Antitumor Activity of a C-raf Antisense Oligonucleotide in Combination with Standard Chemotherapeutic Agents against Various Human Tumors Transplanted Subcutaneously into Nude Mice

Thomas Geiger, Marcel Müller, Brett P. Monia, and Doriano Fabbro
Department of Oncology, Novartis Pharma, Inc., CH-4002 Basel, Switzerland [T. G., M. M., D. F.], and Department of Molecular Pharmacology, ISIS Pharmaceuticals, Carlsbad, California 92008 [B. P. M.]

ABSTRACT

A 20-mer phosphorothioate oligodeoxynucleotide (ODN) directed against human C-raf kinase (CGP 69846A or ISIS 5132) was analyzed for its antitumor activity either alone or in combination therapy. Combination studies with CGP 69846A and standard chemotherapeutic agents (cisplatin, mitomycin C, tamoxifen, or Adriamycin) were performed in nude mice that had been transplanted s.c. with a variety of human tumors (breast, prostate, colon, small cell lung, large cell lung, and squamous lung carcinomas). For the majority of the combinations studied, additive antitumor effects with CGP 69846A and the cytotoxins were found. The combination of CGP 69846A with cisplatin or mitomycin C showed superadditive antitumor activities against NCI-H69 small cell lung carcinomas with complete tumor responses. CGP 69846A, in combination with cisplatin, showed superadditive antitumor effects against PC3 human prostate carcinomas with tumor cures, and in combination with mitomycin C, superadditive antitumor activities of CGP 69846A with tumor cures against NCI-H460 large cell lung carcinoma were found.

These effects appeared to be sequence-specific because a mismatched control ODN was completely without effect as a single agent against NCI-H69 human small cell lung cancers. The combination of the mismatched control ODN with mitomycin C or cisplatin did not influence the antitumor activity of the cytotoxins against NCI-H69 human small cell lung cancers, indicating that the superadditive antitumor effects observed for CGP 69846A in combination with cisplatin or mitomycin C are the result of a sequence-specific mechanism of action in NCI-H69 human small cell lung cancers.

INTRODUCTION

Current and Future Therapy. The present treatment of cancer is based on a combination of surgery, radiotherapy, and chemotherapy. The limitations of chemotherapy reside in the unacceptable low tolerability of chemotherapeutic agents at efficacious doses, with the toxicity of these compounds originating in the lack of specificity in their mechanism of action as transcriptional inhibitors or DNA-damaging agents. It is thus clear that therapeutic alternatives that are based on strategies aiming at clearly defined, disease-relevant molecular targets are urgently required. Such novel strategies are much more likely to lead to the discovery of highly efficacious anticancer agents with much better tolerability. Recent advances in the field of nucleic acid chemistry and biochemistry in combination with an improved understanding of the factors contributing to carcinogenesis may now offer the possibility to design ODN2-based therapeutic agents that are largely devoid of the unspecific toxic side effects shown by traditional anticancer agents. The strategies involved are based on the inhibition of protein expression by the specific binding of synthetic ODNs to single-stranded RNA ("antisense" approach) or double-stranded genomic DNA ("anti-gene" approach). The underlying concepts of the antisense approach have been extensively reviewed recently (1–4).

C-raf Background. The raf family of serine/threonine-specific protein kinases comprises three members: A-raf, B-raf, and C-raf. The enzymes are expressed in a tissue-specific manner and are important mediators of signal transduction involving cell growth, transformation, and differentiation (5–7). Experimental evidence supports a direct role for C-raf kinase in the development and maintenance of human malignancies. The raf kinases are the direct downstream mediators of the ras proteins, whose oncogenic version is associated with >30% of human solid tumor types including lung, colon, and pancreas cancers (8, 9).

Antisense Approach and Previous Results. The antisense approach offers an opportunity to circumvent these problems and to knock-out target gene expression by a highly selective and sequence-specific mechanism. CGP 69846A (ISIS 5132) is a 20-mer phosphorothioate antisense ODN that specifically inhibits the expression of human C-raf mRNA and protein in cancer cells with an IC50 below 100 nM (10). CGP 69846A displayed potent antitumor activity as a single agent against A549 lung, T24 bladder, Colo205 colon, and MDA-MB231...
breast carcinomas in vivo in the dose range of 0.006–6 mg/kg (10).

Present Study. The antitumor activity of CGP 69846A in combination with standard chemotherapeutic agents was investigated in the present study. All chemotherapeutics used represent first- or second-line therapy in clinical oncology such as TMX for breast cancer; ADR for gastric, prostate, and breast cancer; MIT-C for colorectal, gastric, lung, and breast cancer; and CPT for prostate, lung, and gastric cancer. The combination of a C-raf antisense ODN with standard chemotherapeutic agents in vivo is a novel strategy for the treatment of human cancer that has not been described in the literature before.

