c-raf-1 Depletion and Tumor Responses in Patients Treated with the c-raf-1 Antisense Oligodeoxynucleotide ISIS 5132 (CGP 69846A)¹

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ABSTRACT

Abnormally regulated signaling through proliferative signal transduction pathways characterizes many of the common solid tumors. The best described of these involves potentially oncogenic proteins of the Ras family, which activate Raf proteins in the early steps of the mitogen-activated protein kinase cascade. ISIS 5132, a phosphorothioate antisense oligodeoxynucleotide directed to the 3’ untranslated region of the c-raf-1 mRNA, inhibits the growth of human tumor cell lines in vitro and in vivo in association with specific down-regulation of target message expression. Using a semiquantitative reverse transcription-PCR assay, we analyzed changes in c-raf-1 mRNA expression in peripheral blood mononuclear cells collected from patients with advanced cancers treated with ISIS 5132 as part of a clinical trial. Specimens were collected for analysis pretreatment and on days 3, 5, 8, and 15 of the first cycle and on day 1 of each subsequent cycle. We observed significant reductions of c-raf-1 expression from baseline by day 3 in 13 of 14 patients (P = 0.002). The time course and depletion of c-raf-1 message in peripheral blood mononuclear cells paralleled the clinical benefit in two patients. These findings demonstrate that ISIS 5132 specifically reduces target gene expression in treated patients and that peripheral blood mononuclear cells are suitable tissues for biomarker studies in future trials.

INTRODUCTION

The elucidation of oncogenic intracellular signaling pathways provides novel specific targets for antineoplastic intervention. The signaling molecule c-raf-1 is one of three highly conserved members of theraf gene family, which code for a group of serine/threonine protein kinases best known for their role in growth factor receptor-mediated signal transduction through the mitogen-activated protein kinase pathway (1, 2). After recruitment to the plasma membrane by activated Ras, Raf-1 is activated by phosphorylation and begins a sequence of downstream events including the phosphorylation of MEK1 and MEK2 that in turn activate ERK1 and ERK2 (3–5). Activated ERK1 and ERK2 then translocate to the nucleus, where they have multiple effects on gene expression mediated by the induction of transcription factors such as Elk-1 and Ets-2 and by activation of the cyclins D1 and E (2, 6, 7). The targets of activated ERK1 and ERK2 play key roles in cellular proliferation and differentiation (6). The transcription factor nuclear factor-κB can be activated by Raf-1 in the cytoplasm and has additional transforming effects that are independent of the ERK pathway (8). Recent work has shown the antiapoptotic protein Bcl-2 to activate Raf-1 on outer mitochondrial membranes; here Raf-1 inhibits the inactivation of Bcl-2 by BAD, allowing Bcl-2-mediated suppression of apoptosis (9).

Mutations in ras genes have been reported in a range of solid tumors and leukemias, most notably in pancreatic adenocarcinomas in which >80% of patients have mutated K-ras (10). Mutated Ras is constitutively active, with cellular transformation resulting from Raf-1 activation of downstream effectors in the ERK1 and ERK2 pathway (11). However, the complexity of Raf-1 regulation extends beyond its interaction with Ras. Considerable evidence suggests that there are multiple Ras-independent activators of Raf-1, including the tyrosine kinases Src (12) and JAK1 (13), protein kinase Cα (14, 15), ceramide-activated protein kinase (16), as well as Raf-1 oligomerization (17). Raf-1 itself also possesses oncogenic potential; NH2-terminal deletion mutants have transforming activity, andraf gene mutations have been detected in human tumors (18, 19). The establishment of Raf-1 as an important mediator of diverse signaling pathways underscores its involvement in malignant transformation and provides a rationale for Raf-directed novel therapies.

A 20-mer phosphorothioate antisense ODN targeted to the 3’ untranslated region of c-raf-1 mRNA inhibited the growth of tumor cell lines in vitro at concentrations that effect sequence-specific depletion of c-raf-1 mRNA and protein (20, 21). Administration of this drug (ISIS 5132, CGP 69846A) to immuno-deficient rodents bearing human tumor xenografts results in marked tumor growth delay (ISIS 5132 Investigator’s Brochure, 1999).

