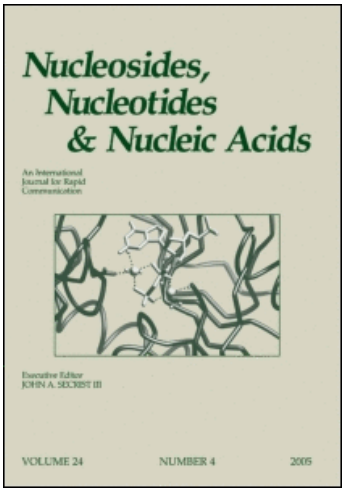


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Chemically Modified Short Interfering Hybrids (siHYBRIDS): Nanoimmunoliposome Delivery *In Vitro* and *In Vivo* for RNAi of HER-2

Richard I. Hogrefe[a]; Alexandre V. Lebedev[a]; Gerald Zon[ab]; Kathleen F. Pirollo[c]; Antonina Rait[c]; Qi Zhou[c]; Wei Yu[c]; Esther H. Chang[c]
[a] Trilink Biotechnologies Inc., San Diego, California, USA [b] Applied Biosystems, Foster City, CA [c] Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, USA

To cite this Article Hogrefe, Richard I. , Lebedev, Alexandre V. , Zon, Gerald , Pirollo, Kathleen F. , Rait, Antonina , Zhou, Qi , Yu, Wei and Chang, Esther H.(2006) 'Chemically Modified Short Interfering Hybrids (siHYBRIDS): Nanoimmunoliposome Delivery *In Vitro* and *In Vivo* for RNAi of HER-2', Nucleosides, Nucleotides and Nucleic Acids, 25: 8, 889 — 907
To link to this Article: DOI: 10.1080/15257770600793885
URL: http://dx.doi.org/10.1080/15257770600793885

Nucleosides, Nucleotides, and Nucleic Acids, 25:889–907, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770600793885

Taylor & Francis
Taylor & Francis Group

CHEMICALLY MODIFIED SHORT INTERFERING HYBRIDS (siHYBRIDS): NANOIMMUNOLIPOSOME DELIVERY *IN VITRO* AND *IN VIVO* FOR RNAi OF HER-2

Richard I. Hogrefe, Alexandre V. Lebedev, and Gerald Zon □ *Trilink Biotechnologies Inc., San Diego, California, USA*

Kathleen F. Pirollo, Antonina Rait, Qi Zhou, Wei Yu, and Esther H. Chang □ *Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, USA*

□ *A blunt-ended 19-mer short interfering hybrid (siHybrid) (**H**) comprised of sense-DNA/antisense-RNA targeting HER-2 mRNA was encapsulated in a liposomal nanoplex with anti-transferrin receptor single-chain antibody fragment (TfRscFv) as the targeting moiety for clinically relevant tumor-specific delivery.* In vitro *delivery to a human pancreatic cell line (PANC-1) was shown to exhibit sequence-specific inhibition of 48-h cell growth with an IC_{50} value of 37 nM. The inhibitory potency of this siHybrid was increased (IC_{50} value of 7.8 nM) using a homologous chemically modified siHybrid (**mH**) in which the 19-mer sense strand had the following pattern of 2′-deoxyinosine (dI) and 2′-O-methylribonucleotide (2′-OMe) residues: 5′-d(TITIT)-2′ OMe(GCGGUGGUU)-d(GICIT). These modifications were intended to favor antisense strand-mediated RNAi while mitigating possible sense strand-mediated off-target effects and RNase H-mediated cleavage of the antisense RNA strand. The presently reported immunoliposomal delivery system was successfully used* in vivo *to inhibit HER-2 expression, and thus induce apoptosis in human breast carcinoma tumors (MDA-MB-435) in mice upon repeated i.v. treatment at a dose of 3 mg/kg of **H** or **mH**. The* in vivo *potency of modified siHybrid **mH** appeared to be qualitatively greater than that of **H**, as was the case* in vitro.

Keywords Synthesis; Short interfering RNA; DNA-RNA hybrid; Chemically modified hybrid; RNAi; HER-2; Nanoimmunoliposome delivery; Pancreatic cancer cell line; PANC-1

Received 23 November 2005; accepted 4 May 2006.

We sincerely appreciate the technical skill of Trilink's Oligonucleotide Production Group for providing the compounds used in the studies reported here. The work is in part supported by a grant from the National Foundation for Cancer Research to EHC and SynerGene Therapuetics, Inc. to KFP.

Current address for Gerald Zon: Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404.

Address correspondence to Gerald Zon, Trilink Biotechnologies Inc., 9955 Mesa Rim Rd., San Diego, CA 92121. E-mail: gzon@trilinkbiotech.com

INTRODUCTION

The remarkably successful use of synthetic small interfering RNA (siRNA) for mammalian gene silencing by RNA interference (RNAi) *in vitro* has quickly led to growing interest in potential therapeutic applications of such compounds.[1,2] In common with attempts to develop therapeutic antisense oligonucleotides (AS-ONs) during the past two decades,[3] major challenges include adequate resistance of siRNAs to serum and cellular nucleases, delivery to target tissue, and uptake by cells. By analogy to what has been found using a diverse array of AS-ON structural moieties,[4] introduction of chemical modifications by synthesis of siRNA analogs has provided compounds that are markedly resistant to nucleases and exhibit improved pharmacokinetics or biodistribution.[5,6] Representative modifications to siRNA that have been investigated include 2′-*O*-methyl- (2′OMe)[7–10] and 2′-fluoro- (2′F)[6,11] ribonucleotides, phosphorothioate (PS)[9,10,12,13] linkages, deoxy nucleic acid (DNA),[9] and locked nucleic acids (LNA).[9,12,14] Not unexpectedly, however, early studies indicated that chemical modifications could adversely influence RNAi potency, depending on the type, number, and location of such modifications.[9,10] Only in rare instances (see below) were chemically modified siRNA analogs found to exhibit enhanced RNAi activity relative to corresponding unmodified siRNA. Nevertheless, structure-activity findings have shed light on the effects of siRNA modification on assembly and function of the RNA-induced silencing complex (RISC), which contains an antisense-RNA guide strand and an endonuclease that cleaves cognate mRNA target.[15]

Our investigation of synthetic analogs of siRNA was initially sparked several years ago by two independent reports[16,17] of RNAi using hybrid duplexes, which by definition herein are comprised of an unmodified antisense-RNA strand and a sense strand that was completely modified as DNA. Surprisingly, it was reported that these antisense-RNA/sense-DNA hybrid duplexes, which were called "siHybrids,"[18] were more potent[16,17] and, moreover, led to longer lasting[17] RNAi, relative to corresponding unmodified siRNA. While these exciting findings for siHybrids lead one to intriguing, if not controversial, mechanistic questions regarding RNAi vs. alternative pathways for inhibition of gene expression, we were equally struck by the following practical possibilities. In contrast to the then emerging trend for design of siRNA analogs wherein more modification is better, the promising properties of siHybrids hinted, perhaps, that less may be more. If so, this could translate into more cost-effective RNAi by virtue of using a sense strand in which relatively inexpensive DNA replaces more costly RNA having chemical modifications. More speculatively, based on molecular appearance to Toll-like receptors in the innate immune system,[19] a double-stranded RNA/DNA siHybrid might be less immunogenic than a homologous double-stranded RNA/RNA siRNA.

