GTI-2040 displays cooperative anti-tumor activity when combined with interferon α against human renal carcinoma xenografts

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Abstract. GTI-2040, an antisense oligonucleotide targeting the small subunit of ribonucleotide reductase, acts as an anti-tumor agent in animal models of human cancer. In the present study, the anti-tumor activity of GTI-2040, in combination with interferon α (IFNα) was investigated against human renal cell carcinoma tumors xenografted into mice. The human renal cell carcinoma cell lines, Caki-1 and A498 were sensitive to IFNα both in vitro and when implanted into mice. In combination with GTI-2040 there were cooperative effects at intermediate doses of the two agents and complete tumor regression at higher combination doses. A control oligonucleotide was not effective as a monotherapy and did not improve the efficacy of IFNα. The effect of combination treatment on apoptosis and proliferation of tumor cells, isolated from xenografted tumors, was examined by histochemistry. GTI-2040 increased the percentage of cells undergoing apoptosis with a concomitant decrease in proliferation. IFNα alone had no effect but in combination with GTI-2040 resulted in increased apoptosis and decreased proliferation compared to GTI-2040 alone. Taken together these results expand the potential clinical applications of GTI-2040 to include combination therapy with IFNα.

Introduction

It is estimated that in 2008 there will be 54,390 new cases of renal cancer in the US with ~13,010 deaths (1). Renal cell carcinoma (RCC) is the most commonly diagnosed renal malignancy accounting for 85-90% of cases (1,2). If diagnosed at the very early stages most forms of RCC can be treated by surgery and in some cases radiation. In contrast, the prognosis for advanced disease is poor with 5-year survival rates currently assessed at <10% (1,2). Once RCC has progressed there are few effective treatment options. RCC has been largely resistant to chemotherapy with chemotherapeutic agents such as vinblastine and FU demonstrating <10% response rates in clinical trials (3,4). High dose IL-2 results in response rates in the range of 10-20% with lasting response of ~5-10% (5,6). As a result, IL-2 has become the standard of care for renal cell carcinoma. Although a significant advancement in treatment, high-dose IL-2 is limited by toxicity resulting in only a small subset of patients being candidates for treatment (2,5,7). Due to the limited patient population, eligible for high-dose IL-2 therapy, interferon α (IFNα) has also emerged as a standard treatment and as a preferred comparator arm in phase III clinical studies evaluating newer drugs (7,8). In the past few years, promising new drug candidates have been developed based on emerging information on the genetic and molecular pathways involved in development of RCC (9,10). These include anti-VEGF monoclonal antibodies (bevacizumab), multi-kinase inhibitors (sorafenib, sunitinib) and inhibitors of the mammalian target of rapamycin (mTOR) pathway (temsirolimus) (11-13). A number of these drug candidates have demonstrated clinical activity with some studies indicating efficacy superior to immunotherapy (11-13). Although some progress has been made in the treatment of renal cell carcinoma there remains a considerable need to develop drugs with good safety profiles that can be added to existing treatments, both standard immunotherapy and emerging treatments.

Ribonucleotide reductase (RNR) catalyzes the reaction in which 2’-deoxyribonucleotides are synthesized from the corresponding ribonucleoside 5’-diphosphates (14,15). This step is the rate-limiting reaction in the production of 2’-deoxyribonucleoside 5’-triphosphates required for DNA
replication and repair. RNR consists of two protein components. R1 is a 160-kDa dimer that contains at least two different effector-binding sites and R2 is a 78-kDa dimer that contains a nonheme iron that participates in catalysis by forming an unusual free radical on the aromatic ring of a tyrosine residue (16,17). Expression of both R1 and R2 are required for enzymatic activity. R1 in proliferating cells is relatively constant throughout the cell cycle, while R2 expression varies with elevated R2 levels observed during late G1 or early S-phase, suggesting that expression of R2 plays a role in regulation of RNR activity, DNA synthesis and cell proliferation (18-24). Recently, an R2 homologue, p53R2, was identified. The human p53R2 gene contains a p53 binding site and is ~80% identical to R2 (25,26). The role of p53R2 is unique from R2 in that it is induced upon exposure to UV light, γ-irradiation or DNA-damaging agents and supplies the dNTPs necessary for DNA repair (26,27). Like R2, p53R2 appears to complex with R1 to form an active RNR (28). RNR activity has been correlated with cancer progression in a number of studies (29,30) and several therapeutic agents are currently used which inhibit RNR as part of their mechanism of action. These include Hydroxyurea (Hydrea®, Bristol-Myers Squibb and Hydroxyurea Capsules, Roxane), Gemcitabine (Gemzar®, Eli Lilly) and Fludarabine (Fludara®, Berlex). These drugs are limited in large part by the development of drug resistance.

