RNA interference targeting the R2 subunit of ribonucleotide reductase inhibits growth of tumor cells *in vitro* and *in vivo*

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RNA interference, a posttranscriptional gene-silencing mechanism, has received considerable attention for its potential as a new therapeutic strategy to treat human diseases and conditions including cancer. Various studies have supported a role for the R2 subunit of ribonucleotide reductase in cancer progression and metastasis. Short interfering siRNA 1284 was designed to target R2. *In vitro* studies, in which three different human tumor cell lines (A498, HT-29 and A2058) were transfected with short interfering siRNA 1284, demonstrate sequence-specific down-regulation of R2, which coincides with a decrease in cell proliferation, and cell cycle inhibition. *In vivo* studies with xenograft mouse models, generated from the same tumor cell lines, indicate that treatment with short interfering siRNA 1284 leads to inhibition of tumor growth and this effect was found to be dose dependent. Taken together, these results suggest that short interfering siRNA 1284, targeting R2, has great potential to serve as a therapeutic agent towards the treatment of human cancers. *Anti-Cancer Drugs* 18:377–388 © 2007 Lippincott Williams & Wilkins.

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Introduction

RNA interference (RNAi) is a highly conserved gene-silencing process occurring in most eukaryotes including humans [1,2]. Originally identified in plants [3], RNAi is thought to have evolved as a cellular defense mechanism activated in response to foreign genes introduced during viral infection or during random integration of transposons or transgenes [4–6]. The RNAi pathway is triggered by long double-stranded RNAs (dsRNAs) that are rapidly cleaved by Dicer, an RNase III endoribonuclease, into short interfering RNAs (siRNAs) [7]. These siRNA duplexes are 21–23 nucleotides in length and each strand contains a two-nucleotide 3′ hydroxyl overhanging end [8–11]. The strand of siRNA containing the thermodynamically less stable 5′ terminus is preferentially incorporated into the RNA-induced silencing complex (RISC) [12,13] and guides activated RISC to the complementary mRNA sequence for sequence-specific endoribonuclease cleavage. The cleaved mRNA then undergoes degradation by cellular ribonucleases [10,14–19].

Several publications have shown that chemically synthesized siRNAs or precursors to siRNAs can be successfully introduced into mammalian cells and be incorporated into the RNAi pathway to down-regulate target genes. As a result, RNAi has proven to be a powerful tool in analyzing and understanding gene function, and in identifying potential drug targets [20–23]. Recent studies have also demonstrated the efficient delivery of siRNAs *in vivo*, along with therapeutic benefit in animal models [24–29]. Although the transfer of RNAi to the clinic is currently in its infancy, RNAi is viewed as a highly specific gene-silencing phenomenon that may prove to be an important new therapeutic tool for treatment of human conditions including chronic infection, cancer and neurodegenerative diseases [30,31].

Ribonucleotide reductase (RNR) catalyzes the conversion of ribonucleoside 5′-diphosphates to their corresponding 2′-deoxyribonucleotides, a rate-limiting step required for the production of 2′-deoxyribonucleoside 5′-triphosphates (dNTPs) that are essential for DNA synthesis and repair [32–34]. The protein components of human RNR include a 160-kDa homodimer of R1 (hRRM1) and a 78-kDa homodimer of R2 (hRRM2). Together, R1 and R2 form the catalytically active RNR enzyme. R1 contains ribonucleotide binding sites and allosteric effector sites, and its expression in proliferating cells is relatively constant throughout the cell cycle [35–39]. R2 contains a tyrosyl-free radical that is stabilized by a nonheme iron center, which is essential for ribonucleotide reduction [40,41]. Studies examining R2 expression indicate that R2 levels are elevated during late G_1 or early S phase, corresponding to the time of DNA synthesis. Thus, the expression and degradation of R2 throughout

the cell cycle plays a role in the regulation of RNR activity, and therefore DNA synthesis and cell proliferation [42,43].

Recently, a R2 homolog, p53R2, was identified. The human p53R2 gene contains a p53 binding site in intron 1 and is approximately 80% identical to R2 [44,45]. The role of p53R2 is quite different from R2 in that p53R2 is induced upon exposure to ultraviolet light, γ-irradiation or DNA-damaging agents and supplies the dNTPs necessary for DNA repair [46,47]. Like R2, p53R2 complexes with R1 to form an active RNR [48].

RNR activity has been strongly correlated with the rate of replication in cancer cells [49,50]. In addition to the function of R2 in modulating RNR activity in DNA synthesis and cell proliferation, several lines of evidence further support a role for R2 in cancer progression and metastasis. R2 cooperates with a variety of oncogenes, including v-*fms*, v-*src*, A-*raf*, v-*fes* and c-*myc*, to act as a determinant in enhancing tumor progression and malignant potential [51]. Overexpression of R2 in H-*ras*-transformed fibroblasts led to a significant increase in membrane-associated Raf-1 protein activation and mitogen-activating protein kinase-2 activity [52]. In addition, elevated expression of R2 enhances the invasive potential of cancer cells [53], reduces radiosensitivity in human solid tumors [54] and increases the drug-resistant properties of cancer cells to a variety of chemotherapeutic reagents, including hydroxyurea and gemcitabine [55–58].

There are several therapeutic agents in use that target RNR and specifically R2. Hydroxyurea is a chemotherapeutic agent that destabilizes the iron center of R2 and quenches the tyrosyl-free radical inactivating RNR activity [59,60]. Use of hydroxyurea is limited in that it has a short half-life and is required in high doses to be effective. Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone) is an iron chelator that sensitizes tumor cells to radiotherapy *in vitro* and *in vivo* [61]. A phase I clinical trial evaluating the use of Triapine in combination with Ara-C (cytarabine) in acute myeloid leukemia patients indicates potential therapeutic benefit for this combination [62]. Some resistance to triapine (3-AP), however, has been observed in leukemia cell lines that overexpress multidrug resistance genes *mdr*1 and *mrp* [63].

Antisense mechanisms targeting R2 have produced positive effects in inhibition of tumor growth and metastasis, and sensitization to chemotherapeutic agents [55,64–66]. RNAi is a novel antisense approach that might offer further therapeutic benefit by potentially requiring lower doses than that required for other antisense strategies. In this study, we have identified a siRNA, termed siRNA 1284 that targets R2 mRNA and have shown sequence-specific down-regulation of R2, which coincides with antiproliferative effects in cancer cells *in vitro* and suppression of tumor growth *in vivo* in xenograft mouse models. Our findings suggest that siRNA 1284, with its ability to down-regulate R2, has potential to serve as a therapeutic agent in the treatment of cancer.