MATERIALS AND METHODS

Antitumor Activity in Vivo. Female or male BALB/c nude mice were obtained from Bomholtgaard (Copenhagen, Denmark) or from the Ciba-Geigy animal farm (Sisseln, Switzerland). For all in vivo experiments, CGP 69846A was dissolved in sterile saline (0.6 mg/ml) and tested at a dose of 6.0 mg/kg by once daily i.v. administration. Tumors were implanted s.c. into female or male BALB/c nude mice. Animals were kept under sterile conditions with free access to food and water. For all in vivo experiments, tumors were serially passaged by a minimum of three consecutive transplantations prior to start of treatment. Tumor fragments (∼25 mg) were implanted s.c. into the left flank of animals with a 13-gauge trocar needle, and for the hormone-dependent breast cancer line MCF-7, an estrogen pellet containing ∼5 mg estradiol (Dow Chemicals, France) was implanted into the right flank of animals under Forene anesthesia (Abbott, Switzerland). Six animals were used per group. Treatments were started when the tumors reached a mean tumor volume of 100–150 mm³. Tumor growth was monitored once to three times weekly and 24 h after the last treatment by measuring perpendicular diameters. Tumor volumes were calculated as described (11). Tumor regression (%) represents the smallest mean tumor volume compared with the mean tumor volume at the start of treatment.

Cell Lines. All cell lines used were obtained from the ATCC and were cultured in the suggested media and additives (ATCC culture conditions) prior to s.c. injection. The following human tumor cells were used for the experiments: estrogen-dependent breast cancer MCF-7 (ATCC: HTB22), colon cancer HCT 116 (ATCC: CCL247), small cell lung carcinoma NCI-H69 (ATCC: HTB119), large cell lung carcinoma NCI-H460 (ATCC: HTB177), squamous cell carcinoma NCI-H520 (ATCC: HTB182), and prostate carcinoma PC3 (ATCC: CRL1435).

Chemotherapeutic Drugs. ADR was purchased from Farmitalia Carlo Erba (Milan, Italy), and MIT-C was from
Fig. 2 Effect of CGP 69846A in combination with MIT-C (A) or CPT (B) on the growth of s.c. transplanted NCI-H69 human small cell lung carcinoma in female BALB/c nude mice. Tumor fragments of approximately 25 mg were transplanted into the left flank of each female BALB/c nude mouse (n = 6 per group). Treatment was started on day 13 after tumor transplantation, and the end of treatment was on day 34. CGP 69846A was administered once daily (days 13-34) at the dose of 6.0 mg/kg i.v. as a single agent as well as in the combination group. A. MIT-C (3.5 mg/kg i.v.) was administered once weekly (days 13, 20, and 27) as a single agent as well as in the combination group. B. CPT (11 mg/kg i.v.) was administered once weekly (days 13 and 20) as a single agent as well as in the combination group. Mean tumor volume is given in cm³; bars, SD.

Kyowa Hakko (Shizuoka, Japan). These chemotherapeutics were dissolved in saline. CPT was from Bristol Meyers Squibb (Princeton, NJ) and given as the pharmacope formulation. Pharmacope was purchased from Bristol Myers Squibb and used exactly as it is used in the clinics to formulate CPT. TMX was from Farmos (Helsinki, Finland) and was suspended in 0.5% methylcellulose prior to treatment.

In combination studies, the chemotherapeutic agents were applied as follows: TMX, 20 mg/kg p.o., three consecutive days weekly; ADR, 9 mg/kg i.v., once weekly; CPT, 11 mg/kg i.v., once weekly in pharmacope; and MIT-C, 3.5 mg/kg i.v., once weekly. These schedulings were the maximal tolerated doses in the mouse strain used for these experiments. Details are given in the legends to the figures. Control animals received the same vehicles either p.o. or i.v. as the animals from the drug-treated groups. CGP 69846A was injected once daily 1 h prior to the application of the chemotherapeutic agents. Tumor size was measured thereafter.

ODNs. The sequences of CGP 69846A and the mismatched control ODN are: CGP 69846A (ISIS 5132), 5'-TCC-CGC-CTG-TGA-CAT-GCA-TT-3'; Control-ODN (seven mismatches), 5'-TCC-CGC-GCA-CTT-GAT-GCA-TT-3'. Phosphorothioate ODNs were synthesized on an Applied Biosystems 380B automated DNA synthesizer essentially as described (12). ODNs were deprotected by overnight incubation at room temperature in methanolic ammonia. ODNs were purified using a C-18 Sep-Pak cartridge (Waters, Milford, MA) followed by ethanol precipitation. CGP 69846A and the control ODN are the fully neutralized sodium salts of a 3'-S'-linked (2-deoxynucleotide phosphorothioate) ODN 20-mer in which each of the 19 internucleotide linkages is a racemic O-linked phosphorothioate.