With these and other possibilities in mind, we embarked on exploratory studies of siHybrids that had two initial objectives. Our primary aim was to evaluate whether RNAi activity of a siHybrid could tolerate DNA sense strand modifications of the type that we postulated might mitigate RNase H-mediated cleavage of the antisense RNA strand, as well as abrogate previously reported[20] sense strand-mediated off-target effects, whether such effects occur through RISC and/or conventional antisense mechanisms. In view of the critical need for efficient delivery of RNAi agents to target tissue, our secondary aim was to determine whether an active siHybrid could be delivered into cells using clinically relevant, targeted liposomes, rather than commonly employed *in vitro* transfection agents[21] that have very limited, if any, therapeutic utility.[22] To address these objectives, we took advantage of reported[23] siRNA-mediated silencing of the HER-2 proto-oncogene, and successful use of immunoliposomal nanoplexes for tumor-targeted delivery of HER-2 AS-ONs both *in vitro* and mouse models of cancer.[24–26] Here, we report that a liposomal nanoplex with anti-transferrin receptor single-chain antibody fragment (TfRscFv) as the targeting moiety delivers an anti-HER-2 DNA/RNA siHybrid having greater potency than similarly delivered corresponding RNA/RNA siRNA. Moreover, this sequence-specific silencing of HER-2 is enhanced in novel siHybrid analogs that are modified with centrally located 2′-OMe moieties, which are intended to block possible RNase H-mediated sense strand off-target cleavage. These novel siHybrid analogs are additionally modified by flanking DNA that contains 2′-deoxyinosine residues, which are intended to further mitigate sense strand off-target cleavage by functioning as sequence diluents. Unbeknownst to us at the time, another potential advantage of these sense strand modifications is abrogation of immunogenic side effects of siRNAs.[11,27] We have reported elsewhere extensive biological and microscopic evidence that demonstrates tumor-targeted delivery and uptake of these novel formulations of modified siHybrid *in vitro* and *in vivo*[28] as a means of investigating the efficacy of these compounds in established mouse models of human cancers.

MATERIALS AND METHODS

Synthetic Oligonucleotides

The nucleotide sequences of the oligonucleotides used in this study are shown in Table 1. The oligonucleotides were chemically synthesized using commercial phosphoramidites (Glen Research, Sterling, VA and Pierce Chemical, Rockland, IL) and ethyl thiotetrazole (AIC) on an 8909 Expedite synthesizer (Applied Biosystems, Foster City, CA) at a 15-μmol scale following manufacturers′ recommended protocols. After standard deprotection procedures, the DNA and mixed DNA/2′OMe/DNA oligonucleotides were purified by reverse-phase HPLC. The RNA oligonucleotides

TABLE 1 Sequences of 19-mer Anti-HER-2 and Control HER-2 Cognate Oligonucleotides[a]

No.	Name	Abbreviation	5′ → 3′ Sense (top) 3′ → 5′ Antisense (bottom)
1	HER-2 duplex 3	**D**	UCUCUGCGGUGGUUGGCAU AGAGACGCCACCAACCGUA
2	HER-2 hybrid 3	**H**	TCTCTGCGGTGGTTGGCAT AGAGACGCCACCAACCGUA
3	HER-2 modified hybrid 3	**mH**	TITITgcggugguuGICIT AGAGACGCCACCAACCGUA
4	Control HER-2 hybrid	**CH**	TTCTCCGAACGTGTCACGT AAGAGGCUUGCACUGAGCA
5	Control HER-2 modified hybrid	**CmH**	TICICcgaacguguCICIT AAGAGGCUUGCACAGUGCA

[a]RNA = capital letters in normal font; DNA = capital letters in bold font; 2′OMe = lowercase letters in normal font.

were deprotected and desilylated using standard procedures, desalted using LH-20 columns (Amersham Biosciences), and then purified by preparative PAGE. All oligonucleotides were precipitated from ethanol as sodium salts and quantified by conventional UV_{260} calculations. Purity of the oligonucleotides was determined by analytical PAGE and HPLC analyse and was estimated to be >90–95% in all cases. Identity of the oligonucleotides was confirmed by mass spectrometry (HT Labs, San Diego). All oligonucleotides were synthesized with 5′-hydroxyl groups, except when stated otherwise.

Thermal Melting (T_m) Measurements

T_m measurements were performed on a Beckman DU640B Spectrophotometer equipped with a water-jacketed UV-cell holder. A water-circulating thermostat provided linear increase of the temperature (1–2°C/min) inside the UV-cell from room temperature to ~80°C. Temperature was controlled by a ThermologR Themistor thermometer. The concentration of each oligonucleotide strand was 2.6 μM. Samples were dissolved in 10 mM sodium phosphate buffer containing 100 mM sodium chloride and 1 mM EDTA, pH 7.4. Before UV measurements, the samples were heated to 90°C for 5 min, then slowly cooled to room temperature and transferred to a 1-mL UV-cell. T_m values for the resultant duplexes were determined from the melting curve as the temperature of the maximum of the first derivative ($\Delta A/\Delta T$) vs. T, where A is absorbance as defined above and T is temperature (°C). The T_m curves and T_m values are given in Figure 1.

Preparation of 1:1 Mixtures of Cognate Oligonucleotides

A mixture of single-stranded antisense oligonucleotide (1 μmol) and its single-stranded cognate oligonucleotide (1 μmol) in water (10 mL) was

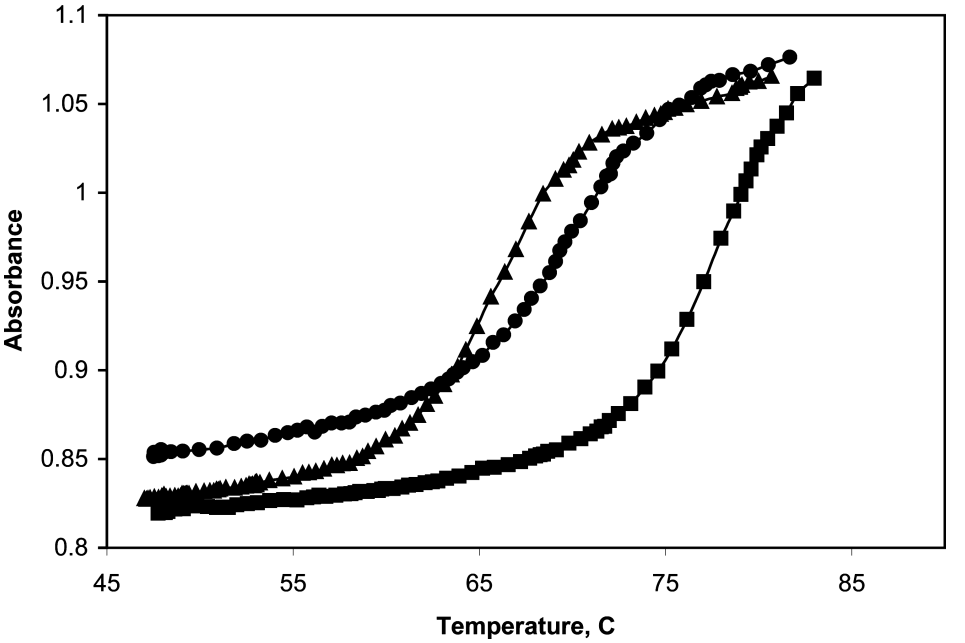


FIGURE 1 Thermal melting (T_m) curves for 19-mer anti-HER-2 cognate oligonucleotides. RNA-RNA duplex 3 (**D**), (■), 79.3 ± 0.3°C; DNA-RNA hybrid 3 (**H**), (▲), 65.7 ± 1.0°C; DNA/2′OMe/DNA-RNA, modified hybrid 3 (**mH**), (●), 69.1 ± 0.5°C. Absorbance values are presented as the difference between the analytical absorbance at 260 nm and the background absorbance at 300 nm.

prepared in a 15-mL screw-cap plastic tube. The capped tube was placed in a beaker containing 100 mL of boiling water and then allowed to slowly cool to room temperature. To ensure that each pair of oligonucleotides formed a duplex, a 5-μL aliquot of the annealed mixture was added to 15 μL of loading buffer (1 × TBE in 50% glycerol). After 10–60 min incubation at room temperature, the mixture was subjected to analytical non-denaturing PAGE together with each single strand loaded in a separate lane as a size marker. During the run the temperature of the gel was maintained below 40°C to prevent thermal melting (see Figure 1). The mixture was stored frozen at –20°C.