Materials and methods

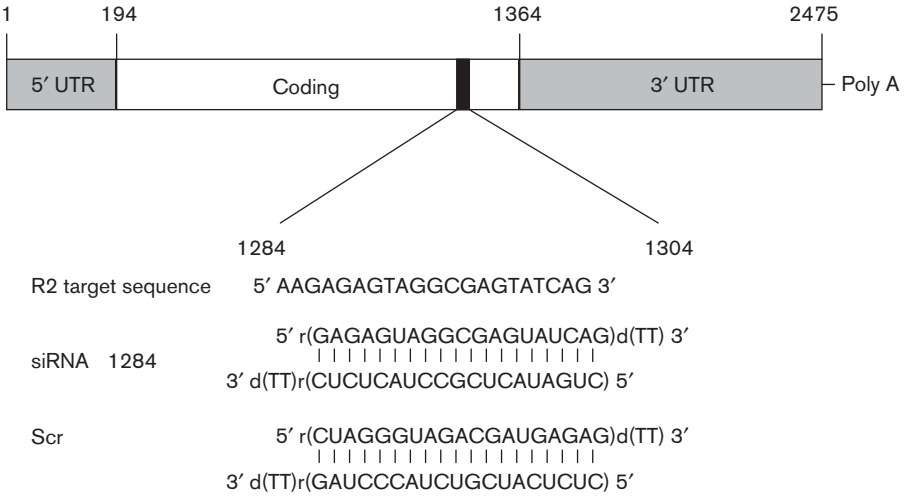
Short interfering RNA (siRNA)

siRNA sequences targeting the mRNA of the R2 subunit of human RNR were selected according to the criteria as previously reported [9,11,14,67,68]. siRNA target sites containing the sequence motif AA(N19) (where N represents any nucleotide) and a G/C content of approximately 50% were selected. Sequences targeting the mRNA within 100 nucleotides of the start codon were not selected. A blast-search of the National Center for Biotechnology Information database was performed to rule out sequences with homology to other regions of the human genome. siRNA 1284 targets the coding region of R2 mRNA (nucleotides 1284–1304) (Fig. 1). The sense sequence is 5′ GAGAGUAGGCGAGUAUCAGdTdT 3′ and the antisense sequence is 5′ CUGAUACUCGCCUA CUCUCdTdT 3′. A scrambled control analog of siRNA 1284, 1284 Scr, contains the same base composition as siRNA 1284, but does not target R2. The sense sequence is 5′ CUAGGGUAGACGAUGAGAGdTdT 3′ and the antisense sequence is 5′ CUCUCAUCGUCUACC CUAGdTdT 3′. siRNAs were purchased from Qiagen (Germantown, Maryland, USA). Upon arrival, the siRNAs were suspended in Qiagen suspension buffer according to the manufacturer's instructions.

Cell culture and transfection

Human tumor cell lines A498 (renal carcinoma), A2058 (melanoma) and HT-29 (colon adenocarcinoma) were purchased from American Type Culture Collection (Manassas, Virginia, USA) and maintained according to American Type Culture Collection specifications, in α-modified Eagle's medium (MEM), Dulbecco's MEM or McCoy's 5a media (Sigma, St. Louis, Missouri, USA) supplemented with 10% heat-inactivated fetal bovine serum, at 37°C in a humidified atmosphere. All media contained an antibiotic–antimycotic solution at a final concentration of 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen Carlsbad, California, USA). Cells were seeded onto 96- or six-well tissue culture plates, 100- or 150-mm tissue culture dishes (as indicated) and grown in appropriate media to subconfluence (30–50%). The cells were washed once with phosphate-buffered saline (PBS) (pH 7.2) and transfected for 6 h (unless otherwise indicated) with siRNAs (12.5, 25 or 200 nmol/l as indicated) in opti-MEM using oligofectamine reagent (Invitrogen), according to the manufacturer's instructions. At the end of transfection,

Fig. 1



Schematic representation of R2 mRNA. The short interfering RNA (siRNA) 1284 target region within the coding region of R2 and its sequence are indicated along with the sequences (both sense and antisense) of siRNAs 1284 and 1284 Scrambled (Scr).

the media containing siRNAs was removed, the cells were washed once with PBS (pH 7.2) and the cells were cultured in growth medium (containing serum) for the duration indicated in the text. A control (C) or mock-transfected sample was included in each experiment, in which case, cells were treated with oligofectamine reagent in the absence of siRNA.

Western blot analysis

To measure the effect of siRNA treatment on R2 protein levels, Western blot analysis was performed. Cells were seeded in six-well plates and transfected as described above. Following the post-transfection incubation period (as indicated in the text), cells were washed once with PBS (pH 7.2) and whole-cell protein extracts were prepared using cell lysis buffer [20 mmol/l Tris (pH 7.5), 140 mmol/l NaCl, 1% NP40, 10% glycerol, 2 mmol/l ethylenediaminetetraacetic acid, 2 mmol/l phenylmethylsulfonyl fluoride and 1 × protease inhibitors (Sigma)]. Extracted protein (10–20 μg) was separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose, as described previously [66]. The R2 protein was detected with a polyclonal goat anti-human R2 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) followed by a horseradish peroxidase-conjugated antigoat IgG secondary antibody (Sigma) and visualized by ECL detection reagents (Amersham Little, Chalfont, Buckinhamshire, UK), where the indicated blots were probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein as a loading control. GAPDH protein was detected with a mouse anti-GAPDH monoclonal antibody (Biodesign International, Saco, Maine, USA) followed by a horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham).

Northern blot analysis

To measure the effect of siRNA treatment on R2 mRNA levels, Northern blot analysis was performed. Cells were seeded in 100-mm dishes transfected as described above. Following the post-transfection incubation period (as indicated in the text), cells were washed once with PBS (pH 7.2) and total cellular RNA was prepared using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total cellular RNA (20 μg) was resolved on a 1% agarose gel containing formaldehyde, followed by transfer to a nylon membrane using the capillary transfer method. The blots were hybridized with an [α-^{32}P]ATP probe synthesized from the R2 cDNA [69]. The probes were labeled by random primer extension, using the DECAprime II kit (Ambion Austin, Texas, USA) according to the manufacturer's instructions. R2 mRNA was detected (using autoradiography) as two bands of 1.6 and 3.4 kb, representing two transcripts produced through alternative polyadenylation [34,70,71]. After R2 mRNA detection, the membranes were stripped and reprobed for detection of mRNA of the 23-kDa highly basic protein as a loading control.