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The abbreviations used are: MEK, MAP kinase kinase; ERK, extracellular-signal-regulated kinase; ODN, oligodeoxynucleotide; PBMC, peripheral blood mononuclear cell; CT, computed tomography; RT-PCR, reverse transcription-PCR; CEA, carcinoembryonic antigen.
Depletion of c-raf-1 mRNA was also observed in the tumors of treated animals. We performed a clinical trial of ISIS 5132 to determine its toxicity profile and clinical effects in humans, to describe its pharmacokinetics, and as reported here to determine whether administration of the antisense construct at tolerable doses could exert a selective biological effect. The effect was measured in a surrogate tissue, the PBMC. We found that this treatment resulted in a pronounced depletion of c-raf-1 kinase mRNA in most of the patients treated in doses \( \geq 2.5 \) mg/kg on a three times weekly schedule. In most of the patients, there was evidence of partial recovery of expression by day 15, despite continued treatment. In two patients, prolonged inhibition of tumor growth was observed in association with these effects. These data demonstrate for the first time that the administration of antisense drugs directed to active oncogenic proliferative pathways may result in both depletion of the target mRNA and a therapeutic antitumor effect in patients.

### PATIENTS AND METHODS

**Patient Population.** Patients treated in this study were at least 18 years of age and had histologically proven cancers that were refractory to standard therapies. All patients had an Eastern Cooperative Oncology Group performance status \( \leq 2 \), a life expectancy \( \geq 12 \) months, and were recovered from previous treatment. Eligibility required adequate bone marrow, renal, and liver function, and no prolongation of the prothrombin time or international normalized ratio. Doses were escalated from 0.5 to 6.0 mg/kg in cohorts with one patient in each cohort. Dosing was resumed and maintained as long as the patient remained free of tumor progression or significant toxicity. Doses were escalated from 0.5 to 6.0 mg/kg in cohorts of three patients. The drug was well tolerated, and no patient required dose reduction. Two patients, one with renal and one with colon cancers, experienced prolonged inhibition of tumor growth lasting more than 8 months.

**Drug Preparation and Administration.** ISIS 5132 is the 19-sodium salt of a 20-base ODN (\( 5'\)-TCCCGCCTTGGACACCTTAT-3'); \( \beta \)-actin primer sequences were: BA (67), \( 5'\)-GGGGAATTCGCTGACATT-3'; and BA (68), \( 5'\)-GATGGAATGAGAGTTTGCATCTTAT-3' as described (23). cDNA synthesis was carried out with 2 \( \mu \)g of total RNA using Ready-to-Go You-Prime First-Strand Bead kits (Pharmacia Biotech, Uppsala, Sweden). PCR was carried out in Ready-to-Go PCR Bead kits (Pharmacia Biotech, Uppsala, Sweden). Both sets of reactions were performed according to the manufacturer’s instructions. The PCR reactions (25 \( \mu \)l total volume, containing 1–10 \( \mu \)l cDNA, 12.5 pmol of each of the c-raf-1 or \( \beta \)-actin primers, and 1 \( \mu \)Ci [\( \alpha ^{32} \)P]dCTP) were heated to 95°C for 5 min and then amplified for 36 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The products were loaded on 8% urea polyacrylamide gels. The gels were dried at 80°C for 1 h under vacuum and exposed to film for several hours at \( -80^\circ \)C.

**RESULTS**

Patient characteristics and study treatment have been described in detail elsewhere (25). Briefly, 29 fully evaluable patients with a range of cancer types received ISIS 5132 as a 2-h infusion three times weekly for 3 weeks. After a 1-week treatment-free interval, dosing was resumed and maintained as long as the patient remained free of tumor progression or significant toxicity. Doses were escalated from 0.5 to 6.0 mg/kg in cohorts of three patients. The drug was well tolerated, and no patient required dose reduction. Two patients, one with renal and one with colon cancers, experienced prolonged inhibition of tumor growth lasting more than 8 months.