Liposomal Nanoplex Formulation and Optimization

Previously described[28–31] materials and procedures were used. Briefly, 1:1 molar ratios of each single-stranded antisense and cognate sense oligonucleotide were annealed. Cationic liposome (dioleoyltrimethylammonium phosphate [DOTAP] and dioleoylphosphatidylethanolamine [DOPE][Avanti Polar Lipids, Alabaster, AL]) was prepared at a 1:1 molar ratio by ethanol injection.[30] The anti-transferrin receptor single-chain antibody fragment (TfRscFv) was mixed with the liposome at the previously established ratio of 1:30 (w/w).[31] The siRNA molecules were subsequently

added to the admixture at a ratio of 1 μg siRNA to 7 nmol liposome, followed by sizing and confirmation of nanosize particle distributions of the final immunoliposome formulations by dynamic light scattering with a Malvern Zetasizer 3000 HS (Malvern, Worcestershire, UK).

In Vitro and *In Vivo* Experiments

In vitro transfections were performed as previously described.[30,31] Briefly, 4×10^3 PANC-1 cells were plated/well of a 96-well plate. After 24 h, the cells were transfected with TfRscFv-LipA complexes, prepared as described above, containing either the hybrid (**H**), control hybrid (**CH**), modified hybrid (**mH**), or control modified hybrid (**CmH**) compounds **2–5**, respectively. The concentration of siRNA analog varied from 0.4 to 250 nM. The optimized (for activity vs. toxicity) ratio of LipA to siRNA analog was 7 to 1 (nmol:μg). A conventional colorimetric cell-viability assay using 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2*H*-tetrazolium hydroxide (XTT)[32] was performed 48 h after transfection. Error bars represent triplicate measurements. All experiments were independently reproduced at least twice and provided substantially the same results (data not given).

For the *in vivo* studies, human breast carcinoma tumors were induced in female athymic nude (NCR nu/nu) mice by subcutaneous inoculation of 6×10^6 MDA-MB-Y35 cells suspended in Matrigel® collogen borement membrane (BD Biosciences, Bedford, MA).

Mice bearing tumors of at least 100 mm^3 were treated with 3 mg/kg anti-HER-2 hybrid (**H**), control hybrid (**CH**), anti-HER-2 modified hybrid (**mH**), or control modified hybrid (**CmH**) compounds **2–5**, respectively, encapsulated in TfRscFv-LipA. The complex was prepared as described above using the ratio of LipA to siRNA of 7 to 1 (nmol:μg) Treatment was by i.v. injection three times over 24 h. Mice were sacrificed 46 h after the first injection and 20 h after the last injection. Forty micrograms of total protein isolated from each tumor was electrophoretically fractionated using a Criterion Precast 4–20% gradient gel transferred to nylon membrane and then immunostained for expression levels of HER-2 (rabbit polyclonal antibody C-18; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphorylated AKT (pAKT) (mouse monoclonal antibody Ser 473; Cell Signaling Technology™, Beverly, MA), phosphorylated mitogen-activated protein kinase (pMAPK) (mouse monoclonal antibody, Thr 202/Tyr 204, E10; Cell Signaling Technology™), cleaved caspase-3 (rabbit polyclonal antibody Asp175; Cell Signaling Tecnology™), antiapoptotic protein BCL-2 (rabbit polyclonal antibody N-19; Santa Cruz Biotechnology, Inc.), and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (rabbit polyclonal antibody; Trevigen, Inc., Gaithersburg, MD).

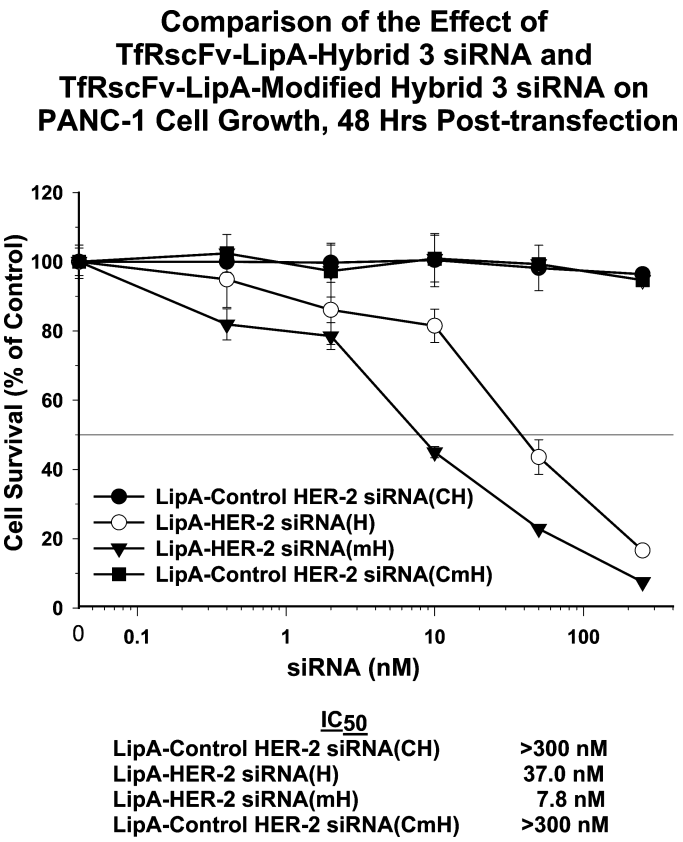
RESULTS

T_m Characterization of siRNA and siHybrids

Compound **1** shown in Table 1 is a 19-mer, blunt-ended version of an RNA/RNA duplex (**D**) that had been previously reported[23] as a 21-mer with 3′ d(TT) overhangs to have RNAi activity against HER-2. This truncated RNA/RNA duplex (**D**), compound **1**, provided a reference T_m of 79.3 ± 0.3°C for the expected melting transition from double- to single-stranded species (Figure 1). The ~14°C decrease in T_m to 65.7 ± 1.0°C found for hybrid (**H**) compound **2**, wherein the RNA sense strand of compound **1** is replaced by DNA, was consistent with the well-known generalization that DNA/RNA hybridization is less stable than RNA/RNA. The ~4°C increase in T_m to 69.1 ± 0.5°C for modified hybrid (**mH**) compound **3**, wherein the DNA sense strand in compound **2** is replaced by a chimeric "5/9/5" motif of DNA/2′OMe/DNA, was consistent with the well-known generalization that introduction of 2′OMe moieties into oligonucleotides increases T_m. Although we did not characterize the corresponding control HER-2 compounds **4** and **5**, we estimate that they have roughly comparable T_m values, relative to **2** and **3**, respectively, based on the presence of 10 vs. 11 GC-basepairs. In any case, these T_m measurements for compounds **1–3** confirmed that the shortened 19-mer RNA/RNA siRNA (**D**) and its DNA/RNA hybrid (**H**) had GC content adequate for encapsulation and intracellular delivery of largely double-stranded species, which also applied to DNA/2′OMe/DNA modified hybrid (**mH**) even though 4 dI residues were incorporated.