In addition to its role in RNR activity, the R2 protein, independent of R1, appears to play a role in determining the malignant potential of cells via synergism with a number of activated oncogenes (31,32). Altered R2 gene expression cooperates with ras in mechanisms of malignant progression (31,32). Increased expression of R2 has been found to increase the drug-resistant properties of cancer cells while a retrovirus vector containing the R2 sequence in an antisense orientation (38). These studies would indicate that, apart from the anti-proliferative effect of RNR inhibition, the specific inhibition of the R2 protein would likely provide additional therapeutic benefit.

Several pre-clinical studies support targeting of R2 in combination with cytotoxic chemotherapeutics. Drugs that inhibit RNR, as part of their mechanism of action (hydroxyurea, FU, trimidox, triapine) have been shown to increase sensitivity to other drugs (39-43). In addition, recent studies have demonstrated that decreasing RNR R2 expression via siRNA treatment results in increased sensitivity to gemcitabine both in vitro and in vivo (44,45). Taken together these results suggest that there may be several mechanisms by which targeting of R2 may increase sensitivity to anti-cancer drugs.

Antisense oligonucleotides (AS-ODN) are short DNA molecules that can interfere with gene expression by forming duplexes with complementary sequences of target messenger RNAs (mRNAs) (46). A previous study screened 102 oligonucleotides (ODNs) complementary to RNR subunit R2 for the ability to decrease R2 mRNA levels in vitro (47). One ODN, GTI-2040, was further characterized and demonstrated specific, dose-dependent down-regulation of R2 with a concomitant decrease in proliferation, tumor growth and metastasis. In particular, GTI-2040 demonstrated excellent activity against two human renal cell carcinoma tumors implanted in mice. A completed phase I trial of GTI-2040 for the treatment of advanced cancers demonstrated a favorable safety profile at therapeutically relevant doses (48) and GTI-2040 is currently being evaluated in clinical trials in a number of cancer indications. A recently completed phase II combination study of GTI-2040 with capecitabine for renal cell carcinoma demonstrated a reasonable toxicity profile and a high incidence of disease stabilization. However, subsequent to initiation of this study capecitabine has been found to lack significant activity for treatment of renal cell carcinoma (49). This suggests that GTI-2040 should be tested in combination with an agent that has clinically proven activity for treatment of renal cell carcinoma.

In the present study, the potential of GTI-2040 as an anti-tumor drug candidate for treatment of renal cell carcinoma, was examined further. The anti-tumor activity of GTI-2040 in combination with IFNα was tested against human RCC cell lines xenografted into mice. The activities were compared to treatment with GTI-2040, alone and in combination with IFNα. Finally, in an effort to understand the mechanism by which GTI-2040 acts in combination with IFNα, cell proliferation and apoptosis in xenograft tumor samples were studied by immunohistochemistry.

Materials and methods

Oligonucleotides. All oligonucleotides used in this study were fully thioated. They were synthesized on an automated DNA synthesizer (Perkin-Elmer, USA) by Boston BioSystem Inc. (Boston, MA). Oligonucleotides were purified and purity assessed by reversed-phase high performance liquid chromatography. Each oligonucleotide preparation was found to be >95% full-length material. GTI-2040 hybridizes to coding region of R2 mRNA. A scrambled control analogue of GTI-2040, GTI-2040Scr, is not complementary to R2, while maintaining the same base composition ratio (47).

Cell lines and cell culture. Unless noted otherwise in the text human tumor cell lines were purchased from the American Type Culture Collections (ATCC) (Rockville, MD). Caki-1 and A498 cell lines were maintained, according to ATCC recommendation, in α-MEM or McCoy’s 5a medium (Gibco BRL, Gaithersburg, MD) supplemented with 10-20% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO2. All media used in these experiments also contain 100 x diluted antibiotic solution that consist of 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL).