**Variability in c-raf-1 Expression** PBMCs were obtained from patients treated at doses of 2.5 mg/kg and above immediately prior to treatment on days 1, 3, 5, 8, and 15 of the first cycle. Samples were obtained from patients who continued on therapy. Because of the low abundance of the c-raf-1 message in PBMCs, mRNA quantitation was performed using a semiquantitative RT-PCR assay. A typical autoradiograph of c-raf-1 and of \( \beta \)-actin is shown (Fig. 1).

Baseline c-raf-1 expression did not relate to patient age, sex, tumor type, or performance status. Similarly, no association
between c-raf-1 expression at baseline or during ISIS 5132 therapy and peripheral blood lymphocyte or monocyte counts was noted.

Depletion of c-raf-1 mRNA after Treatment. At all dose levels tested, depletion of c-raf-1 mRNA was observed within 48 h of the initial dose. Thirteen of 14 patients showed a decrease in message levels on day 3 (P = 0.002). No dose relationship was evident in this effect. The median decrease was to 42% (mean, 53%) of initial values (Table 1). When absolute values were considered, the mean mRNA content decreased from 1497 units at baseline to 602 units (40%) on day 3. Median values continued to be depleted to a median of 26% (mean, 71%) on day 5 (P = 0.017), with some evidence of recovery after 3 days without treatment on day 8 (median, 62%; mean, 81%; P = 0.03) and continued depletion on day 15 (median, 35%; mean, 74%; P = 0.017). The day at which the nadir was reached varied among patients; one reached nadir on day 3, seven on day 5, two on day 8, and four on day 15. The mean nadir value did not differ by dose of ISIS 5132, suggesting that all doses used exhibited similar biological effects (Table 1). Higher doses did not result in more protracted inhibition.

Biological Responses in Responding Patients. Two patients, both of whom had demonstrated tumor progression with previous cytotoxic chemotherapy, exhibited long-term stable disease in response to ISIS 5132 treatment. One was a 68-year-old man with colorectal cancer metastatic to liver who had progressed 2 years after adjuvant therapy with 5-fluorouracil/leucovorin and had evinced further tumor growth during therapy with a 17-1A monoclonal antibody and irinotecan. After treatment with 3 mg/kg of ISIS 5132, minor (20%) shrinkage in a liver metastasis was accompanied by a progressive decline in CEA from 895 to 618 ng/ml. During this time, c-raf-1 mRNA values declined to <10% of the initial value (Fig. 2). After seven cycles of treatment, both the plasma CEA values and the PBMC c-raf-1 mRNA began to increase, and 1 month later, the CT scan revealed progression of the hepatic metastases.

A 46-year-old woman with renal cell cancer metastatic to lung and lymph nodes failed to respond to interleukin-2, IFN-α, and 5-fluorouracil in combination and began treatment with ISIS 5132 at 5 mg/kg. She had immediate symptomatic improvement, but the size of the tumor was unchanged on CT scans. After 10 cycles of treatment, she began to have recurrent pain, and progression was identified radiologically. In this patient, the nadir PBMC c-raf-1 mRNA was 12%, and values remained low until the beginning of the ninth cycle, when a return above baseline was observed, again followed shortly thereafter by progressive disease.

DISCUSSION
Inhibition of proliferative pathways has long been a goal of anticancer therapy, but progress has been hampered by the limited specificity of small molecule inhibitors. The demonstration of dysregulation of the signal transduction pathways of growth signaling as an early and continuing abnormality of carcinogenesis has provided novel targets for the control of tumor growth. Several proliferative mechanisms have been shown to converge on the ras/raf/MEK/ERK signal transduction axis, including ras and raf mutation, overproduction of autocrine growth factors, and inactivation by mutation of parallel growth inhibitory pathways (e.g., transforming growth factor β; Refs. 2, 19, and 26). As the proximate membrane-associated protein in this pathway, Raf-1 is therefore a logical target.

