

## Potent siRNA Inhibitors of Ribonucleotide Reductase Subunit RRM2 Reduce Cell Proliferation *In vitro* and *In vivo*

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**Abstract Purpose:** Ribonucleotide reductase (RR) is a therapeutic target for DNA replication – dependent diseases such as cancer. Here, a potent small interfering RNA (siRNA) duplex against the M2 subunit of RR (RRM2) is developed and shown to reduce the growth potential of cancer cells both *in vitro* and *in vivo*.

**Experimental Design:** Three anti-RRM2 siRNAs were identified via computational methods, and the potency of these and additional “tiling” duplexes was analyzed in cultured cells via cotransfections using a RRM2-luciferase fusion construct. Knockdown of RRM2 by the best duplex candidates was confirmed directly by Western blotting. The effect of potent duplexes on cell growth was investigated by a real-time cell electronic sensing assay. Finally, duplex performance was tested *in vivo* in luciferase-expressing cells via whole animal bioluminescence imaging.

**Results:** Moderate anti-RRM2 effects are observed from the three duplexes identified by computational methods. However, the tiling experiments yielded an extremely potent duplex (siR2B+5). This duplex achieves significant knockdown of RRM2 protein in cultured cells and has pronounced antiproliferative activity. S.c. tumors of cells that had been transfected with siR2B+5 preinjection grew slower than those of control cells.

**Conclusions:** An anti-RRM2 siRNA duplex is identified that exhibits significant antiproliferative activity in cancer cells of varying human type and species (mouse, rat, monkey); these findings suggest that this duplex is a promising candidate for therapeutic development.

Ribonucleotide reductase (RR) catalyzes the conversion of ribonucleoside 5'-diphosphates into their corresponding 2'-deoxyribonucleotides and is a rate-limiting step in the pathway for the production of 2'-deoxyribonucleoside 5'-triphosphates that are necessary for DNA replication. Human RR consists of two subunits, RRM1 and RRM2, and the expression of both proteins is required for enzymatic activity. RRM1 and RRM2 are encoded by different genes on separate chromosomes, and their mRNAs are differentially expressed throughout the cell cycle. The cellular RRM1 protein level is kept relatively stable through the entire cell cycle, whereas RRM2 is only expressed during the late G<sub>1</sub>/early S phase when DNA replication occurs (1).

RR has long been an important target for controlling pathologies that depend on DNA replication. Inhibition of RR activity has been tested as potential therapy in anticancer, antiviral, antibacterial, and other antiparasitic settings. A recent review on RR inhibitors as anticancer agents has appeared (2). Numerous small-molecule inhibitors, such as hydroxyurea (approved for human use) and triapine (3), interact with subunit RRM2. These small molecules are not completely specific to the RRM2 protein, however, and work continues on the design of more specific and effective inhibitors (4).

A recent approach to RR down-regulation involves the use of nucleic acid-based, gene-specific inhibition. Yen et al. (5) and Lee et al. (6) have shown that antisense molecules to RRM2 can significantly reduce the growth of human cancer cells both *in vitro* and *in vivo*. Using GTI-2040, a 20-nucleotide phosphorothioate oligodeoxyribonucleotide that has been reported to inhibit the production of RRM2 (at a concentration of 200 nmol/L) *in vitro* (7), Lee et al. (6) showed significant inhibition of s.c. tumors in nude mice of human colon, pancreas, liver, lung, breast, kidney, ovary, brain, and prostate cancer cells. These researchers also showed that RRM2 protein levels are elevated in cancer cell lines, and their results are consistent with earlier studies that revealed increased levels of RR in tumors and tumor cell lines (8). The concept of using RRM2 mRNA inhibition as an anticancer strategy in humans is being tested by workers at Lorus Therapeutics (9). The results of a phase I trial of GTI-2040 given in patients with advanced solid tumors showed a manageable toxicity profile, and that it

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was generally well tolerated (10), leading to a phase I/II trial, in which GTI-2040 is given in combination with capecitabine to patients with metastatic renal cell carcinoma (11).

Small interfering RNA (siRNA) duplexes are another class of nucleic acids capable of achieving potent, sequence-specific inhibition of gene expression; indeed, their mechanism of action, RNA interference, is quickly becoming the method of choice for the regulation of gene expression over antisense, ribozyme, or DNAzyme technologies (12). Lin et al. (13) reported that suppression of RRM2 by siRNA sensitizes HCT-116 cells to DNA-damaging agents and RR inhibitors. Also, Whang et al. (14) have used siRNA against RRM2 to enhance pancreatic adenocarcinoma chemosensitivity to gemcitabine and retrovirally expressed siRNA against RRM2 to attenuate pancreatic adenocarcinoma cellular invasiveness and diminish its gemcitabine resistance (15). These reports provide motivation for further study of siRNA-mediated inhibition of RRM2 because potent sequences could provide for specific and effective therapies.

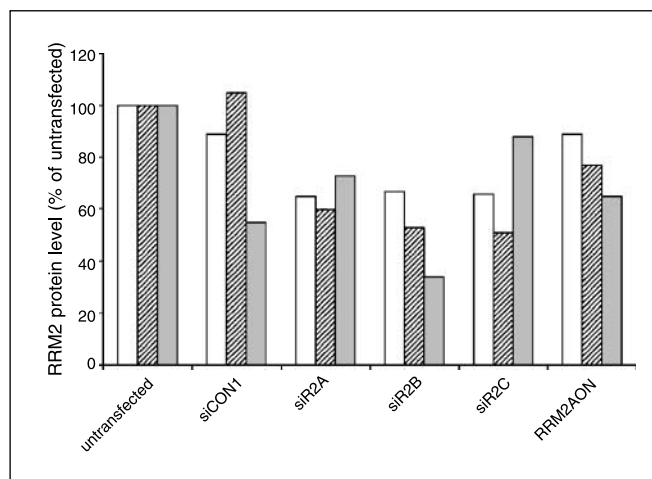
Here, we identify several potent siRNA sequences for RRM2 inhibition and show that these sequences can provide specific and effective silencing of the RRM2 protein. In addition, we show that sequence-specific RRM2 inhibition leads to growth suppression in human cancer cells *in vitro* and tumor growth suppression *in vivo*. Finally, we show that the most potent siRNA sequence found in this work shows complete target site homology and achieves growth suppression of mouse, rat, and monkey cell lines.