Cell proliferation assays

The effect of R2 down-regulation on cell proliferation was examined by two different assays. For the Sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay, cells were seeded in 96-well tissue culture plates and transfected with siRNAs 1284 or Scr (12.5 nmol/l) as

described above. All transfections were carried out in quadruplicate. At 48 h post-transfection, cells were incubated with XTT reaction solution according to the manufacturer's instructions (Roche; Cell Proliferation Kit II Mannheim, Germany) and the absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay plate reader. For the cell count assay, cells were seeded in six-well tissue culture plates (in triplicate for each time point) and transfected with siRNAs 1284 or Scr (12.5 nmol/l) as described above. At 3, 4 and 5 days post-transfection, cells were trypsinized. The cell suspensions were counted and numbers graphed.

Cell cycle analysis

The effect of R2 down-regulation on cell cycle progression was examined by flow cytometry. A498 cells were seeded in 150-mm tissue culture dishes and transfected with siRNAs 1284 or Scr (25 nmol/l) as described above. At 35 h post-transfection, cells were collected, fixed in 75% ethanol, treated with RNase A (Sigma), stained with propidium iodide (Molecular Probes) and analyzed by flow cytometry using the FACScalibur cell analyzer (BD Biosciences, San Jose, California, USA). The cell cycle profile was determined using ModFit LT software (Verity Software House-Topsham, Maine, USA).

In vivo treatment with siRNAs

SCID mice and CD-1 nude mice were purchased from Charles River Laboratories (Montreal, Quebec, Canada). All animal experimentation was performed according to the National Institutes of Health, Sunnybrook and Women College Health Science Center, and Lorus Therapeutics Inc. animal care and use guidelines. Experiments were initiated when mice were 6–7 weeks old and the number of mice per experimental group was eight or 10 as indicated in the text. A498 cells were grown in α-MEM, A2058 cells were grown in Dulbecco's MEM and HT-29 cells were grown in McCoy's media. All media was supplemented with 10% fetal bovine serum. A498 cells (1×10^7 cells in PBS) were subcutaneously implanted into the right flank of SCID mice and A2058 cells (5×10^6 cells in PBS) or HT29 cells (3×10^6 cells in PBS) were subcutaneously implanted into the right flank of athymic CD-1 nude mice. Once tumors had reached an approximate volume of 80–90 mm^3, 17 days posttumor injection for A498 tumors and 70 mm^3, 5 days posttumor implantation for A2058 and HT-29 tumors, siRNA 1284 or Scr were administered by bolus injection via the tail vein three times/week at 125, 250 or 500 μg/kg, as indicated in the text. C groups were treated with the same volume of Qiagen suspension buffer (minus siRNA) in PBS. In each case, tumor volume was measured one to two times/week (as indicated) using a caliper. At the end of the experiment, the animals were sacrificed, and the tumor, spleen, liver and body weights were measured. To examine R2 protein levels in tumors, tumor sections were collected and each sample was separately homogenized in protein extraction buffer [50 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.02% NaN_3, 1 mmol/l phenylmethylsulfonyl fluoride, and 1 × protease inhibitor cocktail (Sigma)] [66]. After centrifugation, Western blot analysis was conducted and the R2 protein was detected as described above. Loading controls for Western blots were included as indicated in the text.

Statistical evaluation

Statistical analyses of tumor growth kinetics and tumor weight data, from in-vivo experiments, were conducted using the analysis of covariance and analysis of variance programs, respectively.

Densitometry

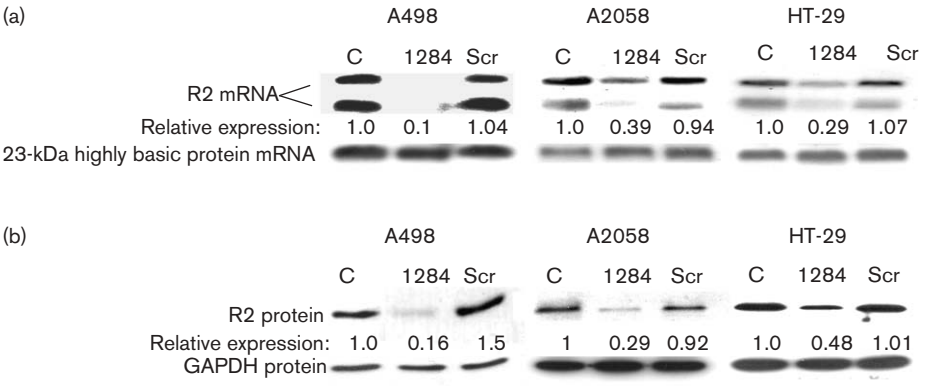
RNA and protein levels from Northern and Western blots, respectively, were quantified using the Bio-Rad GelDoc System and Bio-Rad Quantity One quantification software (version 4.4.0) (Hercules, California, USA).

Results

Specific inhibition of R2 expression in tumor cells by siRNA 1284

Previous studies examining the expression of the R2 subunit of RNR in cancer cell lines indicate that R2 protein levels are elevated in a diverse number of cancer types including renal, skin, colon and breast [66]. The basal level of R2 protein, in A498 (renal carcinoma), HT-29 (colon adenocarcinoma) and A2058 (melanoma) cells, for example, was found to be 6.2, 12.6 and 6.5 times greater, respectively, than R2 protein expression in normal human umbilical vein endothelial cells [66]. To identify a siRNA drug candidate that down-regulates the expression of R2 and reduces tumor growth, seven siRNAs targeting the coding region of R2 were designed, according to the criteria described in Materials and methods. These siRNAs were subjected to *in vitro* screening by Northern and Western blot analyses to identify siRNAs, which reduce R2 expression. Four siRNAs were capable of inhibiting R2 expression (data not shown) and one siRNA, 1284, was consistently most effective in suppressing R2 expression in all cells lines tested. Northern and Western blot results of three different cell lines (A498, HT-29 and A2058) transfected with siRNA 1284 are shown in Fig. 2. Transfection efficiency was assessed by varying the transfection conditions, i.e. cell number, volume of oligofectamine used and transfection time. The optimal transfection conditions for a particular cell line were those in which the highest level of R2 down-regulation possible was achieved for that cell line. Therefore, the length of the transfection time for the three cell lines varied to achieve maximal target down-regulation. In all cases, R2 is significantly down-regulated in comparison to C or mock-transfected samples (see Fig. 2). The relative mRNA and protein expression levels of R2 in siRNA-