In vitro treatment with IFNα and XTT proliferation assay. In order to determine whether A498 and Caki-1 cells are sensitive to direct inhibition of cell proliferation by IFNα, cells were grown in culture as described above and treated with increasing concentrations of IFNα. Cells (1x10^5) per well were
Immunohistochemistry. All procedures preceding the isolation of tumor samples were performed at Lorus Therapeutics. Subsequent immunohistochemistry and image analyses were performed at the Calcium Research Laboratory, Department of Medicine, McGill University, Montreal, Canada. The xenograft tumors were removed and fixed in PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution) overnight at 4˚C. After fixation the samples were dehydrated in graded alcohols and embedded in low melting point paraffin and 5 μm sections were cut on a microtome. Paraffin sections were stained for macrophage and NK1.1 using the avidin-biotin-peroxidase complex technique, as described previously (50). Briefly, mouse anti-macrophage monoclonal antibody diluted 1:100 (Abcam) or mouse anti-macrophage NK1.1 antibody diluted 1:100 (Caltag Laboratories) was applied to sections overnight at room temperature. As a negative control, the preimmune serum (Caltag Laboratories) was applied to sections overnight at room temperature. As a negative control, the preimmune serum. As a negative control, the preimmune serum was substituted for the primary antibody. After washing with high salt buffer (50 mM Tris-HCl, 2.5% NaCl, 0.05% Tween-20, pH 7.6) for 10 min at room temperature following by two 10-min washes with Tris-buffered saline, the sections were incubated with secondary antibody (biotinylated goat anti-rat or mouse IgG), washed as before and processed using the Vectastain ABC-AP kit (Vector Laboratories, Inc.). Red pigmentation to demarcate regions of immunostaining was produced by a 10- to 15-min treatment with Fast Red TR/Naphthol AS-MX phosphate (Sigma), containing 1 mM levamisole as endogenous alkaline phosphatase inhibitor. Sections were counterstained with methyl green and mounted with Kaiser's glycerol jelly.

**Detection of apoptotic cells.** De-waxed paraffin sections were stained with an in situ cell death detection kit (Boehringer Mannheim), as previously described (51). Briefly, following treatment with 3 μg/ml of proteinase K for 20 min at room temperature, the sections were incubated with a reaction mixture for terminal deoxynucleotidyltransferase-mediated nick end-labeling of DNA strand breaks (TUNEL) for 60 min at 37˚C. Sections were then incubated with Converter-AP for 30 min at 37˚C and alkaline phosphatase was visualized after 10-15 min of treatment with Fast Red TR/Naphthol AS-MX phosphate (Sigma), containing 1 mM levamisole as an endogenous alkaline phosphatase inhibitor. Sections were counterstained with methyl green and mounted with Kaiser's glycerol jelly.

**Computer-assisted image analysis.** After immunohistochemical staining or TUNEL assay of sections from saline and drug-treated samples, images of fields were photographed with a Sony digital camera. Images from single sections were digitally recorded using a rectangular template and recordings were processed using Northern Eclipse image analysis software (50). Thresholds were set using green and red channels. The thresholds were determined, as described previously (50).

**Statistical evaluation.** Statistical analyses of tumor volume and weight data, from in vivo experiments, were conducted using data analysis tools in Microsoft Excel. To improve visual clarity, only the positive standard error value is shown for the tumor volume curves. Selected p-values for tumor weight comparisons are presented in the results section with complete statistical analyses available by request from the authors.