## Materials and Methods

**Algorithm for initial target site selection, siRNA duplexes.** The mRNA sequence of human RRM2, denoted hRRM2 (Genbank accession number NM\_001034), was probed for preferred 21-nucleotide target sites based on end-energy differential and predicted mRNA secondary structure using a previously described algorithm (16). Candidates were rejected on the basis of GC content and homology with sequences in additional (off-target) genes (identified via BLAST–Basic Local Alignment Search Tool–search). Three initial candidates (“siR2A,” “siR2B,” and “siR2C”) were tested; numerous duplexes in the immediate vicinities of these candidates were subsequently analyzed (tiling experiment). Complete siRNA sequence information can be found in Supplementary Table S1.

**Cell lines.** The following cell lines were obtained from the American Type Culture Collection (Manassas, VA): HeLa (human cervical adenocarcinoma), HT-29 (human colorectal adenocarcinoma), HepG2 (human hepatocellular carcinoma), Hep3B (human hepatocellular carcinoma), Neuro2A (mouse neuroblastoma), CMMT (monkey mammary gland), and McA-RH7777 (rat hepatoma). Canine osteosarcoma (BW.KOSA.P) cells were the generous gift of Melissa Paoloni (National Cancer Institute, Bethesda, MD). Neuro2A-Luc cells constitutively express firefly luciferase as the result of viral transduction as described previously (17). All cells were cultured in appropriate medium (DMEM, MEM, or McCoy's 5A) supplemented with antibiotics/antimycotics and 10% fetal bovine serum.

**siRNA.** Many of the siRNA duplexes used here were provided as annealed, desalted duplexes from Integrated DNA Technologies (IDT, Coralville, IA); IDT also made the “RRM2AON” antisense molecule. It should be noted that throughout this manuscript, RRM2AON refers to an antisense molecule having the same sequence and linker chemistry as Lorius' GTI-2040 therapeutic (9). All of the duplexes used in the tiling experiments were provided as pairs of single strands by the DNA/RNA and Peptide Synthesis Laboratory of the Beckman Research Institute at the City of Hope (Duarte, CA) that were subsequently annealed.



**Fig. 1.** The levels of RRM2 protein in HeLa (open columns), HepG2 (hatched columns), and Hep3B (solid columns) cells that were lipofected with nontargeting control siRNA (siCON1), one of the three siRNAs against RRM2 (siR2A, siR2B, siR2C), or an antisense deoxynucleotide against RRM2 (GTI-2040) were measured. Each nucleic acid was lipofected at a concentration of 50 nmol/L. At 48 h post-transfection, cells were lysed, and RRM2 levels were measured by Western blotting and quantified via band densitometry. The siRNAs against RRM2 elicited modest reductions in RRM2 protein levels in these three cell lines.

**Transfection of cultured cells with siRNA against hRRM2.** Transfections of inhibitory nucleic acids (siRNA or the RRM2AON antisense oligo) alone were done using either OligofectAMINE or LipofectAMINE RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were typically seeded in six-well plates (250,000 cells per well) 24 h before transfection. Lipoplexes with nucleic acids were prepared in serum-free OptiMEM medium (Invitrogen). Immediately before transfection, complete growth medium was removed from all wells, and wells were rinsed with PBS. Cells were typically exposed to lipoplexes for 4 h before replacement of complete growth medium or trypsinization and replating of the cells, depending on the particular experiment. The concentration of each nucleic acid within lipoplexes is indicated in each experiment.

To accelerate screening of anti-RRM2 siRNAs, a plasmid encoding an hRRM2-luciferase fusion protein (pR2Luc) was prepared. The cDNA of the human RRM2 gene was subcloned into the pApoEHCLuc plasmid that contains the firefly luciferase gene under the control of the liver-specific human  $\alpha_1$ -antitrypsin promoter and apolipoprotein E heterologous control region (18). Cotransfections of pR2Luc and siRNA were done using Lipofectin (Invitrogen) according to the manufacturer's instructions. The levels of fusion protein in lysates of transfected cells were determined using the Luciferase Assay System (Promega, Madison, WI).