Fig. 2



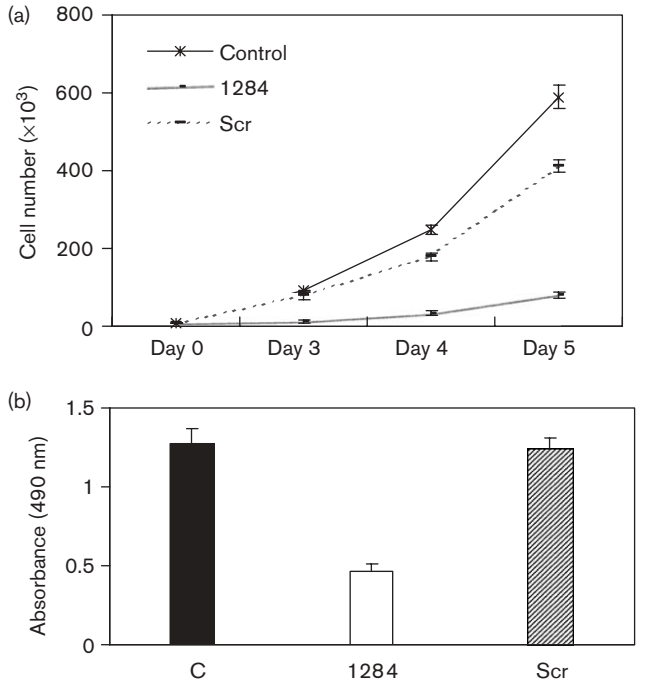
Inhibition of R2 expression by short interfering RNA (siRNA) 1284. A498, A2058 and HT-29 cells were transfected with 200 nmol/l siRNAs, 1284 or 1284 Scrambled (Scr) in the presence of oligofectamine reagent. Control (C) samples were treated with oligofectamine reagent alone. Cells were transfected for 4 h (for A498) or 24 h (for A2058 and HT-29), washed with PBS and subsequently incubated for 24 h in fresh media (complete). RNA and protein extraction were followed by Northern and Western blot analysis, respectively. (a) Northern blot. R2 mRNA is shown and the 23-kDa highly basic protein mRNA was used as a loading control. Relative expression of R2 mRNA of siRNA-transfected cells compared to mRNA expression of the control sample of each cell line is indicated. (b) Western blot. R2 protein is shown and GAPDH was used as a loading control. Relative expression of R2 protein of siRNA-transfected cells compared to protein expression of the control sample of each cell line is indicated. Note that Western and Northern blots represent separate experiments for each cell line

treated samples compared to C are indicated for each cell line. Although comparison of R2 basal levels in the C lanes for A498, HT-29 and A2058 cells in Fig. 2 does not appear to follow the trend indicated by Lee *et al.*, [66], with HT-29 having the highest basal R2 expression (see above), the experiments were performed separately for each cell line and therefore direct comparisons of basal R2 levels between cell lines cannot be made from the data presented in Fig. 2. An Scr sequence of siRNA 1284, Scr had no effect on R2 expression levels indicating that inhibition of R2 mRNA and protein expression by siRNA 1284 is sequence-specific (Fig. 2). This sequence-specific down-regulation of R2 by siRNA 1284 was reproducible in repeated assays using the same cell lines. The initial concentration of siRNA used for screening was 200 nmol/l; however, to minimize the possibility of nonspecific effects of high concentrations of siRNA, it was important to find a low dose of siRNA for transfection that could efficiently silence R2. Titration studies revealed that when A498 cells are transfected, with 12.5 or 25 nmol/l of siRNA 1284, R2 protein expression is suppressed by approximately 40–60% (data not shown). Therefore, these concentrations (12.5 or 25 nmol/l) of siRNA were used in subsequent *in vitro* experiments (discussed below).

siRNA 1284 inhibits cancer cell growth and proliferation *in vitro*

The effect of siRNA 1284-mediated suppression of R2 gene expression on tumor cell growth and proliferation was examined *in vitro* by two different experimental methods. In a cell count experiment, A498 cells (1×10^4 cells) were seeded on day 0 and transfected the following day with siRNA 1284 or Scr as described in Materials and methods. Cell suspensions were collected and counted at 3, 4 and 5 days post-transfection. Cell counts for all 3 days revealed that A498 cells transfected with siRNA 1284 were significantly inhibited in their ability to proliferate as compared with Scr and C samples (Fig. 3a). *P*-values were calculated and found to be less than 0.005 for cell counts of siRNA 1284 transfected samples when compared with cell counts of C or Scr siRNA-transfected samples. This inhibitory effect of R2 depletion on A498 cell growth was confirmed by the XTT colorimetric assay, which measures the ability of viable cells to metabolize tetrazolium salts to a formazan dye. A498 cells were transfected with siRNAs 1284 and Scr and incubated with XTT reagent 48 h post-transfection as described in Materials and methods. The absorbance at 490 nm was determined and taken as a direct indication of the number of surviving cells. As shown in Fig. 3b, OD490 values were significantly lower for siRNA 1284-transfected samples relative to C and Scr samples, indicating the presence of fewer viable cells. Differences in OD490 values between C or Scr and the siRNA 1284-transfected cells were statistically significant as *P*-values were less than 0.05 in both cases (see Fig. 3b). Taken together, both assays (cell count and XTT) confirm that cell proliferation is significantly inhibited after treatment with siRNA 1284 and this inhibition corresponds to a reduction in R2 expression. XTT assays were also conducted with HT-29 and A2058 cells, transfected with siRNA 1284. In both cases, fewer viable cells (as indicated by absorbance at 490 nm) were present in transfected samples relative to the C or mock-transfected samples (data not shown).

Fig. 3



In-vitro antiproliferative effects of short interfering RNA (siRNA) 1284. A498 cells were transfected with 12.5 nmol/l siRNAs, 1284 or 1284 Scrambled (Scr) in the presence of oligofectamine reagent. Control (C) samples were treated with oligofectamine reagent alone. Cells were transfected for 4 h, washed and subsequently incubated in fresh media. (a) Cell count experiment. Cell suspensions were counted at the indicated time points. Day 0 indicates the starting cell number (1×10^4 cells) prior to transfection. The data shown represent the average of three samples for each condition being tested. Differences in cell growth (proliferation) over time between control or Scr and siRNA 1284-transfected cells, were statistically significant with $P < 0.005$ in both cases. (b) XTT assay. XTT reagent was added to each sample (prepared in quadruplicate) at 48 h posttransfection. Absorbance at 490 nm was determined for each. Differences in OD490 values between C or Scr and the siRNA 1284-transfected cells were statistically significant with $P < 0.05$ in both cases.