Immunoliposome-Mediated Delivery of siRNA Analogs *In Vitro* and *In Vivo*

As indicated by the results shown in Figure 2, treatment of PANC-1 cells with a TfRscFv-targeted immunoliposome formulation of anti-HER-2 hybrid (**H**), compound **2**, led to significant killing of this pancreatic cancer cell line. This effect was dose-dependent over the studied range of 0.4 to 250 nM and had an IC_{50} value (the dose resulting in 50% survival) of 37.0 nM. In another experiment (data not given), this hybrid (**H**) had an IC_{50} value in a similar range (68 nM), whereas compound **1**, which is the corresponding RNA/RNA duplex (**D**), gave an $IC_{50} = 100$ nM. This slightly greater potency of the hybrid (**H**) vs. duplex (**D**) composition was consistently reproduced in multiple independent experiments, as was the inactivity ($IC_{50} > 300$ nM) of control hybrid (**CH**), compound **4** (Figure 2), and control duplex (data not given). Increased potency of RNAi upon this type of sense strand RNA replacement with DNA has been previously reported.[17] However, chemical modification of sense strand DNA as embodied in modified hybrid



	IC_{50}
LipA-Control HER-2 siRNA(CH)	>300 nM
LipA-HER-2 siRNA(H)	37.0 nM
LipA-HER-2 siRNA(mH)	7.8 nM
LipA-Control HER-2 siRNA(CmH)	>300 nM

FIGURE 2 Effect of anti-HER-2 and control HER-2 hybrid and modified hybrid siRNA analogues on cell survival. 4×10^3 PANC-1 cells were plated/well of a 96-well plate. After 24 h, the cells were transfected with TfRscFv/LipA complexes containing either the anti-HER-2 hybrid (**H**), anti-HER-2 control hybrid (**CH**), anti-HER-2 modified hybrid (**mH**), or anti-HER-2 control modified hybrid (**CmH**) siRNA analogues; UT = untreated control. The concentration of siRNA analog was varied from 0.4 to 250 nM. The ratio of LipA to siRNA analog was 7 to 1 (nmol : μg). The XTT-based assay cell viability assay was performed 48 h after transfection.

(**mH**), compound **3**, afforded even significantly greater potency, namely, IC_{50} = 7.8 nM (Figure 2). Sequence specificity was supported by the fact that corresponding control modified hybrid (**CmH**), compound **5,** was inactive (IC_{50} > 300 nM). Additional control experiments (data not given) using chemically 5′-phosphorylated versions of compounds **2** and **3** led to essentially unchanged IC_{50} values.

In vivo studies employing a mouse xenograft model of human breast cancer (derived from MDA-MB-435 cells) allowed us to assess the effect of these different siRNA analogues (all directed against HER-2) on the level of expression of selected components in the HER-2 signal transduction pathway in tumors. Immunostaining of electrophoretically separated, tumor-derived proteins shown in Figure 3 indicated that, following equivalent,

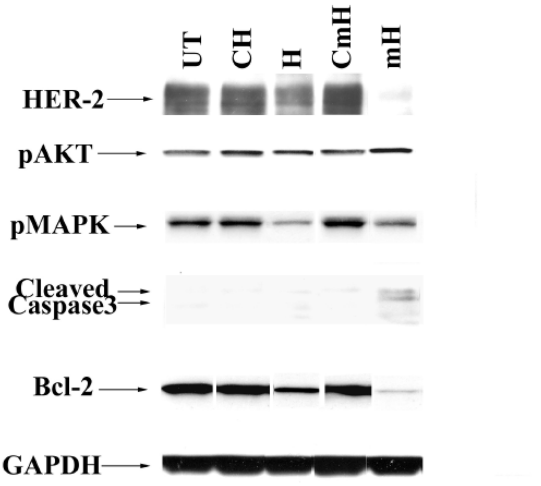


FIGURE 3 Induction of apoptosis in human MDA-MB-435 breast carcinoma tumors treated with anti-HER-2 and control HER-2 hybrid or modified hybrid. Mice bearing human MDA-MB-435 breast carcinoma tumors were treated with 3 mg/kg anti-HER-2 hybrid(**H**), anti-HER-2 control hybrid (**CH**), anti-HER-2 modified hybrid (**mH**), or anti-HER-2 control modified hybrid (**CmH**) siRNA analogs encapsulated with TfRscFv-LipA by i.v. injection three times over 24 h. **UT** = Untransfected cells. Mice were sacrificed 46 h after the first injection and 20 h after the last injection. Forty micrograms of total protein isolated from each tumor was electrophoretically fractionated using a Criterion Precast 4–20% gradient gel and then immunostained for expression levels of HER-2, phosphorylated AKT (pAKT), phosphorylated mitogen-activated protein kinase (pMAPK), cleaved caspase[3] antiapoptotic protein BCL-2, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

repeated i.v. dosing with oligonucleotides at 3 mg/kg, modified hybrid (**mH**, lane 5), compound **3**, induced greater reduction of HER-2, relative to hybrid (**H**, lane 3), compound **2**. In contrast, HER-2 levels in corresponding Controls (**CH**, lane 2 and **CmH**, lane 4), compounds **4** and **5**, respectively, were comparable to that for the untreated control (**UT**, lane 1) sample. For all of these samples, levels of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were essentially the same, indicating equal protein loading. Of the other proteins that were analyzed, phosphorylated AKT (pAKT) appeared to be largely unchanged, whereas levels of phosphorylated mitogen-activated protein kinase (pMAPK) and the antiapoptotic Bcl-2 proteins were decreased by treatment with **H** and **mH**. The presence of cleaved caspase-3, which is a hallmark of apoptosis, was

particularly evident in tumor tissue following treatment with **mH** vs. **H**, as was the reduction of Bcl-2. Consistent with **mH**-mediated RNAi of HER-2 leading to such changes in cleaved caspase-3 and Bcl-2, these proteins were essentially unchanged upon treatment with controls **CmH** or **CH** vs. no treatment. We ascribe these qualitatively different *in vivo* effects of **mH** vs. **H** on the HER-2 protein target and downstream apoptosis-related proteins to the greater RNAi-potency of **mH** vs. **H** that was initially evidenced *in vitro* by IC_{50} values associated with cancer cell viability.

DISCUSSION

HER-2 as a Target for Pancreatic Cancer, and Immunoliposomes for Delivery

Pancreatic cancer (PanCa) still has one of the highest mortality rates of all human malignancies. However, advances in delineating its genetic basis are leading to the development of new strategies for treatment based on molecular mechanisms. These approaches include modulation of genes that are overexpressed, or deficient, in PanCa. The HER-2 gene, a transmembrane tyrosine kinase with homology to the epidermal growth factor receptor (EGFR) is found to be overexpressed in 60–80% of PanCa.[33,34] Overexpression of HER-2 protein has also generally been associated with a more aggressive phenotype and poorer patient prognosis. Because of its established crucial role in signal transduction, HER-2 is an attractive target for anticancer therapies. These include the use of anti-HER-2 antibodies such as the humanized monoclonal antibody (MoAb) Herceptin that, in addition to being approved for treatment of breast cancer, is in clinical trials with PanCa.[35] Investigations have also included treatment with small-molecule kinase inhibitors; however, there are issues of non-specificity toward other kinases.[36] While monoclonal antibodies offer exquisite specificity, the use of Herceptin and other antibodies such as C225[37] are limited to tumors overexpressing HER-2 and EGFR, respectively. We have previously investigated the use of PS-modified AS-ONs targeting HER-2 mRNA in human breast cancer cells lines *in vitro and in vivo*, and in xenograph models in mice.[25,26] This earlier work led to the important observation that anti-HER-2 AS-ONs were able to sensitize breast cancer cells to various chemotherapeutic agents *in vitro* irrespective of their HER-2 status, indicating that use of AS-ONs directed against HER-2 as therapy for breast cancer is not limited to tumors overexpressing the protein. One of the main drawbacks to clinical utility of such AS-ON therapy is lack of an efficient, tumor-targeting, systemic delivery method. We have therefore been developing tumor-specific, ligand-targeted, cationic-liposome delivery systems designed for systemic gene-level therapy of cancer with AS-ONs,[24–26] which may also be applicable for other intended therapeutic agents. As a targeting ligand

in this earlier work, we took advantage of the transferin receptor (TfR), which exhibits elevated levels in various types of cancer cells. The relatively small size (~28 kDa) of the TfRscFv antibody fragment as the targeting moiety attached to a liposome (or other) nanocomplex has advantages in human use over the transferrin (Tf) (~80 kDa) molecule itself or an entire monoclonal antibody (~155 kDa). The scFv-liposome nanocomplex may thus exhibit better penetration into smaller capillaries characteristic of solid tumors. The TfRscFv also has practical advantages related to its production as a recombinant protein, as opposed to a blood product like Tf.