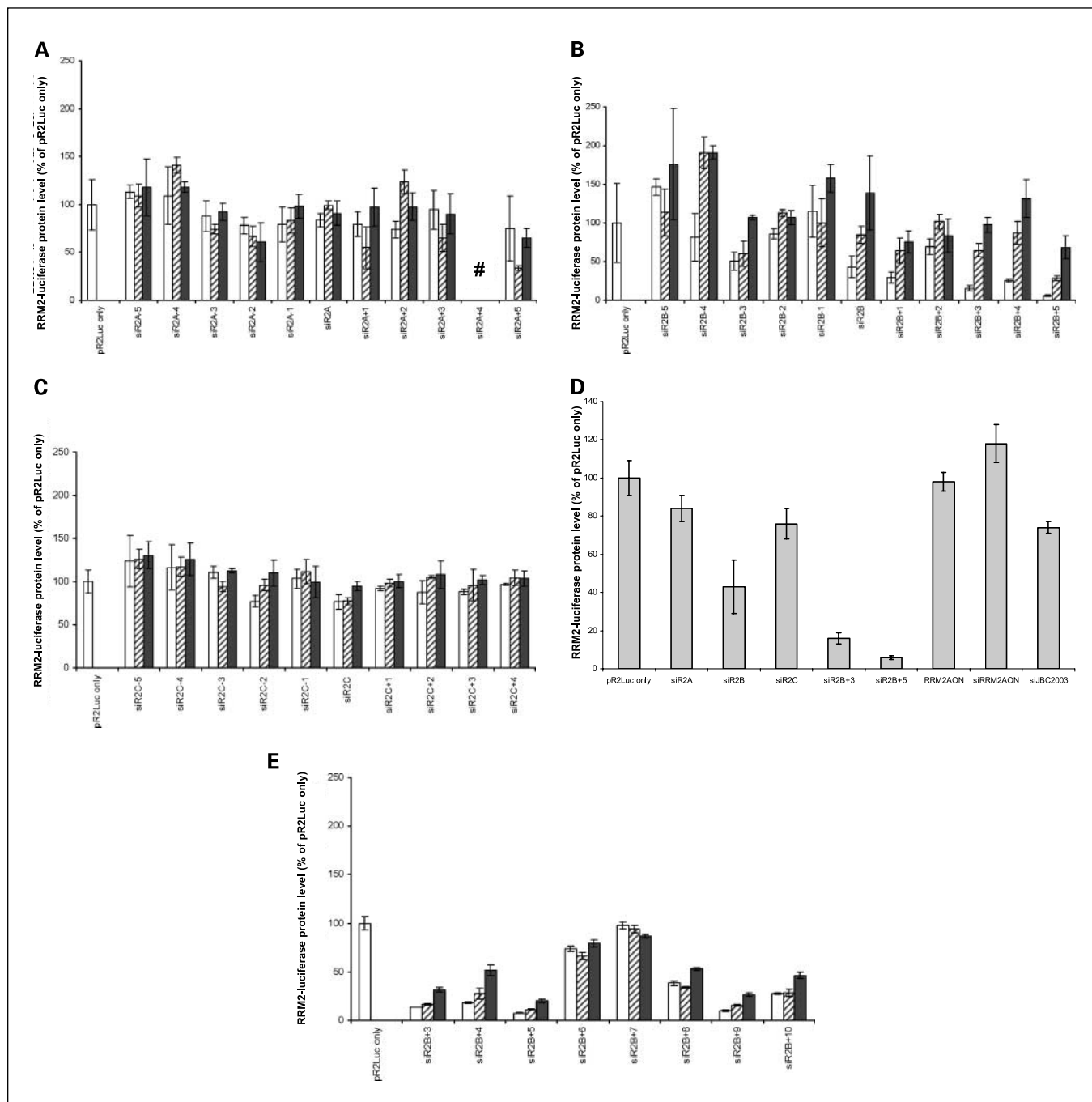
For the tiling experiments,  $5 \times 10^4$  HepG2 cells were seeded per well of a 24-well plate 24 h before transfection. Each well received lipoplexes containing 1  $\mu$ g pR2Luc and siRNA at a concentration of 10, 1, or 0.2 nmol/L as indicated. Lipoplexes were prepared in serum-free OptiMEM medium (Invitrogen). Immediately before transfection, complete growth medium was removed from all wells, and wells were rinsed with PBS. Cells were typically exposed to lipoplexes for 4 h before replacement of complete growth medium.

**Detection of hRRM2 protein levels by Western blot.** At indicated times, transfected cells (in six-well plates) were lysed with  $1 \times$  Cell Culture Lysis Reagent (Promega). Lysates were purified using QIAshredder spin columns (Qiagen, Valencia, CA), and total protein concentrations were determined using the DC Protein Assay (Bio-Rad, Hercules, CA). For each particular experiment, lysates were diluted to the same protein concentration and denatured via addition of  $\beta$ -mercaptoethanol-containing Laemmli sample buffer (Bio-Rad) and incubation at  $100^\circ\text{C}$  for 5 min. The primary antibody was a goat

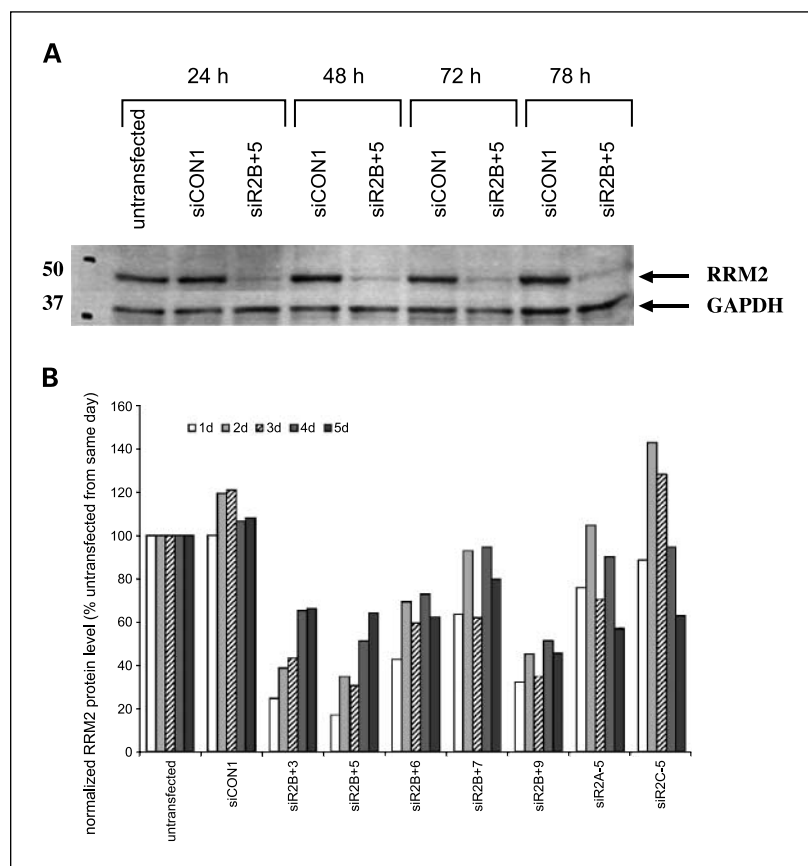
polyclonal anti-R2 antibody (sc-10846; Santa Cruz Biotechnology, Santa Cruz, CA), 1:250 dilution. The secondary antibody was an horseradish peroxidase-conjugated donkey anti-goat immunoglobulin G (Santa Cruz Biotechnology), 1:5,000 dilution. Development was done using the enhanced chemiluminescence detection kit (GE/Amersham Biosciences); band quantification was done using ImageQuant TL software (GE/Amersham Biosciences, Piscataway, NJ).

Normalization of hRRM2 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was done by stripping the hRRM2-probed membrane (REstore stripping buffer; Pierce, Rockford, IL) and reprobing with a goat polyclonal anti-GAPDH antibody (sc-20357, Santa Cruz Biotechnology) as above.