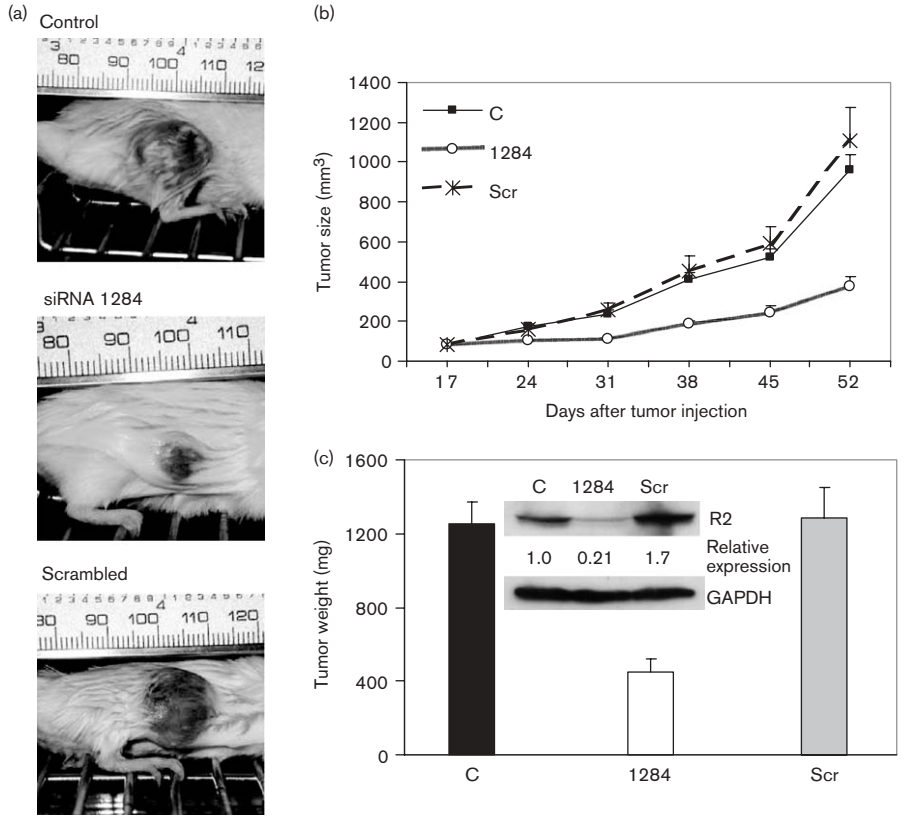
also three times per week. A visual representation of tumors after 3 weeks of treatment is found in Fig. 4a. A ruler was placed above each tumor while photos were taken in Fig. 4a so that tumor diameter could be approximated. The diameter for the C, siRNA 1284- and Scr-treated tumors, is 11, 6 and 13 mm, respectively (depicted in Fig. 4a), indicating that siRNA 1284 treatment inhibits tumor growth in comparison with C or Scr treatment. Tumor volume was monitored weekly and at the end of the experiment, the mice were sacrificed, and the tumor, liver, spleen and body weights were determined. No adverse effects to the liver, spleen or body weight were evident in mice treated with siRNAs 1284 or Scr (data not shown). siRNA 1284 treatment resulted in a significant inhibition to A498 tumor growth (approximately 65%) in comparison with C or Scr, as measured by tumor volume and weight over the 36-day treatment period (Figs. 4b and c). Differences in tumor growth kinetics between C or Scr- and siRNA 1284- treated groups were statistically significant with *P*-values less than 0.0001 in both cases. In addition, differences in tumor weight between C or Scr- and the siRNA 1284-treated group were also statistically significant as *P*-values were determined to be equal to 0.0002 or less than 0.0001, respectively (Fig. 4c). Tumor samples were collected as described in Materials and methods, and R2 protein levels from one sample for each treatment group were examined. Western blot analysis indicates that R2 protein expression is reduced in the tumor sample of the siRNA 1284 treatment group in comparison with tumor samples of the C and Scr treatment groups (see inset in Fig. 4b). The degree of R2 down-regulation in the four tumor samples collected from the siRNA 1284 treatment group varied from 40 to 80%, relative to R2 levels in tumor samples collected from the C group (not shown). R2 down-regulation was not observed in tumor samples of the Scr treatment group (not shown).

siRNA 1284 suppresses R2 expression and has dose-dependent antitumor activity in A498 (human renal carcinoma) SCID mouse xenografts models

In vitro studies clearly demonstrate a potent inhibitory effect of siRNA 1284 on R2 expression and tumor cell proliferation. The antitumor potential of siRNA 1284 was therefore evaluated in A498 SCID mouse xenograft models. A498 tumors were selected for initial *in vivo* studies because R2 expression is significantly elevated in A498 cells and previous studies indicate that this tumor model is sensitive to treatment with agents that affect R2 expression [66]. A498 cells were subcutaneously implanted into SCID mice and when tumors became palpable, siRNA 1284 and Scr were administered via the tail vein, three times per week at 250 μg/kg for approximately 5 weeks. Control animals received suspension buffer alone in PBS (see Materials and methods),

In a subsequent study, siRNA 1284 demonstrated dose-dependent antitumor activity against A498 tumors (Fig. 5a). The data presented in Fig. 5a indicate that at the highest dose tested (500 μg/kg, three times/week) siRNA 1284 produced the greatest inhibition of tumor growth and the lowest dose of siRNA 1284 tested (125 μg/kg, three times/week) had little effect on tumor growth. The inhibition of tumor growth, in the 500 and 250 μg/kg treatment groups, was statistically significant in comparison with the C group ($P < 0.0001$ and $P < 0.001$, respectively). In addition, the inhibition of tumor growth in the siRNA 1284 group (500 μg/kg) was statistically significant in comparison with the Scr group ($P < 0.005$), that also received 500 μg/kg/treatment (Fig. 5a). Suppression of R2 protein expression also appeared to be dose dependent (Fig. 5b). Figure 5b represents R2 expression data taken from one tumor sample for each treatment