**Results**

Preliminary screening of GTI-2040 in combination with standard therapies in a murine xenograft model of tumor growth. Early screening studies with GTI-2040 were carried out on a broad range of cancers and a number of combinations with standard chemotherapeutic and immunotherapeutic drugs. For RCC, GTI-2040 was tested in combination with standard chemotherapeutic and immunotherapeutic drugs. For RCC, GTI-2040 was tested in combination with Flu, vinblastine and IL-2 in murine xenograft tumor growth assays. All agents given as single therapies demonstrated a significant decrease in the growth of tumors compared to saline-treated controls and the combination with GTI-2040 resulted in improvement in the efficacy of test agents resulting in complete regression of tumors in all animals for all combinations (data not shown). The combination treatments were statistically significantly better than the respective single agent chemotherapy treatments and although combination treatment was reproducibly better than treatment with GTI-2040 alone it was not statistically significant due in large part to effectiveness of the GTI-2040 treatment. GTI-2040 was administered at an optimal dose of 10 mg/kg every other day and this dose was previously shown to cause regression of
Caki-1 tumors in some but not all mice within a treatment group (47). In the GTI-2040 treatment groups 30-40% of animals had complete regression of the implanted tumor, whereas in combination groups tumor regression exceeded 90% and in most cases reached 100%. Although GTI-2040 cooperates with a number of standard chemotherapies there is little clinical evidence to support the use of chemotherapy for advanced RCC (3,4,52). IL-2 is a standard option for treatment of advanced RCC, however clinical application is often limited by toxicity leading to only a small percentage of patients being eligible for treatment resulting in IFNα increasingly being used for treatment of RCC (7,8).

Caki-1 and A498 human renal carcinoma cell lines are sensitive to interferon α by in vitro proliferation assays. A number of studies have demonstrated that many RCC cell lines are sensitive to direct cytotoxic effects of IFNα including Caki-1 and A498 renal cell carcinoma cell lines (53-55). In order to confirm and compare the sensitivities of Caki-1 and A498 cell lines, in vitro cell proliferation assays were performed after treatment with IFNα. The results shown in Fig. 1 demonstrate that both Caki and A498 cells are sensitive to IFNα and that the response appears to be dose-dependent at the higher doses of INFα (1000-10000 U/ml). These data indicated that Caki-1 and A498 cell lines are relatively similar in their sensitivity to IFNα and would be amenable to assessment of combination effects with IFNα and GTI-2040 in vivo.

GTI-2040 cooperates with interferon α in two murine xenograft models of renal cell carcinoma tumor growth. Previously, GTI-2040 was shown to be a highly effective treatment in xenograft tumor growth models of human cancers, including two renal cell carcinoma models. At the highest concentration of GTI-2040 tested, 10 mg/kg/48 h, there was highly effective anti-tumor activity including complete regression in a significant number of test animals. In the current study, intermediate (or sub-optimal) doses of GTI-2040 and IFNα were tested in xenograft models of human renal tumor growth. Tumor growth was assessed by measurement of tumor volume over the course of the experiment as well as tumor weights taken at the experimental endpoint. Growth of Caki-1 and A498 human renal cell carcinoma cells implanted in SCID mice was effectively inhibited by both GTI-2040 and IFNα treatments (Figs. 2 and 3, respectively). As shown, the response to these agents is dose-dependent with increasing dose resulting in improved anti-tumor activity. A number of dose combinations of these two agents resulted in anti-tumor activity that exceeded the effects of either agent alone. The following statistical analyses are for the tumor weights measured at the experimental endpoint shown in Figs. 2B and 3B. For Caki-1, the lowest doses of both agents had little effect on tumor growth either alone or in combination, but at the highest doses the single agents reduced tumor growth compared to saline although only GTI-2040 reached statistical significance (p=0.007). In addition, the combination treatment at the higher dose levels was significantly better than saline and GTI-2040 alone (p=0.003 compared to saline, p=0.007 compared to GTI-2040). Although the high dose treatment was reproducibly superior to IFNα alone it did not meet significance (p=0.07), due in large part to the high variability in the IFNα high-dose mono-therapy. Although neither low dose GTI-2040 nor high dose IFNα demonstrated statistically significant anti-tumor efficacy alone (p=0.6 and p=0.2 compared to saline, respectively) the combination demonstrated better anti-tumor efficacy than either agent alone and reached significance compared to saline (p=0.003 compared to saline). Some regression was observed in the high-dose combination treatment group (5 of 10 animals).