**Cell growth assay (real-time cell electronic sensing).** Cells were plated in six-well plates and lipofected with siRNA (20 nmol/L) as



**Fig. 2.** The levels of RRM2-luciferase fusion protein in HepG2 cells cotransfected with pR2Luc and one of various siRNAs at a concentration of 10 nmol/L (open columns), 1 nmol/L (hatched columns), and 0.2 nmol/L (solid columns) cells were measured. At 48 h post-transfection, cells were lysed, and fusion protein levels were measured by luciferase assay. **A**, siRNAs around site A were examined. **B**, siRNAs around site B were examined. **C**, siRNAs around site C were examined. One of the siRNAs examined, siR2B+5, showed superior down-regulation compared with all of the others initially tested. **D**, both siR2B+3 and siR2B+5 showed superior down-regulation of R2-luciferase protein to the original three siRNAs against RRM2, as well as the RRM2AON antisense oligo, an siRNA having the same target site as RRM2AON (siRRM2AON), and a previously published siRNA against RRM2 (siJBC2003). All nucleic acids were at a concentration of 10 nmol/L. **E**, additional siRNAs around site B were examined. Of all siRNAs evaluated, siR2B+5 showed the most potent RRM2-luciferase down-regulation. Columns, average of three replicate wells; bars, SD. #, not determined.



**Fig. 3.** *A*, the levels of endogenous RRM2 protein (normalized to GAPDH) in Hep3B cells that were untransfected or transfected with siR2B+5 or a nontargeting control siRNA (siCON1) at a concentration of 20 nmol/L were measured. RRM2 levels were examined via Western blot at  $t = 24, 48, 72,$  and  $78$  h post-transfection. Very strong sequence-specific reduction of RRM2 protein levels was seen at all time points. *B*, the levels of endogenous RRM2 protein (normalized to GAPDH) in Hep3B cells were determined at  $t = 1, 2, 3, 4,$  and  $5$  d post-transfection. Cells were untransfected or transfected with siR2B+3, siR2B+5, siR2B+6, siR2B+7, siR2B+9, siR2A-5, or siR2C-5 at a concentration of 20 nmol/L. RRM2 and GAPDH band intensities were determined by densitometry, and the ratio of RRM2/GAPDH intensities was calculated and normalized to that determined for untransfected cells at each time point. siR2B+3, siR2B+5, and siR2B+9 showed reduced relative RRM2 levels compared with other treatments at early time points (through 3 d), with siR2B+5 inducing the greatest extent of down-regulation at each of these time points, but the differences between these treatments and the others were no longer evident by the final (5 d) time point.

described above. After exposure to lipoplexes for 4 h, cells were trypsinized, counted, and replated into wells of 16-well devices compatible with a W200 real-time cell electronic sensing (RT-CES) analyzer and 16 $\times$  station (Acea Biosciences, San Diego, CA). Cell growth was monitored periodically (typically, every 0.5 or 1 h) for indicated durations via calculation of a "cell index" (normalized impedance) for each well. Unless otherwise indicated, cells from each well of the original six-well plates were replated into three replicate wells for cell index measurement.

**HPTV injections of mice with pR2Luc alone or with siRNA.** Female BALB/c mice (6-8 weeks of age; The Jackson Laboratory, Bar Harbor, ME) received a single high-pressure tail vein (HPTV) injection. A solution of pR2Luc (either alone or with an indicated siRNA) was prepared in D5W (5% wt/vol glucose in water) such that a 10% (vol/wt) injection provided doses of 0.25 and 2.5 mg/kg for pR2Luc and siRNA, respectively. Before injection, mouse tails were warmed with a heating pad; injections ( $\sim 2$  mL for a  $\sim 20$ -g mouse) were made over  $\sim 3$  to 5 s. Four groups of five mice received pR2Luc alone, pR2Luc + siCON1 (nontargeting control siRNA), pR2Luc + Luc105-21 (anti-luciferase siRNA), or pR2Luc + siR2B+5, respectively. To determine RRM2-luciferase protein levels, 0.2 mL of a 15 mg/mL solution of D-luciferin (Xenogen, Alameda, CA; in PBS) was injected i.p. 10 min before imaging. Bioluminescence signals were quantified using LivingImage software (Xenogen); imaging was done 2, 6, 11, and 17 days postinjection.

**Growth of s.c. Neuro2A-Luc tumors (prelipofected with siRNA) in A/J mice.** Neuro2A-Luc cells were grown in T75 flasks and either untransfected or lipofected with siCON1, siR2B+5, or siR2B+6 (20 nmol/L) as described above. After 4 h exposure to lipoplexes, cells were trypsinized, counted, centrifuged, and resuspended at a concentration of  $4 \times 10^6$  cells/mL in DMEM without serum or antibiotics.

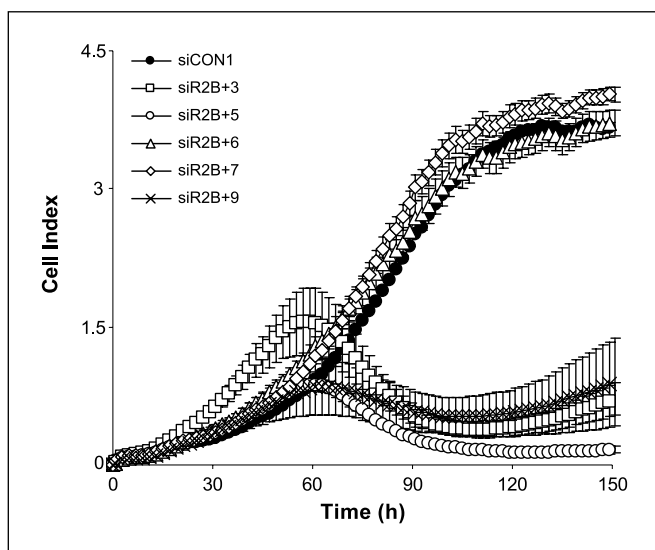
Four groups of female A/J mice (8 weeks of age; The Jackson Laboratory) then received a single injection (0.5 mL,  $2 \times 10^6$  cells) of the suspension of cells treated with siR2B+5 or siR2B+6, respectively, in the right flank (all groups consisted of five mice, except that only four mice received cells transfected with siR2B+6). To monitor tumor growth over time, 0.2 mL of a 15 mg/mL solution of D-luciferin (Xenogen; in PBS) was injected i.p. 10 min before imaging. Bioluminescence signals were quantified using Living Image software (Xenogen); imaging was done 2, 4, 6, 8, 10, and 13 days postinjection.

