), polyethylenimine/branched (Polyscience, Warrington, United States), polyethylinmine/linear (Polyscience, Warrington, United States), poly-L-lysine (Sigma–Aldrich, Munich, Germany), PAMAM (Dendritech, Midland, United States) and Superfect (Qiagen, Hilden, Germany) were solved – if necessary – according to manufacturer's protocols. Delivery reagents are listed in Table 2.

2.2. Peripheral blood mononuclear cells

Peripheral blood mononuclear cell (PBMC) preparations from healthy male and female human donors were obtained from the Institute for Hemostaseology and Transfusion Medicine of the University of Düsseldorf (Germany). PBMCs were purified by centrifugation over Ficoll-Hypaque (Sigma–Aldrich, Munich, Germany). Purified PBMCs were washed twice with 1× PBS and resuspended in RPMI 1640 culture medium supplemented with 5% (v/v) heat inactivated human AB serum (BioWhittaker, Belgium) or 10% (v/v) heat inactivated FCS, 1.5 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Sigma–Aldrich, Munich, Germany).

2.3. Cytokine and chemokine production

Freshly isolated PBMCs were resuspended at a concentration of 3×10^6/ml to 5×10^6/ml and added to 96-well round-bottomed plates (200 μl/well), which had previously received nothing or siRNA or ORN complexed to the indicated formulation or the formulation alone. Cells were cultured in a humidified incubator at 37 °C for the indicated time points. Culture supernatants (SN) were collected and, if not used immediately, were frozen at −20 °C until required. Amounts of cytokines in the SN were assessed using commercially available ELISA kits (TNF-α, Diaclone; Germany) or in-house ELISAs (IFN-α) developed using commercially available antibodies (from BD Pharmingen, Heidelberg, Germany or PBL, New Brunswick, NJ, respectively).

2.4. Reporter assay

Human embryonic-kidney cells (HEK293) containing a NFκB-luciferase reporter construct and expressing human TLR7 or TLR8 or without TLR expression were used as described before [29–31]. Cells were plated on 96-well plates at 1.5×10^4/well and allowed to attach overnight. The cells were subsequently incubated for 16 h with the indicated amount of ORNs complexed to DOTAP (Roche, Mannheim, Germany), DOTAP alone, small molecules, poly rI:rC or CpG ODNs and then tested for luciferase expression. Each data point was done in duplicate.

2.5. Particle size and surface charge

2 μM siRNA si-002 was mixed in 1 ml 1× PBS at different N/P (negative/positive) ratios of lipid-based (DOTAP, ESCORT,

Table 1
siRNA sequences used.

Number	Sense (5′–3′)	Antisense (5′–3′)	Length	Description
si-405	GCUGACCCUGAAGUUCAdTdT	UGAACUUCAGGGUCAGCdTdT	17 + 2	GFP 17 + 2 dTdT
si-406	GCUGACCCUGAAGUUCAUCUU	GAUGAACUUCAGGGUCAGCUU	19 + 2	GFP 19 + 2 UU
si-407	GCUGACCCUGAAGUUCAUCdTdT	GAUGAACUUCAGGGUCAGCdTdT	19 + 2	GFP 19 + 2 dTdT
si-408	AAGCUGACCCUGAAGUUCAUC	CAGAUGAACUUCAGGGUCAGCUU	21 + 0	GFP 21 + 0
si-409	AAGCUGACCCUGAAGUUCAUCUGCACC	GGUGCAGAUGAACUUCAGGGUCAGCUU	27 + 0	GFP 27 + 0
si-410	GCUGACCCUGAAGUUCAUCUGCACCACUU	GUGGUGCAGAUGAACUUCAGGGUCAGCUU	27 + 2	GFP 27 + 2 UU
si-411	GCUGACCCUGAAGUUCAUCUGCACCACdTdT	GUGGUGCAGAUGAACUUCAGGGUCAGCdTdT	27 + 2	GFP 27 + 2 dTdT
si-413	GAAGGC CAGAC GC GAAUUAUU	UAAUUCGCGUCUGGCCUUCUU	19 + 2	B-GAL 19 + 2 UU
si-414	GAAGGC CAGAC GC GAAUUAd T d T	UAAUUCGCGUCUGGCCUUCdTdT	19 + 2	B-GAL 19 + 2 dTdT
si-415	GAAGGC CAGAC GC GAAUUAUU	AAUAAUUCGCGUCUGGCCUUC	21 + 0	B-GAL 21 + 0
si-417	CUACAGGAAGGCCAGACGCGAAUUAUU	AAUAAUUCGCGUCUGGCCUUCCUGUAG	27 + 0	B-GAL 27 + 0
si-418	GGCUACAGGAAGGCCAGACGCGAAUUAUU	AAUAAUUCGCGUCUGGCCUUCCUGUAGUU	27 + 2	B-GAL 27 + 2 UU
si-419	GGCUACAGGAAGGCCAGACGCGAAUUAdTdT	AAUAAUUCGCGUCUGGCCUUCCUGUAGdTdT	27 + 2	B-GAL 27 + 2 dTdT
si-002	GCUACAUUCUGGAGACAUAUU	UAUGUCUCCAGAAUGUAGCUU	19 + 2	luc-2 19 + 2 UU
si-003	GCUACAUUCUGGAGACAUAdTdT	UAUGUCUCCAGAAUGUAGCdTdT	19 + 2	luc-2 19 + 2 dTdT
si-420	UGGCUACAUUCUGGAGACAUA	UAUGUCUCCAGAAUGUAGCCA	21 + 0	luc-2 21 + 0
si-421	GAUGGAUGGCUACAUUCUGGAGACAUA	UAUGUCUCCAGAAUGUAGCCAUCCAUC	27 + 0	luc-2 27 + 0
si-422	GAUGGAUGGCUACAUUCUGGAGACAUAUU	UAUGUCUCCAGAAUGUAGCCAUCCAUCUU	27 + 2	luc-2 27 + 2 UU
si-423	GAUGGAUGGCUACAUUCUGGAGACAUAdTdT	UAUGUCUCCAGAAUGUAGCCAUCCAUCdTdT	27 + 2	luc-2 27 + 2 dTdT
Control	CUUACGCUGAGUACUUCGAUU	UCGAAGUACUCAGCGUAAGUU	19 + 2	Control luciferase

Number	Sequence (5′–3′)	Length	Description
R-0002	UUAUUAUUAUUAUUAUUAUU	20	TLR8 ORN
R-0006	UUGUUGUUGUUGUUGUUGUU	20	TLR7/8 ORN
R-1263	GCCACCGAGCC GAAGGCAC C	20	Control ORN

All siRNA (sense, antisense and double strand) were phosphorodiester (d refers to desoxynucleotides) and all ORN contain phosphorothioate backbone. Control siRNA refers to the luciferase series and was designed from a non-coding region.