A498 tumors were consistently more sensitive to treatment by all agents with the exception of the lowest dose of IFNα (Fig. 3A and B). For A498 tumors, there was significant anti-tumor activity of GTI-2040 and IFNα compared to saline at the higher dose levels (p=0.003, p<0.001, respectively). The combination with lower doses of IFNα and GTI-2040 did increase anti-tumor activity compared to the corresponding mono-therapies but it was not statistically significant compared to mono-therapy. Although neither agent as a mono-therapy at the lower doses demonstrated significant activity compared to saline, in combination significance was reached (p=0.009 compared to saline). There was significant improvement compared to mono-therapy at all higher dose IFNα combinations ([10^4 IFNα + 2.5 mg/kg/48 h GTI-2040]: p=0.002 compared to GTI-2040 alone, p<0.001 compared
with IFN alone), \((10^5 \text{ IFN} + 1 \text{ mg/kg/48 h GTI-2040: p=0.001 compared to GTI-2040 alone, p=0.002 compared with IFN alone})\). Nine of 10 complete regressions were observed at the highest dose combination in A498 cells while neither single agent treatment resulted in tumor regressions. In addition, high-dose IFNs combined with the lower dose GTI-2040 also resulted in 5 of 10 regressions.

Although almost complete regression was achieved in A498 experiments shown in Fig. 3, this was not achieved for Caki-1 tumor growth (Fig. 2) at the initial concentrations tested. Shown in Fig. 4A are the results of Caliper measurements at 1-week intervals were used to calculate tumor volumes. Each point represents mean tumor volume and standard error calculated from 10 animals per experiment. (B) Mice were sacrificed 51 days after tumor cell implantation and tumor weight for each was determined. Mean tumor weight and standard error (SE) are shown for each experimental group of 10 mice.

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of 10000 units in 0.05 ml saline for two weeks. Treatment with IFN doses in 0.1 ml saline as indicated. IFN administered by bolus infusion into the tail vein every other day at 5 mg/kg administered as either intravenous or intratumoral injections. GTI-2040 was administered by bolus infusion into the tail vein every other day at 2.5 mg/kg doses in 0.1 ml saline. IFN treatment continued to experimental endpoint to assess maximal cooperative effect. The combination treatments are as indicated above for the single agent treatments. Mice were sacrificed 56 days after tumor cell implantation and tumor weight for each was determined. Mean tumor weight and standard error (SE) are shown for each experimental group of 10 mice.

As shown in Fig. 6 and Table I, GTI-2040 as a monotherapy has anti-tumor activity (p=0.003) and improves the effects of IFN in combination (p=0.005 compared to IFN alone). Interestingly IFN, at the same suboptimal concentration as Fig. 2 but given continuously without the one week break, demonstrated significant activity compared to saline reiterating the importance of both dose and schedule in designing future studies.

GTI-2040 treatment results in increased apoptosis and decreased proliferation of renal cell carcinoma cells xenografted into mice. The data presented above indicate that treatment with IFN and GTI-2040 is highly effective as a combination therapy. Initial histochemical analyses were undertaken in order to understand the mechanism of anti-tumor activity and to assess whether the effects of GTI-2040 are due in part to immunostimulation, as has been observed with PS-ODNs containing a CpG motif in a specific hexameric context. Mice bearing Caki-1 tumor xenografts were treated as indicated in the figure legend to Fig. 6 and tumor samples were collected as described in the methods at the times indicated and analyzed for rate of proliferation and apoptosis. As shown in Fig. 6 and Table I, GTI-2040 as a monotherapy significantly decreases the rate of proliferation and increases the rate of apoptosis in tumor cells from xenograft samples (p<0.001 for both analyses compared to saline). The intermediate sub-optimal dose of IFN used in the study did not result in changes to either proliferation rate or apoptosis. The control ODN did not demonstrate any effects on proliferation or apoptosis when given as a single agent.

A combination of the GTI-2040 and IFN resulted in an additional increase in apoptosis and a decrease in proliferative rate, suggesting that IFN may have sensitized tumor cells to GTI-2040 (Apoptosis: p<0.001 compared to saline and IFN alone and p=0.016 compared to GTI-2040 alone)
A number of in vitro and in vivo studies have demonstrated a role for RNR in progression and metastasis of cancer (introduction above). Previous studies demonstrated that inhibiting expression of the R2 subunit with GTI-2040 results in significant anti-tumor activity measured in human xenograft models in mice (47). Renal cell carcinoma tumors implanted into mice demonstrated high sensitivity to GTI-2040 treatment resulting in regression of some tumors at high doses of GTI-2040 (47). The results presented here provide compelling pre-clinical evidence in support of combination therapy for renal cell carcinoma using GTI-2040. Although GTI-2040 treatment enhanced the anti-tumor efficacy of every drug tested, the most notable results from a clinical perspective were with IFNα and IL-2, both immunological agents that have become standard therapies for advanced RCC (5,6). Furthermore, the cooperative effects were sequence-specific since treatment with scrambled ODN did not result in enhanced action of interferon α. The broad-spectrum cooperative activity of GTI-2040 may be a reflection of RNR inhibition leading to a decrease in the rate of proliferation and increased apoptosis