**Statistical analyses.** Statistical evaluations of significance in this work were done using Student's  $t$  test (paired, two-tailed), with  $P < 0.05$  considered statistically significant.

## Results

**Three initial siRNAs achieve similar, modest levels of RRM2 knockdown in various human cancer cell lines.** Exploration of the hRRM2 mRNA sequence for potential siRNA targets revealed three candidates for which siRNA duplexes were synthesized (siR2A, siR2B, and siR2C). Sequence information for these duplexes, and all other nucleic acid molecules used in this paper, can be found in Supplementary Table S1. Each of these three siRNAs was lipofected into three different human cell lines (HeLa, HepG2, and Hep3B) and RRM2 protein knockdown was evaluated 48 h later by Western blotting. RRM2 bands were quantified for cells that were either untransfected or transfected with 50 nmol/L of nontargeting control siRNA (siCON1), an antisense molecule against RRM2 (RRM2AON), or these three siRNAs against RRM2; the results





**Fig. 4.** HT-29 cells were transfected with each of five siRNAs (siR2B+3, siR2B+5, siR2B+6, siR2B+7, and siR2B+9; each at 20 nmol/L) for 4 h and then replated into a device that allows real-time measurement of well impedance (presented as cell index as described in ref. 19; measurements made every 1 h after replating). Duplexes that were previously shown to be more potent down-regulators of RRM2 (siR2B+3, siR2B+5, siR2B+9) reduced the growth of cells compared with a nontargeting control duplex (siCON1) and duplexes, which were poor RRM2 down-regulators (siR2B+6 and siR2B+7). Columns, the average of three replicate wells; bars, SD. Analogous plots for other cell lines (Neuro2A, CMMT, HepG2, BW.KOSA.P, and McA-RH7777) can be found in Supplementary Fig. S1.

are summarized in Fig. 1. In nearly all cases, each of the siRNAs against RRM2 elicited modest reduction of intracellular RRM2 protein levels compared with untreated cells and those treated with siCON1 or RRM2AON. These results confirm that this algorithm is suitable for preliminary identification of siRNA duplexes that achieve down-regulation of the desired target protein.

**Tiling experiment reveals more potent duplexes in vicinity of those initially identified.** In an attempt to identify possible siRNA duplexes with higher potency for RRM2 knockdown than the original three, a number of siRNAs (at least 10 new duplexes) immediately upstream and downstream of each of the original three anti-RRM2 duplexes were synthesized. To assist in the evaluation of these new duplexes, an expression construct for a RRM2-luciferase fusion protein was prepared (pR2Luc). Cotransfection of human hepatocytes with siRNA and the resulting fusion construct (pR2Luc) allowed for

measurement of firefly luciferase levels as a surrogate for RRM2 knockdown.

HepG2 cells were cotransfected with pR2Luc and each of the aforementioned tiling siRNAs around target sites A, B, and C at each of three concentrations (10, 1, and 0.2 nmol/L). The firefly luciferase expression in lysates 48 h post-transfection was measured and is summarized in Fig. 2A to C. Two of the duplexes around site B (siR2B+3 and siR2B+5) showed superior potency to the other duplexes. These two duplexes were compared head to head against an siRNA having the same target site as RRM2AON (siRRM2AON) and a previously published siRNA against RRM2 (siJBC2003). As shown by the data given in Fig. 2D, siR2B+5 displayed the most potent knockdown of endogenous RRM2 of all duplexes tested. Note that siRRM2AON does not provide for down-regulation, demonstrating that a good antisense target site is not necessarily also a good target site for siRNA. The observations obtained from the data in Fig. 2A to D led to the evaluation of additional duplexes around site B (siR2B+6 through siR2B+10). As shown by the data listed in Fig. 2E, siR2B+5 achieved the most significant knockdown of the RRM2-luciferase fusion protein of all duplexes tested, although siR2B+9 and siR2B+3 also were highly potent.

**siR2B+5 achieves potent knockdown of endogenous RRM2 in human (Hep3B) cells.** Having amassed this fusion construct knockdown data for dozens of siRNAs, a correlation in the ability of these duplexes to achieve down-regulation of both the RRM2-luciferase fusion target and the endogenous RRM2 target must be shown. The most potent siRNA duplex tested in cotransfection studies with pR2Luc, siR2B+5, was then examined for its ability to knockdown endogenous RRM2 protein in Hep3B cells (Fig. 3A). Cells were lipofected with 20 nmol/L siRNA and lysed at various time points (24, 48, 72, and 78 h) post-transfection; RRM2 levels were quantified by Western blot. In comparison to untransfected cells and cells transfected with a nontargeting control siRNA (siCON1), siR2B+5 elicited strong knockdown of the RRM2 protein at all time points examined.