Fig. 4



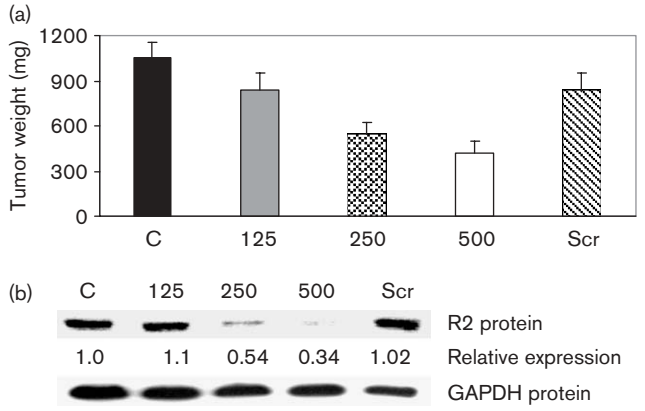
Antitumor effect of short interfering RNA (siRNA) 1284 in A498 xenografts. A498 cells (1×10^7 cells in 100 µl PBS) were subcutaneously implanted into the right flank of 6–7-week-old SCID mice. After tumors reached an approximate volume of 80–90 mm^3, 17 days after tumor cell implantation, siRNAs 1284 or Scr were administered (250 µg/kg, i.v., three times per week). The control group (C) received suspension buffer alone in PBS. (a) Photos of A498 xenografts after 3 weeks treatment with siRNAs. Tumors from each group were photographed after 3 weeks of treatment. A ruler was placed above each tumor while photos were taken so that tumor diameter could be determined in millimeter units. (b) Tumor growth curve. Caliper measurements, taken once per week, were used to calculate tumor volumes. Each point represents the mean tumor volume calculated from eight animals per experimental group. Differences in tumor growth kinetics, between C or Scr- and siRNA 1284-treated groups, were statistically significant with $P<0.0001$ in both cases. (c) Tumor weight. Mice were sacrificed after 36 days of treatment and tumor weight for each was determined. Mean tumor weight and standard error of the mean (SEM) are shown for each experimental group. Differences in tumor weight between C or Scr- and siRNA 1284-treated groups were statistically significant with $P=0.0002$ or $P<0.0001$, respectively. Excised tumor samples (four per treatment group) of a similar size were separately homogenized in protein extraction buffer and Western blot analysis was performed as described in Materials and methods. R2 protein from one tumor sample for each treatment group is shown and GAPDH was used as a loading control. Relative expression of R2 protein in tumor samples of siRNA treatment groups compared to R2 protein expression in the tumor sample of the C group is indicated (see inset of graph).

group. R2 expression was examined in three different tumor samples for each treatment group (not shown) and the degree of down-regulation from tumor samples of the 250 and 500 µg/kg treatment groups varied from 37 to 70 and 46 to 96%, respectively, compared with samples from the C group. R2 down-regulation was not observed in tumor samples collected from the 125 µg/kg and Scr treatment groups (i.e. less then 10% down-regulation in each case compared with control). Taken together, the *in vivo* data from A498 tumor xenograft models indicate that the effect of siRNA 1284 on gene expression is sequence-specific and dose-dependent, and siRNA 1284-mediated R2 down-regulation correlates well with anti-tumor activity.

Antitumor activity of siRNA 1284 in other tumor xenograft models

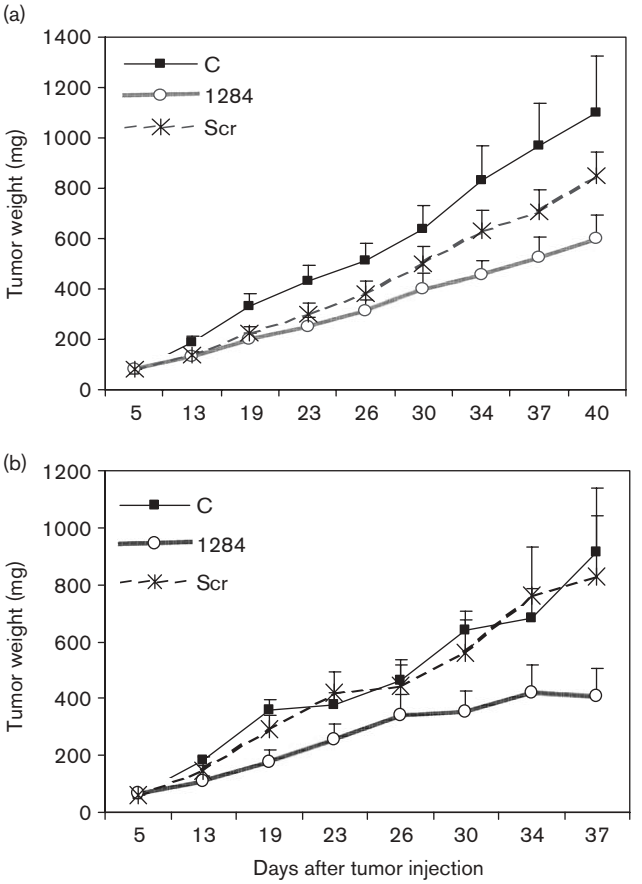
R2 is overexpressed in several cancer cells including A2058 and HT-29 cells [66]. Given the ability of siRNA 1284 to down-regulate R2 in a sequence-specific manner *in vitro* (Fig. 2), the antitumor activity of siRNA 1284 was also examined in both A2058 and HT-29 xenograft models to determine its therapeutic potential in a range of cancer types. A2058 or HT-29 cells were implanted into CD-1 nude mice as described in Materials and methods. Once tumors had developed, 250 µg/kg of siRNAs 1284 or Scr were administered via the tail vein, three times/week. C groups received suspension buffer alone in PBS (see Materials and methods). In both cases, siRNA 1284

Fig. 5



Dose-dependent antitumor effects of short interfering RNA (siRNA) 1284. A498 cells were subcutaneously implanted into the right flank of SCID mice. After tumors reached an approximate volume of 80–90 mm^3, 17 days after tumor cell injection, siRNA 1284 was administered (125, 250 or 500 μg/kg, intravenously, three times per week). The Scr group received 500 μg/kg, intravenously, also three times per week. Control group (C) received suspension buffer alone in PBS. (a) Tumor weight. Mice were sacrificed after 36 days of treatment and tumor weight for each was determined. Mean tumor weight (calculated from 10 animals per experimental group) and SEM are shown for each group. Differences in tumor weight between control and siRNA 1284-treated groups, 250 and 500 μg/kg, were statistically significant with $P<0.001$ or $P<0.0001$, respectively. The difference in tumor weight between Scr and siRNA 1284 (both 500 μg/kg per treatment) was also statistically significant with $P<0.005$. (b) R2 protein expression. Excised tumor samples of a similar size (four per treatment group) were separately homogenized in protein extraction buffer and Western blot analysis was performed as described in Materials and methods. R2 protein data for one tumor sample for each treatment group is shown and GAPDH was used as a loading control. Relative expression level of R2 protein in siRNA treatment groups compared to the expression level in the C group is indicated.