Table I. Rate of apoptosis and proliferation of renal cell carcinoma cells xenografted into mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline (i.v.)</th>
<th>Saline (i.t.)</th>
<th>GTI-2040</th>
<th>IFNα</th>
<th>GTI-2040scr</th>
<th>IFNα</th>
<th>GTI-2040scr</th>
<th>IFNα</th>
<th>GTI-2040</th>
<th>IFNα</th>
<th>GTI-2040scr</th>
<th>IFNα</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Apoptotic cells</td>
<td>2.21±0.79</td>
<td>2.3±0.8</td>
<td>8.34±2.26</td>
<td>2.25±0.92</td>
<td>2.3±1.13</td>
<td>2.83±1.21</td>
<td>13.56±5.46</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% BrdU-positive cells</td>
<td>20.12±3.39</td>
<td>19.7±2.56</td>
<td>12.11±2.19</td>
<td>20.16±2.51</td>
<td>20.87±2.21</td>
<td>13.95±3.85</td>
<td>7.25±1.99</td>
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(Proliferation: p<0.0001 for all comparisons). This would be consistent with a number of studies that demonstrate that IFNα induces and/or sensitizes cells to apoptosis (58,59). The control scrambled oligonucleotide had no effect on either apoptosis or proliferation as a single agent but did result in a combined effect with IFNα when measuring proliferation (p=0.001 compared to both saline and IFNα alone). This is likely due to the known non-specific effects of PS-ODNs as there was not a concomitant increase in apoptosis. Although the combined effect was significantly better than the monotherapy groups it was not as good at decreasing proliferation as the combination with GTI-2040 (p<0.001 comparing the two combinations).

Although the SCID mice used in this study are deficient in T and B cell function tumor samples were assessed for macrophage and NK cell infiltration into tumors upon treatment with either agent and in combination. There were no observable NK or macrophage infiltrates in the tumor samples analyzed suggesting that the mechanism of action observed is not the result of immune stimulation (not shown). Tumor infiltrates have been demonstrated in GTI-2040-treated CD-1 mice (intact B cell function) suggesting that although immune stimulation can be a contributing factor in GTI-2040 anti-tumor activity it is not involved in the effects seen in SCID mice used for these studies (data not shown). These results are consistent with previous studies that demonstrated efficacy in SCID beige mice lacking NK cell function in addition to B and T cell deficiency (47).

Discussion

A number of in vitro and in vivo studies have demonstrated a role for RNR in progression and metastasis of cancer (introduction above). Previous studies demonstrated that inhibiting expression of the R2 subunit with GTI-2040 results in significant anti-tumor activity measured in human xenograft models in mice (47). Renal cell carcinoma tumors implanted into mice demonstrated high sensitivity to GTI-2040 treatment resulting in regression of some tumors at high doses of GTI-2040 (47). The results presented here provide compelling pre-clinical evidence in support of combination therapy for renal cell carcinoma using GTI-2040. Although GTI-2040 treatment enhanced the anti-tumor efficacy of every drug tested, the most notable results from a clinical perspective were with IFNα and IL-2, both immunological agents that have become standard therapies for advanced RCC (5,6). Furthermore, the cooperative effects were sequence-specific since treatment with scrambled ODN did not result in enhanced action of interferon α. The broad-spectrum cooperative activity of GTI-2040 may be a reflection of RNR inhibition leading to a decrease in the rate of proliferation and increased apoptosis
observed in the histochemistry and immunohistochemistry analyses. The observed effect of the scrambled oligonucleotide on proliferation in combination with IFNα but not on apoptosis or as a single agent is likely the result of previously described non-specific effects of PS-ODN. Control PS-ODNs can exhibit minor biological activity in vitro and in vivo, but usually at lower efficacy than observed for the specific PS-ODN [examples (60-62)]. This is usually related to immunostimulatory or serum protein binding properties of the PS-ODNs (56,57).