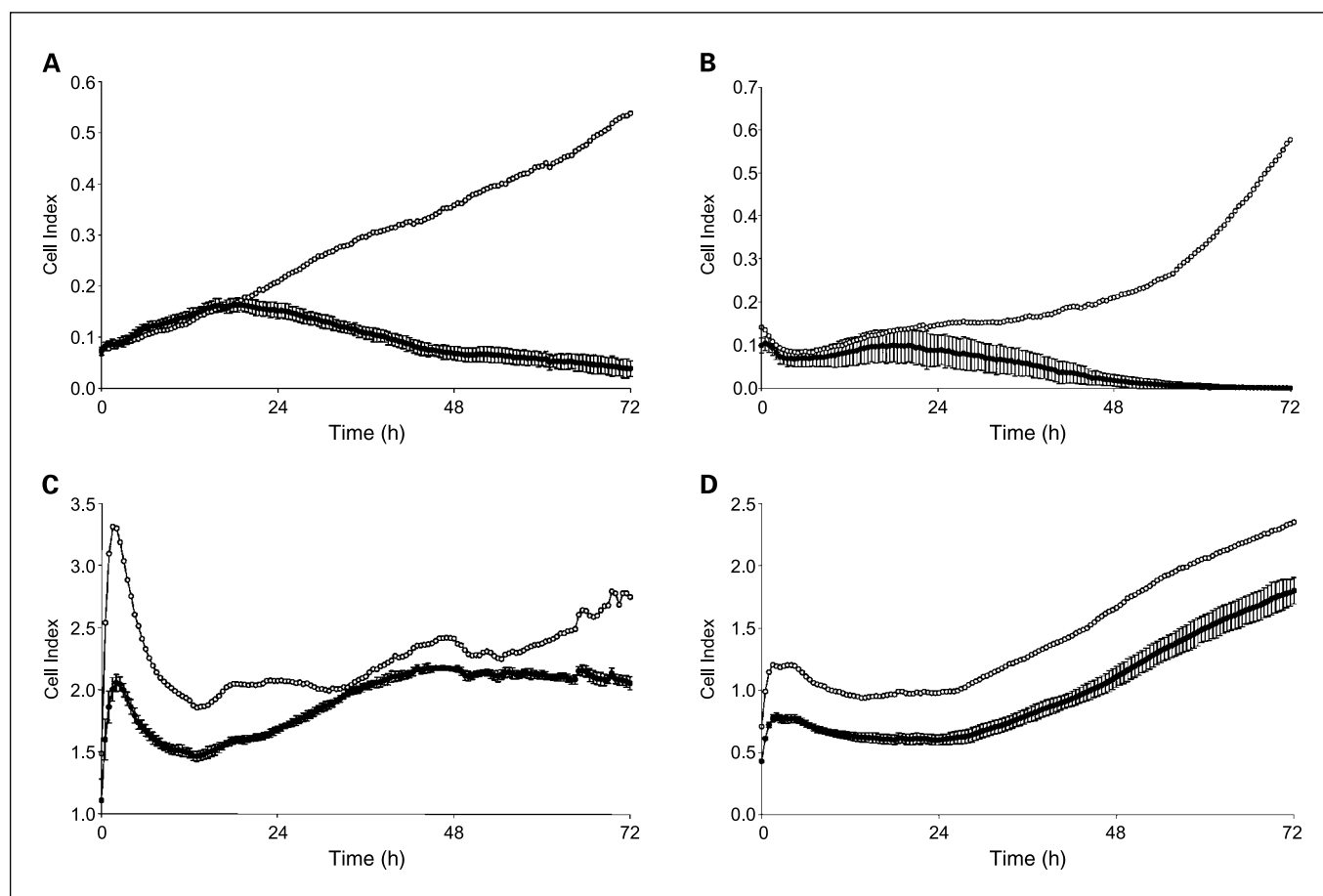
This experiment was then expanded to include additional time points (1, 2, 3, 4, and 5 days) and duplexes (siCON1, siR2B+3, siR2B+5, siR2B+6, siR2B+7, siR2B+9, siR2A-5, and siR2C-5); band density was quantified and normalized and is presented in Fig. 3B. siR2B+3, siR2B+5, and siR2B+9 showed reduced relative RRM2 levels compared with other treatments at early time points (through 3 days), with siR2B+5 inducing the greatest extent of down-regulation at each of these time points, but the differences between these treatments and the others were no longer evident by the final (5 days) time point.

**Table 1.** The homology of target site (within RRM2 mRNA) of the siR2B+5 duplex was explored across various species

Species	Genbank accession number	Target site location	Target sequence	Homology (to human)
Human	NM_001034	nt 637-655	5' gauuuagccaagaaguca 3'	19/19 (100%)
Mouse	NM_009104	nt 500-518	5' gauuuagccaagaaguca 3'	19/19 (100%)
Rat	NM_001025740	nt 497-515	5' gauuuagccaagaaguca 3'	19/19 (100%)
Dog	XM_540076	nt 488-506	5' <u>g</u> guuuagccaagaaguca 3'	18/19 (94.7%)
Monkey	*	nt 40285-40303	5' gauuuagccaagaaguca 3'	19/19 (100%)

NOTE: The mismatched base in the canine sequence is identified by an underline.

\*Sequence alignment was done between hRRM2 mRNA and a Rhesus sequence scaffold (10568:34896-42414) at <http://genome.ucsc.edu/>.



**Fig. 5.** Cells were transfected with a potent anti-RRM2 siRNA (siR2B+5, ●) or a nontargeting control siRNA (siCON1, ○), each at 10 nmol/L, for 4 h and then replated into a device that allows real-time measurement of well impedance (presented as cell index as described in ref. 19; measurements made every 0.5 h after replating). *A*, mouse (Neuro2A) cells. *B*, rat (McA-RH7777) cells. *C*, monkey (CMMT) cells. *D*, dog (BW.KOSA.P) cells. As predicted from results in human cells (see Fig. 4) and sequence homology analysis (see Table 1), siR2B+5 achieves significant reduction in the proliferation of mouse, rat, and monkey cells. In contrast, siR2B+5 shows no evidence of antiproliferation in dog cells, suggesting that the mismatch that exists between the sequence of siR2B+5 and the canine RRM2 mRNA sequence may alter mRNA secondary structure in such a way that renders it a poor target site for siR2B+5. Points, average cell index of three replicate wells; bars, SD.

The results from these experiments show that inhibition of the RRM2 protein can persist for many days from a single transfection, confirm the utility of the R2-luciferase fusion construct in the initial siRNA screening process, and show that the siR2B+5 duplex is a potent inhibitor of RRM2.

**Knockdown of RRM2 in human cells via siR2B+5 reduces cell growth potential.** The ultimate objective of finding a potent anti-RRM2 siRNA duplex is to use it as a therapeutic to treat human cancer. Consequently, whereas strong knockdown of the RRM2 protein within human cancer cells is certainly a critical requirement for such a duplex, so is a concomitant reduction in cellular growth. The ability of siR2B+5 to promote growth arrest of human cells was examined via real-time monitoring of cell viability.