Fig. 6



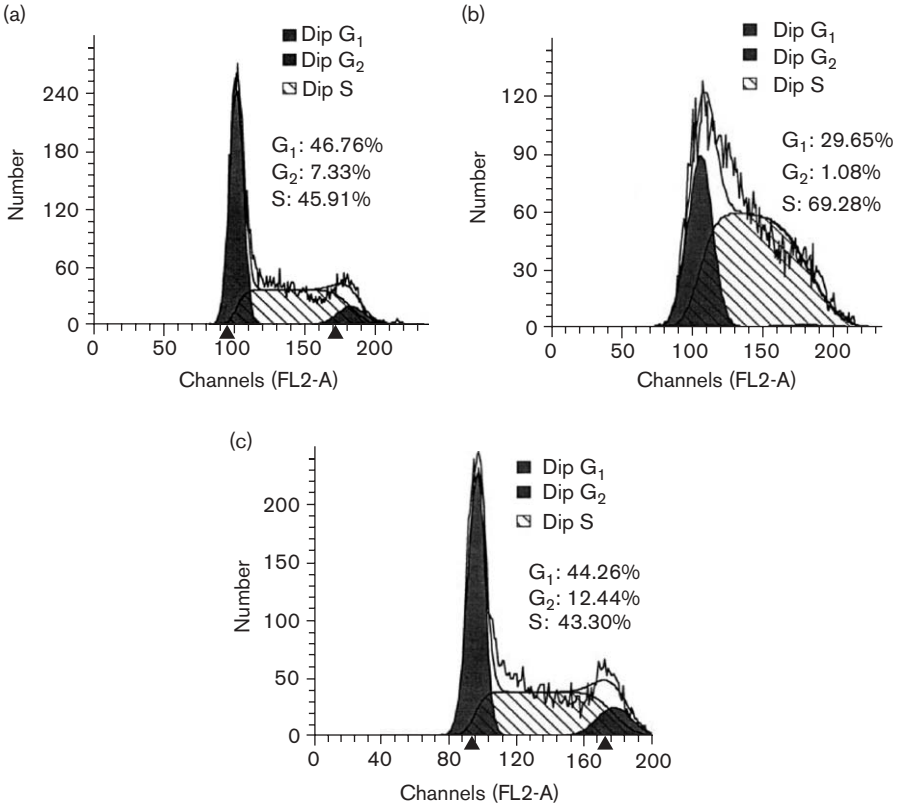
Antitumor effects of short interfering RNA (siRNA) 1284 in HT-29 and A2058 xenografts. HT-29 cells (3×10^6 cells in 100 μl PBS) or A2058 cells (5×10^6 cells in 100 μl PBS) were subcutaneously implanted into the right flank of CD-1 athymic mice. After tumors reached an approximate volume of 70 mm^3, 5 days after tumor cell injection, siRNAs, 1284 or Scr were administered (250 μg/kg, i.v., three times per week). The control group (C) received suspension buffer alone in PBS. Caliper measurements, taken once per week, were used to calculate tumor volumes. Each point represents the mean tumor volume calculated from 10 animals per experimental group. (a) HT-29. Differences in tumor growth kinetics between C or Scr- and siRNA 1284-treated groups were statistically significant with $P<0.0001$ in both cases. (b) A2058. Differences in tumor growth kinetics between C or Scr- and siRNA 1284-treated groups were statistically significant with $P<0.0001$ or $P=0.0001$, respectively.

treatment resulted in a significant inhibition of tumor growth in comparison with C and Scr groups (*P*-values were equal to or less than 0.0001), as measured over the treatment period (Fig. 6). Some nonspecific effect of Scr was evident with HT-29 tumors. A greater effect was, however, observed with siRNA 1284, which was significantly different from that caused by Scr. Considering this effect was not observed in either A2058 or A498 xenograft models, it appears to be tumor cell related.

siRNA 1284 produces a block in S phase of the cell cycle

As one of the two subunits of RNR, R2 is important for the production of deoxyribonucleoside diphosphates that are essential for DNA synthesis and therefore cell proliferation [32,42]. Thus, down-regulation of R2 is likely to deplete cells of their dNTPs and inhibit cell cycle progression. To examine whether the growth inhibitory effect of siRNA 1284 is the result of cell cycle inhibition related to specific R2 down-regulation, cell cycle analysis was conducted. A498 cells were transfected with siRNAs 1284 or Scr, and at 35 h post-transfection cells were collected, fixed and stained with propidium iodide as described in Materials and methods. Flow cytometric analysis indicated that the majority of A498 cells (69.28%) were blocked in S phase when transfected with siRNA 1284. The percentage of cells in S phase for the mock-transfected C and Scr samples were 45.91 and 43.30%, respectively (Fig. 7). Cell cycle experiments were repeated and the data from three such experiments are summarized in Table 1. The mean percentage (± SEM) of A498 cells blocked in G_1, G_2 and S phases of the cell cycle for control, siRNA 1284 and Scr siRNA

Fig. 7



S phase block induced by short interfering RNA (siRNA) 1284. A498 cells were transfected with 25 nmol/l siRNAs, 1284 or Scr in the presence of oligofectamine reagent. Control (C) samples were treated with oligofectamine reagent alone. Cells were transfected for 4 h, washed and subsequently incubated in fresh media. At 35 h posttransfection, cells were fixed, stained with propidium iodide and analyzed by flow cytometry as described in Materials and methods. The cell cycle profiles for (a) control, (b) siRNA 1284- and (c) Scr-treated cells are shown, and the percentages of cells blocked in G_1, G_2 and S are also indicated.

Table 1 Summary of cell cycle data for A498 transfected cells

	Phase of cell cycle[a]		
Sample	S (%)	G_1(%)	G_2(%)
Control	43.13 (±3.33)	46.85 (±2.92)	10.02 (±1.35)
siRNA 1284	64.88 (±2.22)	28.78 (±0.68)	5.34 (±2.64)
Scrambled siRNA	46.91 (±1.81)	40.77 (±2.07)	12.32 (±1.02)

siRNA, short interfering RNA.
[a]Values are mean ± SEM from three separate experiments.

treatments are shown, and further indicate that a block in S phase occurs when cells are transfected with siRNA 1284 (see Table 1). *P*-values were calculated using data from Table 1 and found to be less than 0.05 for the percentage of siRNA 1284 transfected cells in S phase when compared with the percentage of C or Scr siRNA-transfected cells in S phase. Untreated samples were also evaluated and indicate that the presence or absence of oligofectamine reagent did not contribute to the observed alterations in cell cycle progression (data not shown). Sub-G_1 values that represent the apoptotic population within a particular sample were consistently below 1% for all samples suggesting that inhibition of R2 expression by siRNA 1284 does not lead to apoptosis within the time frame evaluated. Early apoptotic events were also not evident when cells were transfected and stained with Annexin V (data not shown).