For a number of reasons including; consistent response rate, tolerability and ease of administration, IFNα has become the preferred agent for comparison in phase III trials and for evaluation of emerging drug candidates either as single agents or in combination with standard therapies (7,8). As a result, the current study focused on IFNα as a comparator drug and as the combination treatment for GTI-2040. IFNα has been extensively studied in pre-clinical models of cancer progression. IFNα is a biological agent that has diverse functions and may act as an anti-tumor agent via multiple direct and indirect pathways including immune stimulation of acquired and innate immune effector cells, direct anti-proliferative effects on tumor cells, induction of apoptosis and attenuation of tumor angiogenesis (58,59,63-67). The detailed molecular mechanism by which the combination of GTI-2040 and IFNα induces apoptosis and decreases proliferation remains to be elucidated and may involve multiple and/or overlapping pathways. Given what is known about R2 and its role in tumorigenesis there exist a number of pathways by which these agents cooperate. Future studies will attempt to elucidate the molecular components of the combined effect of IFNα and GTI-2040 with an emphasis on whether the cooperative effects observed are the result of over-lapping or non-overlapping molecular pathways. Of particular interest and warranting further analysis is that an intermediate sub-optimal dose of IFNα did not increase apoptosis or decrease proliferation in RCC tumor cells on its own, but the combination with GTI-2040 resulted in sensitization of RCC tumor cells to both increased apoptosis and decreased proliferation. In this study, IFNα demonstrated dose-dependent inhibition of proliferation in vitro and so further studies are necessary to determine whether higher doses of IFNα affect proliferation and apoptosis in vivo. IFNα is known to induce and or sensitize cells to apoptosis via a number of pathways including JAK/STAT and PI3 signaling and involving activation of multiple caspases and proapoptotic molecules (58). In addition, recent studies on RCC and hepatocellular carcinoma cells with IFNα in combination with 5-FU implicate p53 in the synergy demonstrated by these compounds (68,69). Both of the RCC cell lines studied here are wild-type for p53 (ATCC) and so additional studies with p53 mutant cell lines or by expression of a dominant-negative p53 in Caki-1 and A498 cells may be useful in determining whether p53 is involved in the synergy observed with GTI-2040. Separate from its role as a subunit of RNR R2 has been implicated in tumor progression via interaction with a number of oncogenes including synergism with the Ras pathway (31,32). Given that the EGF/Ras/Raf-1/Erk survival pathway has been shown to counteract the effects of IFNα, there is a compelling possibility that inhibition of R2 may augment IFNα-mediated anti-tumor effects via down-regulation of the Ras/Raf 1 signalling pathway (58,70-72).

Although IFNα was administered intratumorally in the current study, additional studies will address the potential for alternative modes of administration. Preliminary studies suggest that systemic administration of IFNα (subcutaneous on opposite flank) in the mouse model of human RCC may be effective in decreasing tumor growth both as a single agent and in combination with GTI-2040. Further studies are required to establish optimal dosing and to compare the therapeutic benefit to intratumoral administration.

Immunotherapy has been standard treatment for RCC for many years but more recently there is a growing list of mechanism-based drugs that have promising therapeutic activity based on an increased understanding of how RCC develops and progresses. Given the current paradigm in cancer management it is expected that these new drugs will be used in combination with each other and/or with immunotherapy. As research expands the knowledge of how RCC differs between patients the hope is that the combination therapy will be tailored to individual or sub-groups of patients that stand to benefit most from each therapy or combination. Given that GTI-2040 has the potential to cooperate with both chemotherapy and immunotherapy it may also prove useful with newer treatments. For this reason the GTI-2040 research program will be expanded to include these emerging therapies.

GTI-2040 has been evaluated in a number of clinical trials and has demonstrated reasonable tolerability both as a single agent and in combination with chemotherapeutic agents (48,73). Although limited to early stages of clinical investigation there is accumulating evidence that GTI-2040 specifically down-regulates the R2 target and may have therapeutic activity (74). The data presented here and preliminary animal studies on a number of indications and combinations (our unpublished observations) support the future use of GTI-2040 in combination with standard drugs in clinical studies.

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References