The phosphorothioate antisense drug ISIS 5132 is targeted to a 20-base sequence of the 3′ untranslated region of c-raf-1 mRNA, which was demonstrated in cell culture to result in maximal depletion of the target message (20). In mice bearing human tumor xenografts, inhibition of tumor growth was associated with selective depletion of both mRNA and protein (21). A similar phosphorothioate drug targeted to the murine c-raf-1 gene was associated with minimal toxicity at doses that were effective in tumor growth inhibition (27). It was then incumbent upon the initial human studies to: (a) demonstrate that at dose levels associated with minimal toxicity a specific effect on c-raf-1 mRNA content could be achieved; and (b) suggest that the biological effect may result in some therapeutic benefit. Evidence is presented that both of these requirements have been met in a clinical trial.

As detailed fully elsewhere (25), the side effects associated with ISIS 5132 administration were mild and well tolerated. Fever and fatigue were the only symptoms, and laboratory abnormalities included anemia, short-lived elevation of the activated partial thromboplastin time, and transient dose-dependent elevation of the alternative pathway complement protein C3a. Dose escalation was halted at 6 mg/kg, because higher doses in simian models were associated with complement activation (28). None of the effects are believed to be specific to the antisense sequence, and similar constitutional symptoms have been reported with polynucleotides investigated in the 1970s and with other antisense constructs in early clinical trials (29–31).

To demonstrate an effect on c-raf-1 expression, we chose PBMCs as a surrogate tissue, based primarily on their availability and ease of repeated sampling, and their immediate exposure to ISIS 5132 in plasma. In previous studies, we have found that PBMC expression of certain detoxication genes correlates well with that in colon mucosa (25). An anticipated limitation was the known variability in antisense ODN uptake among various
tissue types (32) and the recognized lower permeability of this cell type to antisense constructs in general (33). Therefore, the demonstration of a biological effect in this cell type is encouraging.

Almost all of the patients showed some evidence of down-regulation of c-raf-1 in PBMCs. The effect was observed at all doses tested, and neither nadir values nor the duration of the effect varied with dose (Table 1). In six patients who demonstrated progressive depletion in the first week of therapy, evidence of recovery was observed in samples obtained on days 8 and 15. It is unclear if this pattern resulted from the latter samples being drawn 3 rather than 2 days after dosing (which may suggest recovery within 72 h of an individual dose), or if these findings may be indicative of an adaptive response. In the two patients who went on to receive multiple cycles of treatment, no such adaptive response was evident, but the possibility of generating an acquired resistant phenotype in certain individuals may reasonably be hypothesized. The basis for resistance to antisense therapy has not been described, but a parallel may exist with conventional cytotoxic agents (24, 34, 35). Down-regulation of protein expression might also contribute to resistance. Benimetskaya et al. (36) have recently identified an antigen (Mac-1) on the surface of neutrophils that acts as a receptor that can mediate ODN internalization. Another potential source of resistance might be inferred from the response of the patient with colorectal cancer; despite continuing treatment, c-raf-1 mRNA levels in the PBMCs rose concomitantly with progression in plasma CEA values, followed shortly by CT scan evidence of tumor regrowth (Fig. 2). This time course may have a number of explanations, including a pharmacokinetic cause for the escape from inhibition (accelerated plasma clearance), or induction of resistance through alterations in cell signaling pathways.