Sequence Selection of Oligonucleotides

The primary objective of the oligonucleotide syntheses described in this report was to determine whether siHybrids had more potent RNAi activity than conventional siRNA, as previously reported by others,[17] and whether chemically modified siHybrids, which to our knowledge had never been reported, could be designed to further improve RNAi activity. We also wished to confirm whether an active siHybrid could be delivered into cells using clinically relevant, targeted liposomes rather than commonly employed *in vitro* transfection agents[21] that have very limited, if any, therapeutic utility.[22] The biological model system and delivery methodology that we chose to use to investigate these questions were predicated on our previous findings for AS-ON-mediated inhibition of expression of HER-2 mRNA and use of immunoliposomes for targeted delivery of the AS-ON in cell culture and, more importantly, in mouse models of human cancer.[24–26] Clinical relevance of immunoliposome-based delivery of nucleic acid "cargo," in general, had been presaged by promising findings in the case of gene therapy with p53.[38]

At the outset of the presently described studies, which began several years ago, we were well aware of the potential importance of siRNA sequence selection.[39] Analysis of HER-2 mRNA by a then freely web-accessible algorithm (from Dharmacon, Chicago, IL) indicated ~60 candidate siRNA sequences to empirically evaluate. Due to resource limitations, we chose to instead take advantage of an earlier report[23] in which several conventional siRNA compounds were found to exhibit sequence-specific activity against HER-2 in breast (SKBr3 and MCF-7) and ovarian (SKOV-3) cancer cell lines. In that work,[23] delivery was achieved by use of TransIT-TKO®, which is a commercially available (Mirus Bio Corp., Madison, WI) transfection agent, and RNAi activity was measured at the level of protein by fluorescence activated cell sorting (FACS), and mRNA by real-time PCR with SYBR green detection. The least active compound (called "HER-2 construct 1"),[23] the most active compound ("HER-2 construct 3"), and an inactive compound ("Control construct") that were reported by these earlier investigators were used in our pilot studies aimed at determining whether these exact same

conventional siRNAs, all with 3′ d(TT) overhangs, would give analogous results in our cellular assay system upon delivery by immunoliposomes. Although no sequence-selection criteria were given,[23] HER-2 construct 1 and HER-2 construct 3 were stated as being targeted to HER-2 mRNA at nt 1962–1982 and 1–21, respectively. Neither of these target regions was either among, or proximate to, the ~60 target regions mentioned above, and no sequence-selection criteria were given for the control construct.[23] However, these sequence-design uncertainties were rendered moot by our very first test results that confirmed greater inhibition of cell growth by HER-2 construct 3 vs. HER-2 construct 1, and inactivity of the control construct (data not given). Based on these limited confirmatory studies, we proceeded to use HER-2 construct 3 and control construct as the basis for more detailed structure-activity studies of siHybrids and chemical modifications thereof.

Chemical Design of Oligonucleotides

The first stage of our structural design studies involved testing HER-2 duplex 3 (**D**), compound **1,** and HER-2 hybrid 3 (**H**), compound **2** (Table 1), which were blunt-ended 19-mer versions of 21-mer HER-2 construct 3[23] discussed above. Blunt-ended 19-mer RNA/RNA siRNAs were known at the time to exhibit RNAi activity;[8] however, to our knowledge, this shortening had not been previously reported for sense-DNA/antisense-RNA siHybrids. The T_m findings given in Figure 1 indicated that there was adequate hybridization for encapsulation of largely double-stranded species in immunoliposomes. As shown in Figure 2, the resultant nanosize formulations of **H** (and **D**, not shown) led to dose-dependent inhibition of survival of PANC-1 cells, the sequence specificity of which was supported by absence of significant activity for control HER-2 hybrid (**CH**), compound **4** (and its RNA/RNA counterpart; data not given).

Having thus established intersystem consistency of comparative data for earlier reported[23] lipofectin-delivered conventional siRNA against HER-2 breast cancer cells, and our immunoliposome-delivered siHybrid counterpart (**H**, compound **2**) in pancreatic cancer cells, chemical modifications were designed as follows. The antisense strand of siHybrid compound **2** would be kept as unmodified RNA, while dI residues would be introduced into the sense strand with the intention of serving as sequence diluents. In other words, the well-known ability of dI to hybridize (albeit not strictly equivalently) to all four bases in DNA or RNA in primers and probes leads to fourfold increases in the number of possible cognate target sequences per dI residue. Modified hybrid (**mH**) compound **3** (Table 1) has a total of four dIs in the 5′ and 3′ flanking regions of the sense strand, which therefore has $4^4 = 256$ possible cognate target sequences. Consequently, off-target hybridization by the sense strand of **mH**, compound **3**, is statistically diluted 256-fold in terms of the number of cognate target sequences, regardless of

whether this hybridization is mediated by RNA-induced silencing complex (RISC)[15] or not. We therefore speculated that the relative concentration of any one of these cognate sense strand/target mRNA complexes would be below a threshold value for measurable inhibitory activity, and thus mitigate sense strand off-target effects. To our knowledge, this siRNA design concept has not been reported but has some indirect support in the AS-ON literature where oligonucleotides referred to as "mixed" or "random" sequence and having all four bases at each position are used as negative controls. Our investigations of the predicted decrease in off-target effects upon incorporation of dI have been deferred to future studies that require global gene expression analysis with microarrays.[20] Such studies would ideally include comparisons of our dI/2′OMe constructs with non-dI-containing constructs that have been reported as all-DNA,[17] all-2′OMe,[40] and alternating-[41] 2′OMe compositions, which in principle may have more off-target effects due to mRNA cleavage mechanisms that are RNase H-mediated (for all-DNA) or by physical blocking, i.e., non-RNase H-mediated (for all- or alternating-2′OMe). Unbeknownst to us at the time, another potential advantage of our sense strand modifications is abrogation of immunogenic side effects of siRNAs that have been recently reported to be diminished, if not eliminated, by introduction of certain chemical modifications.[11,27] This, too, will be a subject of our future studies.

It was of more immediate interest to us at the time to investigate an additional design feature in prototypal modified hybrid (**mH**) compound **3**, namely, use of centrally located 2′OMe residues to prevent possible RNase H-mediated degradation of the antisense-RNA strand in **3**.[3] The footprint of RNase H, albeit bacterial rather than human, is sufficiently compact so as to be able to accommodate even a relatively short 12-mer DNA/RNA substrate,[42] and could thus cleave the 19-mer siHybrid DNA/RNA, compound **1**. Such RNase H-mediated degradation of an unmodified DNA/RNA siHybrid would be expected to decrease RNAi potency. Consequently, the 2′OMe-protected modified hybrid, compound **3** was anticipated to be more potent than its unmodified counterpart, siHybrid, compound **2**, assuming for the sake of simplicity that all other factors governing RNAi activity were equal. Despite the incorporation of four dIs, the T_m (Figure 1) of modifed hybrid compound **3** was actually somewhat greater than **h**ybrid compound **2**, presumably due to well-known stabilizing effects of 2′OMe moieties. We did not assess whether increased end-fraying in **mH**, compound **3**, leads to increased degradation by nucleases. In any case, the T_m for **mH**, compound **3** appeared to be adequate for encapsulation and delivery by our immunoliposome formulation, which led to cell growth inhibition that was dose-dependent and sequence specific compared to control modified hybrid compound **5** (Figure 2). More importantly, in this assay, modified hybrid, compound **3** (IC_{50} = 37 nM) was ~5-fold more potent compared to unmodified siHybrid **H,** compound **2** (IC_{50} = 7.8), which

was consistent with the aforementioned design concept for incorporating 2′OMe residues. This greater RNAi activity of modified hybrid, compound **3** compared to siHybrid **H,** compound **2**, was also supported by *in vivo* results summarized in Figure 3, namely, more pronounced diminution of HER-2 protein and increased apoptosis upon treatment with modified hybrid compared to siHybrid **H**. We recognized that a number of other factors would have to be investigated in order to establish the root cause(s) of this apparently higher potency. In this initial work, it was decided to test whether differential 5′ phosphorylation by intracellular kinase activity was somehow involved, given the apparently critical role of such phosphorylation in the mechanism of siRNA-mediated RNAi.[9,10] 5′-Phosphorylated versions of siHybrid **H** and modified hybrid, compounds **2** and **3**, were therefore synthesized, formulated, and tested, as described above, but led to essentially unchanged RNAi activity. Differential 5′ phosphorylation therefore does not seem to be a determining factor with these compounds. We note, however, that RNA deaminases might be among the other mechanistic factors to consider. RNA deaminases have been recently suggested as enzymes that limit siRNA efficiency in mammalian cells by inadvertently binding to siRNA or otherwise depleting the effective concentration of the siRNA antisense strand for incorporation into RISC.[43]