Real-time monitoring of cell viability was done using an RT-CES instrument (Acea Biosciences; ref. 19). HT-29 cells were lipofected in six-well plates with each of five duplexes (siR2B+3, siR2B+5, siR2B+6, siR2B+7, and siR2B+9) at 20 nmol/L. At 4 h postlipofection, the cells were trypsinized, counted, and replated into RT-CES devices (10,000 cells per well equivalent to that of a 96-well plate). Cell viability was determined as a cell index calculated from the impedance of each well measured

every 0.5 h for ~6 days thereafter. Transfection with siR2B+3, siR2B+5, or siR2B+9 led to a significant reduction in cell index as compared with siR2B+6 or siR2B+7 (Fig. 4). These results suggest that duplex potency (RRM2 down-regulation) strongly correlates to its ability to reduce the growth of human cancer cells.

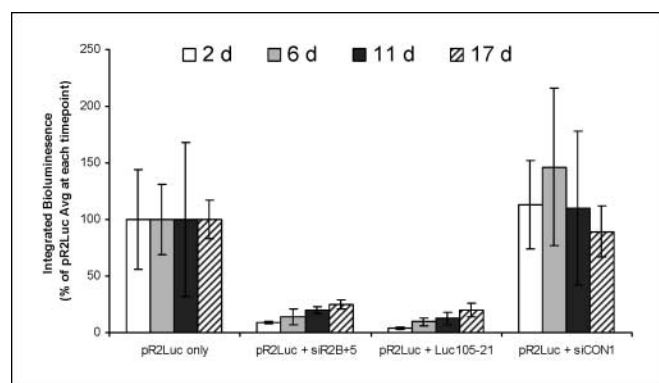
**siR2B+5 sequence homology and effect on growth potential across species.** Although initial experimental investigations of siR2B+5 were limited to human cells, the homology of the 19-nucleotide target site to the R2 mRNA of other species was explored. A summary of the target site homology across a number of species can be found in Table 1.

Complete (19/19 nucleotides) homology was found between human and mouse, rat, and rhesus orthologs; this suggests that siR2B+5 may be able to achieve down-regulation of RRM2 in cells of these species. In contrast, incomplete homology (18/19 nucleotides) was found between human and dog, although the mismatch site (base 2 with respect to the 5' end of the target sequence) is at a location that is expected to have minimal, if any, impact on siRNA potency (20). Similar homology investigations were done for other siRNA duplexes (siR2B+3, siR2B+6, siR2B+7, and siR2B+9); the results are listed in Supplementary Table S2.

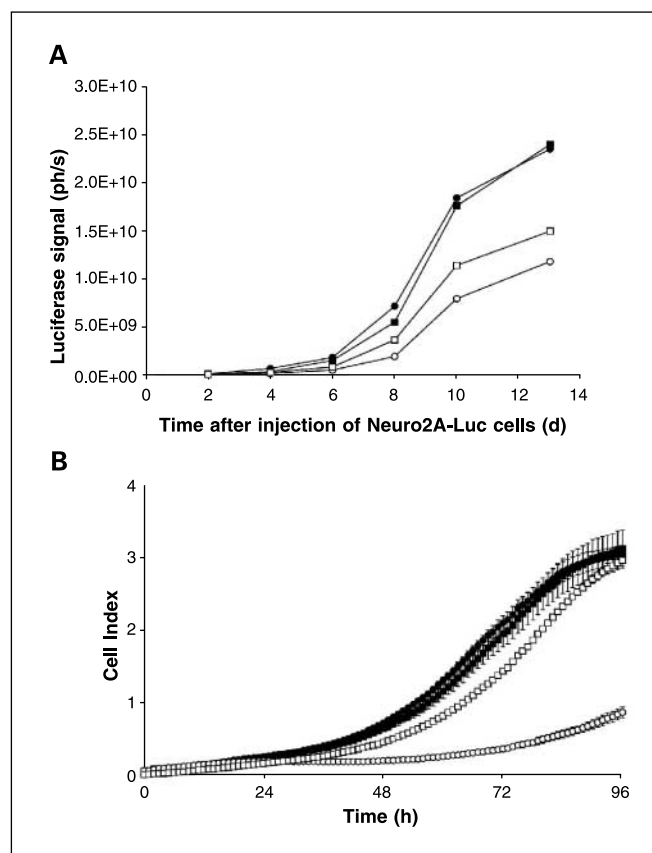
To determine the effects of these duplexes on the growth of cells of other species, RT-CES cell growth profiles were established for mouse (Neuro2A), rat (McA-RH7777), dog (BW.KOSA.P), and monkey (CMMT) cells transfected with a variety of anti-RRM2 siRNAs. The results obtained for the siR2B+5 duplex are shown in Fig. 5; those for all other duplexes are presented in Supplementary Fig. S1. Significant reduction in cell growth (versus untransfected cells) is observed for siR2B+5 in mouse, rat, and monkey cells; however, such reduction is not observed in dog cells. We hypothesize that the mismatch between siR2B+5 and dog RRM2 mRNA may cause an alteration in mRNA secondary structure that makes it a poor target site for siR2B+5. These results suggest that both the rat and the mouse might be suitable species in which to conduct preclinical evaluations of formulations containing siR2B+5. Similarly, monkeys, but not dogs, would be appropriate large animal species for these evaluations.

**siR2B+5 reduces expression of coadministered RRM2-luciferase fusion gene in murine liver.** With the ability of siR2B+5 to reduce RRM2 protein levels and the growth rates of cultured cells having been established, the function of this siRNA *in vivo* was then evaluated. Female BALB/c mice received a single high-pressure (hydrodynamic) tail vein injection of pR2Luc alone or in combination with siR2B+5, a nontargeting control duplex (siCON1), or an siRNA against luciferase (Luc105-21). The expression of the RRM2-luciferase fusion protein in the liver was monitored over time via whole animal bioluminescence imaging (Fig. 6). Coinjection of pR2Luc with siCON1 showed insignificant differences in expression in comparison to injection of pR2Luc alone. In contrast, coinjection with siR2B+5 showed marked (up to >90%) signal reduction that was comparable to that of Luc105-21 (positive control) and persisted over the length of imaging (17 days postinjection). These results indicate that siR2B+5 can function in the *in vivo* environment, here, within mouse hepatocytes.