Discussion

In recent years, with the discovery of RNAi as a gene-silencing mechanism, considerable effort has been made to delineate the RNAi pathway and define the protein complexes involved in mRNA cleavage and silencing. These complexes include an RNase III-like endoribonuclease named Dicer, that cleaves long strands of dsRNA into siRNA duplexes and a second multiprotein complex RISC that contains the catalytic subunit argonant 2 that is responsible for mRNA cleavage [16]. RNAi is viewed as a powerful tool to define gene function, and a novel therapeutic strategy for treatment of human diseases and conditions including cancer. Gene products contributing to the transformation of cells, tumor growth and metastasis are all potential drug targets for RNAi.

In mammalian cells, two forms of RNR exist. The first, consists of the large R1 subunit and a small R2 subunit, and supports S-phase-specific DNA replication [32]. The second RNR consists of a R2 variant, p53R2, that like R2, complexes with R1 to form an active RNR. This RNR supports DNA damage repair [46]. RNR, containing R2, is an essential and rate-limiting enzyme responsible for the production of dNTPs for DNA synthesis and cell proliferation [32]. The R2 protein is a gene product that clearly demonstrates a role in cancer progression. R2 forms a complex with R1, to produce a functional RNR, and R2 expression levels modulate RNR activity during cell proliferation [43]. In addition, R2 is a determinant of malignant potential of human cancers and overexpression contributes to metastasis and resistance of cancer cells to chemotherapy and radiotherapy [51,53,55–58]. R2 as an antitumor target is a suitable candidate for RNAi-mediated gene silencing.

In this study, we have identified a siRNA (siRNA 1284) that targets the R2 mRNA. siRNA 1284 successfully inhibited the expression of R2 in cancer cell lines leading to inhibition of cell proliferation *in vitro*. *In vivo*, siRNA 1284 treatment resulted in a significant inhibition of tumor growth in three different tumor models (renal carcinoma, melanoma and colon adenocarcinoma). Dose-dependent antitumor effects correlating with dose-dependent target down-regulation were also demonstrated in at least one tumor model. The observed inhibition of tumor growth correlated with R2 suppression. In addition, an effect on tumor growth was not evident when mice were treated with a siRNA (different from siRNA 1284) that targets R2, but does not decrease R2 expression (data not shown), clearly demonstrating the importance of R2 down-regulation for the inhibition of tumor growth. Cell cycle analysis indicated that cells transfected with siRNA 1284 were blocked in S phase. Cell cycle arrest was not observed for the C or Scr-transfected cells. This arrest in S phase likely contributes to the observed antiproliferative and antitumor effects in siRNA 1284-treated tumor cells and xenograft models and is a consequence of suppression of R2 and therefore inhibition of RNR activity.

The lack of activity of Scr *in vitro* and *in vivo* (in A2058 and A498 xenograft models) provides further evidence that the antiproliferative effects of siRNA 1284 occur in an R2-specific manner. In one xenograft model (HT-29), however, moderate antitumor activity was evident for Scr treatment groups. This effect is not likely to be mediated through R2, but is likely a nonspecific RNAi effect specific to HT-29 tumors. Two lines of evidence support this statement. First, as discussed above, an effect of Scr on cell cycle progression (i.e. an arrest in S phase characteristic of inhibition of R2 and RNR activity) was not observed when cells were transfected with Scr. Second, R2 protein and mRNA levels were not modified when cells were transfected with Scr. Off-target effects of RNAi on gene expression have been observed in microarray studies [72] and need to be considered in the development of RNAi-based therapeutic drugs. Off-target effects imply silencing of unintended genes. Such nonspecific RNAi effects might occur as a result of siRNA recognition of mRNAs of only partial homology and appear to be concentration dependent. Various algorithms and tools for identifying siRNAs are available to assist in the design of highly effective and specific siRNAs that can be used at low concentrations. The interferon response may also be triggered by siRNAs; however, this response is said to be dependent on the length of the siRNA duplexes such that siRNAs less than 30 bp will not activate interferon. An interferon response is more likely to be a consequence of short hairpin RNA vector delivery, discussed below [73,74]. It is currently not clear why a nonspecific RNAi effect was observed only in the HT-29 xenograft model.

Recently Duxbury *et al.* [65] identified a siRNA, different from siRNA 1284, that suppresses R2 expression in tumors and enhances chemosensitivity of pancreatic adenocarcinoma models to gemcitabine. Retroviral delivery of this same siRNA suppressed invasive potential and gemcitabine resistance of pancreatic adenocarcinoma cells *in vitro* [64]. These studies further validate the therapeutic potential of a siRNA that suppresses R2. Antisense oligonucleotides (ODNs) are also potent gene silencers. Indeed, Lee *et al.* [66] demonstrated that an ODN, GTI-2040, containing a phosphorothioate backbone and directed against R2 mRNA, is highly effective in suppressing tumor growth and metastasis [66]. siRNAs could potentially be used at concentrations of 100–1000-fold less than phosphorothioate ODNs directed against the same target [75,76]. Although direct comparison was not made, substantial antitumor efficacy with siRNA 1284 was obtained at a lower dose than that used for GTI-2040, suggesting that therapeutic use of siRNA may have the advantage of being required in lower and therefore less toxic doses, than phosphorothioate oligonucleotide antisense strategies. Clinical usefulness, however, will depend largely on the identification of an efficient delivery system for siRNA.

A major hurdle in the development of RNAi-based therapeutics is in the delivery of the siRNAs to the target tissues and organs. Among many approaches to siRNA delivery, two have received the most attention [77–82]. The first involves a gene therapeutic approach in which viruses (retro, lenti and adeno) have been engineered to deliver and stably express short hairpin RNAs (shRNAs) or precursors to siRNAs from viral expression vectors. Limitations to vector-based siRNA delivery, however, include the possibility of insertional

mutagenesis and malignant transformation. The second approach to siRNA delivery involves their encasement in lipid complexes or other types of delivery complexes. The use of siRNAs over small hairpin RNA-based vectors would avoid problems associated with viral expression vectors, however, stability and uptake of encased siRNAs in mammalian cells is poor. Various groups are currently exploring the possibility of chemically modifying siRNA duplexes, to make them more stable and resistant to RNases [83–85]. In our study, siRNA 1284 showed potent antitumor activity when administered as a naked dsRNA complex upon intravenous bolus injection. Although highly effective in preclinical animal models, chemical modification of siRNA 1284, along with the identification of an efficient delivery system, may be required to achieve the same efficacy in the clinic.

The excellent efficacy of siRNA 1284 described in this study underscores the therapeutic potential of an RNAi-based agent to target R2 that contributes to tumor formation and cancer progression. With the advancement of RNAi research, a better understanding of siRNA modifications and delivery systems will likely bring siRNA 1284 closer to the clinic.

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