Subsequently Reported Anti-HER-2 RNAi Studies

After we began the work described in this report, two experimental investigations of RNAi-mediated inhibition of HER-2 were published.[44,45] The first of these studies[44] utilized a retrovirus designed to produce a 22-mer siRNA targeting nt 171–192 of Her-2 mRNA that was overexpressed in breast (BT-474 and MDA-MB-453) and ovarian (SKOV-3) cell lines. Retrovirus-infected cells exhibited lower levels of HER-2 protein, slower proliferation, increased apoptosis, and decreased tumor growth upon transplantation in mice. The second study[45] also involved breast cancer cells (SKBr3, BT-474, MCF-7, and MDA-MB-468) and is more relevant to the presently described work in that it involved the *in vitro* use of non-retroviral, synthetic 21-mer siRNAs, and delivery by a cationic lipid (Oligofectamine), albeit not cell-targeted. No *in vivo* experiments were reported. Of the HER-2 mRNA targets thus investigated, which were nt 1255–1277, 3386–3408, 3563–3585, and 3682–3704, the second and fourth were said to be the most active; however, all of these exhibited some level of sequence-specific activity compared to control siRNAs.

More recently, there have been three additional reports of RNAi of HER-2.[46–48] One of these studies[46] involves synthetic 21-mer siRNAs; however, in contrast to *in vitro* delivery to SKOV-3 cells by TransIT-TKO®, which was said to be ineffective in the presence of added serum, complexation with polyethyleneimine (PEI) was successfully used. Custom design

(Dharmacon) of three anti-HER-2 siRNAs was cited but without nt-target information. Our BLAST search of these reported sequences indicated matches to HER-2 mRNA nt 415–36, 1144–64, and 2282–2302, none of which correspond to any of the aforementioned active siRNAs against HER-2, or nt 1962–82 targeted by Choudhury *et al.*[23] and ourselves. In any event, results for RNAi in that study[46] are reported for use of the three siRNAs as an equimolar "cocktail," and therefore do not provide information about the relative activity of each compound. Although these investigators used PEI-complexation of this mixture of three siRNAs for systemic delivery to successfully reduce growth of SKOV-3 tumor xenografts in athymic nude mice, PEI-mediated delivery *in vivo* is known to encounter various toxicities that are not completely understood.[49] A patent application[47] reports RNAi of HER-2 using siRNA and chemically modified analogs that were transfected by unspecified cationic lipids into SKOV-3 cells and A549 human pulmonary epithelial cells. Significant inhibition was shown to be sequence specific and dose dependent for nt 2344 and 3706 targeting sites. The final study of RNAi of HER-2 that we are aware of was available to us only as a translated abstract,[48] which indicated that vector-encoded siRNA targeting unspecified target(s) in HER-2 mRNA led to significant inhibition of growth and apoptosis in a human lung adenocarcinoma cell line (calu-3), relative to empty vector and siRNA-sequence controls.

It is evident from all of the aforementioned examples of RNAi of HER-2 that numerous siRNAs have been found to exhibit significant activity. This is akin to what has been typically found for *in vitro* screening of siRNA sequences against other mRNA targets, which is a primary reason for the rapid, widespread adoption of siRNA as reagents for gene silencing experiments in cell culture. Since optimization of siRNA sequences for potency and duration of RNAi should, ideally, also take into account cominimization of off-target and immunogenic side effects, considerably more sequences and other *in vitro* information is needed to rank order anti-HER-2 siRNA clinical candidates.

CONCLUSIONS

The presently reported findings confirm earlier reports[17,18] that siHybrids are more potent than corresponding siRNAs. While these findings are encouraging, they are limited to a specific case involving HER-2 and may not be general. From a chemistry perspective, relatively simple chemical modifications of the sense strand of siHybrids were shown to further enhance the potency of RNAi. Achieving this with chemically modified siRNAs has, by contrast, required relatively exotic and extensive structural alterations to both the sense and antisense strands.[6] Our results have also shown, for the first time, that potency advantages of chemically modified siHybrids

observed *in vitro* can be found *in vivo*. More investigations will obviously be needed to assess the extent to which these findings apply to other mRNA targets and siHybrid delivery systems in different animal models. Nevertheless, the presently reported use of clinically relevant nanoimmunoliposomes for targeted delivery of a siHybrid, or sense strand-modified siHybrid, to tumors in an animal model of human cancer is significant in itself and has been described in detail in a companion study.[28] Our initial selection of HER-2 mRNA as an RNAi-based pharmacological target for treatment of various cancers has been buttressed by a number of subsequent reports by others.[44–48] Further preclinical investigations of nanoimmunoliposomes for delivery of siHybrids, or other RNAi agents, that target HER-2, or other cancer genes, either alone or in combination with small-molecule chemotherapeutic agents are warranted. The later possibility is currently under investigation in our labs in the case of gemcitabine, which is a well-known approved drug for cancer. Another promising *in vivo* example of delivery to specific target tissue or cells using immunoliposomes that has been very recently reported involves a Fab antibody fragment directed against HIV-1 in conjunction with a conventional siRNA.[50] Other recent reports of siRNA delivery using various types of liposomal formulations have also been described recently.[11,51–54] While many questions need to be investigated in order to elucidate the biochemical mechanism(s) of action of the presently reported chemically modified siHybrids, we believe that these analogs represent a novel and intriguing class of siRNA mimics for further study.

REFERENCES

1. Leung, R.K.; Whittaker, P.A. RNA Interference: From Gene Silencing to Gene-Specific Therapeutics. *Pharmacology & Therapeutics* **2005**, 107, 222–239.
2. Dorsett, Y.; Tuschl, T. siRNAs: Applications in Functional Genomics and Potential as Therapeutics. *Nature Reviews Drug Discovery* **2004**, 3, 318–329.
3. Crooke, S.T. Antisense Strategies. *Current Molecular Medicine* **2004**, 4, 465–487.
4. Kurreck, J. Antisense Technologies. Improvement through Novel Chemical Modifications. *European Journal of Biochemistry* **2003**, 270, 1628–1644.
5. Soutschek, J.; Akinc, A.; Bramlage, B.; Charisse, K.; Constien, R.; Donoghue, M.; Elbashir, S.; Geick, A.; Hadwiger, P.; Harborth, J.; John, M.; Kesavan, V.; Lavine, G.; Pandey, R.K.; Racie, T.; Rajeev, K.G.; Rohl, I.; Toudjarska, I.; Wang, G.; Wuschko, S.; Bumcrot, D.; Koteliansky, V.; Limmer, S.; Manoharan, M.; Vornlocher, H.P. Therapeutic Silencing of an Endogenous Gene by Systemic Administration of Modified siRNAs. *Nature* **2004**, 432, 173–178.
6. Allerson, C.R.; Sioufi, N.; Jarres, R.; Prakash, T.P.; Naik, N.; Berdeja, A.; Wanders, L.; Griffey, R.H.; Swayze, E.E.; Bhat, B. Fully 2′-Modified Oligonucleotide Duplexes with Improved In Vitro Potency and Stability Compared to Unmodified Small Interfering RNA. *Journal of Medicinal Chemistry* **2005**, 48, 901–904.
7. Amarzguioui, M.; Holen, T.; Babaie, E.; Prydz, H. Tolerance for Mutations and Chemical Modifications in a siRNA. *Nucleic Acids Research* **2003**, 31, 589–595.
8. Czauderna, F.; Fechtner, M.; Dames, S.; Aygun, H.; Klippel, A.; Pronk, G.J.; Giese, K.; Kaufmann, J. Structural Variations and Stabilising Modifications of Synthetic siRNAs in Mammalian Cells. *Nucleic Acids Research* **2003**, 31, 2705–2716.