**Transfection with siR2B+5 before injection reduces growth rate of s.c. murine (Neuro2A-Luc) tumors in A/J mice.** The ability of siR2B+5 to reduce RRM2 protein levels and growth rates in



**Fig. 6.** Female BALB/c mice received a single high-pressure (10% v/w) injection of pR2Luc (0.25 mg/kg) alone or with one of three different siRNAs (1.25 mg/kg): siR2B+5, an siRNA against luciferase (Luc105-21), or a nontargeting control siRNA (siCON1). The expression of the RRM2-luciferase protein in these mice was monitored at various time points (2, 6, 11, and 17 d) postinjection via whole animal bioluminescence imaging. Results are presented as integrated whole animal bioluminescence normalized to the average for mice injected with pR2Luc alone at each time point. A significant ( $P < 0.05$ ) reduction in bioluminescence is seen for mice coinjected with siR2B+5 or Luc105-21 (versus siCON1) at all time points. Columns, average of five mice; bars, SD.



**Fig. 7.** **A**, luciferase-expressing murine neuroblastoma (Neuro2A-Luc) cells were untransfected or lipofected with siRNA (siCON1, siR2B+5, or siR2B+6; 20 nmol/L) for 4 h before s.c. injection ( $2 \times 10^6$  cells per mouse) in the right flank of A/J mice. Median integrated bioluminescence signal (photons per second) is plotted as a function of time (days after cell injection) for cells that were untransfected ( $n = 5$ , ●) or transfected with siCON1 ( $n = 5$ , ■), siR2B+5 ( $n = 5$ , ○), or siR2B+6 ( $n = 4$ , □). **B**, additional Neuro2A-Luc cells from the treatments in **A** were replated (4 h post-transfection) into a device that allows real-time measurement of well impedance (presented as cell index as described in ref. 19) every hour for 96 h. Points, the average of triplicate samples at each time point; bars, SD. Significant ( $P < 0.05$ ) reduction in cell index is observed by 96 h for cells transfected with siR2B+5 versus all other treatment groups.

several cell lines has been shown, as has its potency to reduce expression of the RRM2-luciferase protein *in vivo*. These results suggested that tumor growth *in vivo* could be retarded via RRM2 knockdown induced by siR2B+5. To examine this, luciferase-expressing Neuro2A (Neuro2A-Luc) cells were untransfected or lipofected with either siCON1, siR2B+5, or siR2B+6 before trypsinization and s.c. injection in female A/J mice. The growth of the resulting tumors was monitored for nearly 2 weeks postinjection via bioluminescence imaging (Fig. 7A). At all time points examined, median tumor signal was lower for cells treated with siR2B+5 than for all other treatment groups. This reduced tumor growth rate is consistent with RT-CES growth data obtained for additional cells that received these treatments (Fig. 7B). In both analyses, the cells transfected with siR2B+5 showed modest reductions in growth (compared with untransfected and siCON1-transfected controls), but these were inferior to those seen with siR2B+5. These results suggest that siR2B+5, if appropriately delivered to tumor cells *in vivo*, has the capacity to reduce tumor growth.

## Discussion

There has been tremendous interest in the pursuit of siRNA-based therapeutic strategies for the treatment of cancer in recent years (for a recent review, see ref. 21). For such a strategy to be successful, it must involve (a) the determination of a "drugable" (in this case, amenable to siRNA-induced silencing) target that is critical for the proliferation of cancer cells; (b) the discovery of one or more siRNA duplexes capable of achieving target silencing and concomitantly reduced tumor proliferation; and (c) the ability to deliver this siRNA to cancer cells *in vivo*. Here, we have focused on a target that has already been identified by researchers using small-molecule and antisense oligodeoxyribonucleotide inhibitors. Indeed, the RRM2 protein and its role in numerous cancers have been well characterized, but the potential for development of a potent siRNA inhibitor for RRM2 remains largely untapped.

Our efforts began by scanning the RRM2 mRNA sequence for a few hotspots at which to commence the process of siRNA evaluation. The three resulting duplexes all showed modest reduction of RRM2 protein levels in a few different human cancer cell lines, but further exploration was necessary to discover a duplex with superior potency. A plasmid encoding an RRM2-luciferase fusion protein was prepared to accelerate a tiling experiment in which ~10 duplexes in the immediate vicinity of each of the original three targets were tested in human hepatocellular carcinoma cells. This tiling experiment revealed the potency of siR2B+5, which reduced the expression of a cotransfected RRM2-luciferase protein by 80% even when lipofected at only a 200 pmol/L (0.2 nmol/L) concentration. Interestingly, by comparison, a duplex having a target site just a single base downstream of this highly potent duplex, siR2B+6, had virtually no anti-RRM2 activity, illustrating the necessarily empirical nature of siRNA evaluation. Additionally, for the hRRM2 target, a sequence known to yield effective antisense inhibition gave essentially no RRM2 knockdown when constructed as a siRNA. Thus, direct conversion of antisense sequences is not a viable route to the development of potent siRNAs.

Subsequent experimentation focused on the siR2B+5 duplex, revealing both its strong silencing of endogenous RRM2 and its ability to reduce cellular proliferation rates. Furthermore, investigation of species homology of the target site was shown to be predictive of its antiproliferative effect in cell lines from different species and suggestive of appropriate and inappropriate species for future *in vivo* work. Treatment of murine neuroblastoma (Neuro2A) cells with siR2B+5 before s.c. injection in mice was shown to reduce tumor growth.

The level of RRM2 expression (Western blot analyses) has been shown to be low in most of the major organs, e.g., heart, liver, lung, etc. (22). To test whether or not functional siRNA against RRM2 has any deleterious effects on animals, a plasmid containing the RRM2-luciferase fusion gene (pR2Luc) was given to mice using the HPTV injection method to provide uptake into hepatocytes. Coinjection of siR2B+5 and the pR2Luc plasmid revealed that the siR2B+5 remains functional in mouse hepatocytes (inhibition of the fusion protein) and did not cause acute or chronic (over ~2.5 weeks) problems to the animals.

Collectively, these results suggest that siR2B+5 is a worthy candidate for further development as an anticancer therapeutic. In light of the third essential element of a successful therapeutic strategy mentioned above (delivery of siRNA to cancer cells *in vivo*), we intend to combine siR2B+5 with a targeted, nonviral siRNA delivery system that has previously been shown to deliver an siRNA systemically that resulted in reduced tumor growth (17). Tumor reduction studies with such a siR2B+5-containing formulation are in progress.

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