The effects on c-raf-1 expression are suggestive of but do not prove an antisense effect. The observed time course is supportive, but the absence of specimens from the lower dose levels is unfortunate because all doses from 2.5 mg/kg demonstrated a biological effect. The difficulty of demonstrating an antisense effect has been emphasized by Stein (37), and guidelines for its analysis have been published (38, 39). Drug re-

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### Table 1  c-raf-1 mRNA expression in peripheral mononuclear cells of patients who had sampling performed during cycle 1 of ISIS 5132 treatment (expressed as a percentage of baseline activity)

<table>
<thead>
<tr>
<th>Dose</th>
<th>Patient no.</th>
<th>Baseline activity %</th>
<th>Day 3 %</th>
<th>Day 5 %</th>
<th>Day 8 %</th>
<th>Day 15 %</th>
<th>Nadir %</th>
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<tr>
<td>2.5 mg/kg</td>
<td>1</td>
<td>2770</td>
<td>33</td>
<td>26</td>
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<td>32</td>
<td>26 (5)</td>
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<td></td>
<td>2</td>
<td>2449</td>
<td>64</td>
<td>198</td>
<td>52</td>
<td>7</td>
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<tr>
<td></td>
<td>3</td>
<td>2162</td>
<td>66</td>
<td>20</td>
<td>64</td>
<td>31</td>
<td>20 (5)</td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>4</td>
<td>2253</td>
<td>22</td>
<td>8</td>
<td>111</td>
<td>17</td>
<td>8 (5)</td>
</tr>
<tr>
<td></td>
<td>5</td>
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<td>12</td>
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<td>46</td>
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<tr>
<td>4 mg/kg</td>
<td>6</td>
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<td></td>
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<td>1503</td>
<td>12</td>
<td>8</td>
<td>23</td>
<td>39</td>
<td>8 (5)</td>
</tr>
<tr>
<td>5 mg/kg</td>
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<td>671</td>
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<td>135</td>
<td>78</td>
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<td>841</td>
<td>47</td>
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<td>131</td>
<td>0</td>
<td>0 (15)</td>
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<tr>
<td></td>
<td>11</td>
<td>272</td>
<td>213</td>
<td>204</td>
<td>21</td>
<td>165</td>
<td>21 (8)</td>
</tr>
<tr>
<td>6 mg/kg</td>
<td>12</td>
<td>1008</td>
<td>72</td>
<td>55</td>
<td>6</td>
<td>106</td>
<td>6 (8)</td>
</tr>
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<td></td>
<td>13</td>
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<td>65</td>
<td>41</td>
<td>62</td>
<td>99</td>
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</tr>
<tr>
<td>Median %</td>
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<td></td>
<td>42</td>
<td>26</td>
<td>62</td>
<td>36</td>
<td></td>
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<tr>
<td>Mean %</td>
<td></td>
<td></td>
<td>53</td>
<td>68</td>
<td>81</td>
<td>76</td>
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</tr>
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</table>

*NA, no sample available.*

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**Fig. 2** Changes in plasma CEA level and c-raf-1 mRNA expression during ISIS 5132 treatment in a patient with colon cancer whose disease was inhibited for 8 months.
responses may be nonantisense but sequence selective (40–42) or nonantisense and sequence independent (43). The former type of response may occur with phosphorothioate antisense drugs bearing a CpG dinucleotide (as does ISIS 5132); stimulation of B and natural killer cells by these drugs has been reported (38). However, it has been demonstrated that ISIS 5132 does not stimulate natural killer activity, because it lacks CpG flanking sequences that are required for this effect (44). It seems unlikely, too, that an immune stimulatory effect would underlie the patient responses observed, especially in the patient with colorectal cancer, a tumor that is notably resistant to immunological modulation. Among the sequence-independent effects, it is recognized that binding of polyanionic antisense drugs to a variety of peptides including some growth factors may occur. c-raf-1 expression at the mRNA level is independent of growth factor activity, however, and the very specific effect upon c-raf-1 mRNA levels in PBMCs supports a direct antisense mechanism, at least in this animal.

These findings also raise the question of the optimal scheduling of ISIS 5132. Another Phase I trial using a 21-day continuous infusion has also reported antitumor effects (45). The broad range of dose-response using the short-term infusion suggests that an infusional schedule may achieve a more uniform biological and antitumor effect. Studies to address this issue are in progress.

REFERENCES


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