9. Chiu, Y.L.; Rana, T.M. siRNA Function in RNAi: A Chemical Modification Analysis. *RNA* **2003**, 9, 1034–1048.
10. Harborth, J.; Elbashir, S.M.; Vandenburgh, K.; Manninga, H.; Scaringe, S.A.; Weber, K.; Tuschl, T. Sequence, Chemical, and Structural Variation of Small Interfering RNAs and Short Hairpin RNAs and the Effect on Mammalian Gene Silencing. *Antisense and Nucleic Acid Drug Development* **2003**, 13, 83–105.
11. Morrissey, D.V.; Lockridge, J.A.; Shaw, L.; Blanchard, K.; Jensen, K.; Breen, W.; Hartsough, K.; Machemer, L.; Radka, S.; Jadhav, V.; Vaish, N.; Zinnen, S.; Vargeese, C.; Bowman, K.; Shaffer, C.S.; Jeffs, L.B.; Judge, A.; MacLachlan, I.; Polisky, B. Potent and Persistent In Vivo Anti-HBV Activity of Chemically Modified siRNAs. *Nature Biotechnology* **2005**, 23, 1002–1007.
12. Braasch, D.A.; Jensen, S.; Liu, Y.; Kaur, K.; Arar, K.; White, M.A.; Corey, D.R. RNA Interference in Mammalian Cells by Chemically-Modified RNA. *Biochemistry* **2003**, 42, 7967–7975.
13. Li, Z.Y.; Mao, H.; Kallick, D.A.; Gorenstein, D.G. The Effects of Thiophosphate Substitutions on Native siRNA Gene Silencing. *Biochemical and Biophysical Research Communications* **2005**, 329, 1026–1030.
14. Elmen, J.; Thonberg, H.; Ljungberg, K.; Frieden, M.; Westergaard, M.; Xu, Y.; Wahren, B.; Liang, Z.; Orum, H.; Koch, T.; Wahlestedt, C. Locked Nucleic Acid (LNA) Mediated Improvements in siRNA Stability and Functionality. *Nucleic Acids Research* **2005**, 33, 439–447.
15. Pham, J.W.; Sontheimer, E.J. Molecular Requirements for RNA-Induced Silencing Complex Assembly in the *Drosophila* RNA Interference Pathway. *Journal of Biological Chemistry* **2005**, 280, 39278–39293.
16. Hohjoh, H. and RNA Interference (RNA(i)) Induction with Various Types of Synthetic Oligonucleotide Duplexes in Cultured Human Cells. *FEBS Letters* **2002**, 521, 195–199.
17. Lamberton, J.S.; Christian, A.T. Varying the Nucleic Acid Composition of siRNA Molecules Dramatically Varies the Duration and Degree of Gene Silencing. *Molecular Biotechnology* **2003**, 24, 111–120.
18. Christian, A.T.; Lamberton, J.S. Short Interfering Nucleic Acid Hybrids and Methods Thereof. 2004, WO 022771 A2.
19. Judge, A.D.; Sood, V.; Shaw, J.R.; Fang, D.; McClintock, K.; MacLachlan, I. Sequence-Dependent Stimulation of the Mammalian Innate Immune Response by Synthetic siRNA. *Nature Biotechnology* **2005**, 23, 457–462.
20. Jackson, A.L.; Bartz, S.R.; Schelter, J.; Kobayashi, S.V.; Burchard, J.; Mao, M.; Li, B.; Cavet, G.; Linsley, P.S. Expression Profiling Reveals Off-Target Gene Regulation by RNAi. *Nature Biotechnology* **2003**, 21, 635–637.
21. Brazas, R.M.; Hagstrom, J.E. Delivery of Small Interfering RNA to Mammalian Cells in Culture by Using Cationic Lipid/Polymer-Based Transfection Reagents. *Methods in Enzymology* **2005**, 392, 112–124.
22. Chien, P.Y.; Wang, J.; Carbonaro, D.; Lei, S.; Miller, B.; Sheikh, S.; Ali, S.M.; Ahmad, M.U.; Ahmad, I. Novel Cationic Cardiolipin Analogue-Based Liposome for Efficient DNA and Small Interfering RNA Delivery In Vitro and In Vivo. *Cancer Gene Therapy* **2005**, 12, 321–328.
23. Choudhury, A.; Charo, J.; Parapuram, S.K.; Hunt, R.C.; Hunt, D.M.; Seliger, B.; Kiessling, R. Small Interfering RNA (siRNA) Inhibits the Expression of the Her2/neu Gene, Upregulates HLA Class I and Induces Apoptosis of Her2/neu Positive Tumor Cell Lines. *International Journal of Cancer* **2004**, 108, 71–77.
24. Rait, A.S.; Pirollo, K.F.; Ulick, D.; Cullen, K.; Chang, E.H. HER-2-Targeted Antisense Oligonucleotide Results in Sensitization of Head and Neck Cancer Cells to Chemotherapeutic Agents. *Annals of the New York Academy of Sciences* **2003**, 1002, 78–89.
25. Rait, A.S.; Pirollo, K.F.; Xiang, L.; Ulick, D.; Chang, E.H. Tumor-Targeting, Systemically Delivered Antisense HER-2 Chemosensitizes Human Breast Cancer Xenografts Irrespective of HER-2 Levels. *Molecular Medicine* **2002**, 8, 475–486.
26. Rait, A.S.; Pirollo, K.F.; Rait, V.; Krygier, J.E.; Xiang, L.; Chang, E.H. Inhibitory Effects of the Combination of HER-2 Antisense Oligonucleotide and Chemotherapeutic Agents Used for the Treatment of Human Breast Cancer. *Cancer Gene Therapy* **2001**, 8, 728–739.
27. Kariko, K.; Buckstein, M.; Ni, H.; Weissman, D. Suppression of RNA Recognition by Toll-Like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA. *Immunity* **2005**, 23, 165–175.
28. Pirollo, K.F.; Zon, G.; Rait, A.; Zhou, Q.; Yu, W. Hogrefe, R.I., and Chang, E.H. A Tumor Targeting Nanoimmunoliposome Complex for siRNA Delivery. *Human Gene Therapy* **2006**, 17, 117–124.

29. Xu, L.; Huang, C.C.; Huang, W.; Tang, W.H.; Rait, A.; Yin, Y.Z.; Cruz, I.; Xiang, L.M.; Pirollo, K.F.; Chang, E.H. Systemic Tumor-Targeted Gene Delivery by Anti-Transferrin Receptor scFv-Immunoliposomes. *Molecular Cancer Therapeutics* **2002**, 1, 337–346.
30. Xu, L.; Tang, W.H.; Huang, C.C.; Alexander, W.; Xiang, L.M.; Pirollo, K.F.; Rait, A.; Chang, E.H. Systemic p53 Gene Therapy of Cancer with Immunolipoplexes Targeted by Anti-Transferrin Receptor scFv. *Molecular Medicine* **2001**, 7, 723–734.
31. Yu, W.; Pirollo, K.F.; Yu, B.; Rait, A.; Xiang, L.; Huang, W.; Zhou, Q.; Ertem, G.; Chang, E.H. Enhanced Transfection Efficiency of a Systemically Delivered Tumor-Targeting Immunolipoplex by Inclusion of a pH-Sensitive Histidylated Oligolysine Peptide. *Nucleic Acids Research* **2004**, 32, e48.
32. Hawser, S.P.; Norris, H.; Jessup, C.J.; Ghannoum, M.A. Comparison of a 2,3-*bis*(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino)Carbonyl]-2H-tetrazolium Hydroxide (XTT) Colorimetric Method with the Standardized National Committee for Clinical Laboratory Standards Method of Testing Clinical Yeast Isolates for Susceptibility to Antifungal Agents. *Journal of Clinical Microbiology* **1998**, 36, 1450–1452.
33. Day, J.D.; Digiuseppe, J.A.; Yeo, C.; Lai-Goldman, M.; Anderson, S.M.; Goodman, S.N.; Kern, S.E.; Hruban, R.H. Immunohistochemical Evaluation of HER-2/neu Expression in Pancreatic Adenocarcinoma and Pancreatic Intraepithelial Neoplasms. *Human Pathology* **1996**, 27, 119–124.
34. Apple, S.K.; Hecht, J.R.; Lewin, D.N.; Jahromi, S.A.; Grody, W.W.; Nieberg, R.K. Immunohistochemical Evaluation of K-ras, p53, and HER-2/neu Expression in Hyperplastic, Dysplastic, and Carcinomatous Lesions of the Pancreas: Evidence for Multistep Carcinogenesis. *Human Pathology* **1999**, 30, 123–129.
35. Lieberman, S.M.; Horig, H.; Kaufman, H.L. Innovative Treatments for Pancreatic Cancer. *Surgical Clinics of North America* **2001**, 81, 715–739.
36. Rabindran, S.K. Antitumor Activity of HER-2 Inhibitors. *Cancer Letters* **2005**, 227, 9–23.
37. Luwor, R.B.; Lu, Y.; Li, X.; Mendelsohn, J.; Fan, Z. The Antiepidermal Growth Factor Receptor Monoclonal Antibody Cetuximab/C225 Reduces Hypoxia-Inducible Factor-1 Alpha, Leading to Transcriptional Inhibition of Vascular Endothelial Growth Factor Expression. *Oncogene* **2005**, 24, 4433–4441.
38. Yu, W.; Pirollo, K.F.; Rait, A.; Yu, B.; Xiang, L.M.; Huang, W.Q.; Zhou, Q.; Ertem, G.; Chang, E.H. A Sterically Stabilized Immunolipoplex for Systemic Administration of a Therapeutic Gene. *Gene Therapy* **2006**, 11, 1434–1440.
39. Khvorova, A.; Reynolds, A.; Jayasena, S.D. Functional siRNAs and miRNAs Exhibit Strand Bias. *Cell* **2003**, 115, 209–216.
40. Ge, Q.; McManus, M.T.; Nguyen, T.; Shen, C.H.; Sharp, P.A.; Eisen, H.N.; Chen, J. RNA Interference of Influenza Virus Production by Directly Targeting mRNA for Degradation and Indirectly Inhibiting All Viral RNA Transcription. *Proceedings of the National Academy of Sciences, USA* **2003**, 100, 2718–2723.
41. Allerson, C.R.; Bhat, B.; Eldrup, A.B.; Monoharan, M.; Griffey, R.H.; Baker, B.F.; Swayze, E.E. Compositions Comprising Alternating 2′-Modified Nucleosides for Use in Gene Modulation. *US 2005/0187178 A1.*
42. Nowotny, M.; Gaidamakov, S.A.; Crouch, R.J.; Yang, W. Crystal Structures of RNase H Bound to an RNA/DNA Hybrid: Substrate Specificity and Metal-Dependent Catalysis. *Cell* **2005**, 121, 1005–1016.
43. Yang, W.; Wang, Q.; Howell, K.L.; Lee, J.T.; Cho, D.S.; Murray, J.M.; Nishikura, K. ADAR1 RNA Deaminase Limits Short Interfering RNA Efficacy in Mammalian Cells. *Journal of Biological Chemistry* **2005**, 280, 3946–3953.
44. Yang, G.; Cai, K.Q.; Thompson-Lanza, J.A.;Jr., Bast, R.C.; Liu, J. Inhibition of Breast and Ovarian Tumor Growth Through Multiple Signaling Pathways by Using Retrovirus-Mediated Small Interfering RNA against Her-2/neu Gene Expression. *Journal of Biological Chemistry* **2004**, 279, 4339–4345.
45. Faltus, T.; Yuan, J.; Zimmer, B.; Kramer, A.; Loibl, S.; Kaufmann, M.; Strebhardt, K. Silencing of the HER2/neu Gene by siRNA Inhibits Proliferation and Induces Apoptosis in HER2/neu-Overexpressing Breast Cancer Cells. *Neoplasia* **2004**, 6, 786–795.
46. Urban-Klein, B.; Werth, S.; Abuharbeid, S.; Czubayko, F.; Aigner, A. RNAi-Mediated Gene-Targeting through Systemic Aapplication of Polyethylenimine (PEI)-Complexed siRNA In Vivo. *Gene Therapy* **2005**, 12, 461–466.
47. McSwiggen, J.; Beigelman, L.; Pavco, P.; Fosnaugh, K.; Jamison, S. RNA Interference Mediated Inhibition of Epidermal Growth Factor Receptor (EGFR) Gene Expression Using Short Interfering Nucleic Acid (SINA). 2005, US 0176024 A1.

48. Ren, S.H.; Zhang, W.; Qu, P.; Liu, Y.; Wang, J.W.; Zhang, L. Effect of RNAi-Mediated Gene Silencing of C-erbB-2 on Proliferation of Lung Adenocarcinoma Cell Line calu-3. *Ai Zheng* **2005**, 24, 1173–1178.
49. Moghimi, S.M.; Symonds, P.; Murray, J.C.; Hunter, A.C.; Debska, G.; Szewczyk, A. A Two-Stage Poly(Ethylenimine)-Mediated Cytotoxicity: Implications for Gene Transfer/Therapy. *Molecular Therapy* **2005**, 11, 990–995.
50. Song, E.; Zhu, P.; Lee, S.K.; Chowdhury, D.; Kussman, S.; Dykxhoorn, D.M.; Feng, Y.; Palliser, D.; Weiner, D.B.; Shankar, P.; Marasco, W.A.; Lieberman, J. Antibody Mediated In Vivo Delivery of Small Interfering RNAs via Cell-Surface Receptors. *Nature Biotechnology* **2005**, 23, 709–717.
51. Schiffelers, R.M.; Ansari, A.; Xu, J.; Zhou, Q.; Tang, Q.; Storm, G.; Molema, G.; Lu, P.Y.; Scaria, P.V.; Woodle, M.C. Cancer siRNA Therapy by Tumor Selective Delivery with Ligand-Targeted Sterically Stabilized Nanoparticle. *Nucleic Acids Research* **2004**, 32, e149.
52. Jr., Landen, C.N.; Chavez-Reyes, A.; Bucana, C.; Schmandt, R.; Deavers, M.T.; Lopez-Berestein, G.; Sood, A.K. Therapeutic EphA2 Gene Targeting In Vivo Using Neutral Liposomal Small Interfering RNA Delivery. *Cancer Research* **2005**, 65, 6910–6918.
53. Kunisawa, J.; Masuda, T.; Katayama, K.; Yoshikawa, T.; Tsutsumi, Y.; Akashi, M.; Mayumi, T.; Nakagawa, S. Fusogenic Liposome Delivers Encapsulated Nanoparticles for Cytosolic Controlled Gene Release. *Journal of Controlled Release* **2005**, 105, 344–353.
54. Pal, A.; Ahmad, A.; Khan, S.; Sakabe, I.; Zhang, C.; Kasid, U.N.; Ahmad, I. Systemic Delivery of RafsiRNA Using Cationic Cardiolipin Liposomes Silences Raf-1 Expression and Inhibits Tumor Growth in Xenograft Model of Human Prostate Cancer. *International Journal of Oncology* **2005**, 26, 1087–1091.