RESULTS

Human Breast Carcinomas. The antitumor activity of CGP 69846A was tested in combination therapy with standard chemotherapeutic agents against various human tumors transplanted s.c. into nude mice. CGP 69846A was tested in combination with TMX against estrogen receptor-positive MCF-7 tumors in female BALB/c nude mice. CGP 69846A was as active as a single agent as TMX in inhibiting the growth of MCF-7 tumors in vivo (Fig. 1A). The combination of CGP 69846A with TMX resulted in an additive antitumor effect, as shown in Fig. 1A.
Fig. 3   Effect of CGP 69846A in combination with MIT-C on the growth of s.c. transplanted NCI-H460 human large cell lung carcinoma (A) and NCI-H520 human squamous cell lung carcinoma (B) in female BALB/c nude mice. A tumor fragments of approximately 25 mg were transplanted into the left flank of each female BALB/c nude mouse (n = 6 per group). Treatment was started on day 4 after tumor transplantation, and the end of treatment was on day 30. CGP 69846A was administered once daily (days 4–18) at the dose of 6.0 mg/kg i.v. as a single agent as well as in the combination group. MIT-C (3.5 mg/kg i.v.) was administered once weekly (days 4, 11, and 18) as a single agent as well as in the combination group.

B. tumor fragments of approximately 30 mg were transplanted s.c. into female BALB/c nude mice (n = 6 per group). Treatment was started at day 8, and the end of treatment was on day 19. CGP 69846A was administered once daily (days 8–19) at a dose of 6 mg/kg i.v. as a single agent as well as in the combination group. MIT-C (3.5 mg/kg i.v.) was administered once weekly (days 4, 11, and 18) as a single agent as well as in the combination group. Mean tumor volume is given in cm³; bars, SD.

ADR was more effective than CGP 69846A (Fig. 1B). Additive antitumor effects against MCF-7 breast carcinomas were found when CGP 69846A was applied in combination with ADR, and complete inhibition of tumor growth up to day 38 was observed, as shown in Fig. 1B.

**Human Lung Carcinomas.** The antitumor activity of CGP 69846A in combination with MIT-C was studied against NCI-H69 human small cell lung carcinomas. MIT-C was more active as a single agent than CGP 69846A and resulted in tumor regressions of 60%, as shown in Fig. 2A. For the combination of CGP 69846A with MIT-C, a superadditive antitumor activity with complete tumor responses around day 32 was found against NCI-H69 small cell lung carcinomas (Fig. 2A).

The animals with tumor cures were carefully observed up to day 41, and no indications of tumor growth were observed. CGP 69846A as a single agent was less efficacious than CPT against NCI-H69 human small cell lung cancers, as shown in Fig. 2B. The combination of CGP 69846A with CPT resulted in superadditive antitumor activity against NCI-H69 human small cell lung carcinomas; however, no cures were found when CGP 69846A was applied in combination with CPT (Fig. 2B).

CGP 69846A was tested in combination with MIT-C in NCI-H460 human large cell lung carcinomas. MIT-C was more effective than CGP 69846A as a single agent in inhibiting growth of NCI-H460 carcinomas in nude mice and resulted in partial tumor regressions from days 4 to 29. Thereafter, however, regrowth of the tumors was observed under treatment with MIT-C (Fig. 3A). CGP 69846A was only slightly active as a single agent against NCI-H460 large cell lung carcinomas. CGP 69846A in combination with MIT-C resulted in a superadditive antitumor activity with complete tumor responses around day 18 (Fig. 3A). The animals with tumor cures were carefully observed up to day 36, and no indications for tumor regrowth were found.

CGP 69846A was tested in combination with MIT-C in NIH-H520 human squamous lung carcinomas. MIT-C was more active than CGP 69846A as a single agent and resulted in an almost complete inhibition of tumor growth; however, no tumor regression was observed (Fig. 3B). The combination of CGP 69846A with MIT-C resulted in an additive antitumor effect against human squamous lung carcinomas with a complete inhibition of tumor growth up to day 20 (Fig. 3B).
Human Prostate Carcinomas. The antitumor activity of CGP 69846A was studied as a single agent and in combination with CPT against PC3 human prostate carcinomas. CPT as a single agent was more efficacious than CGP 69846A (Fig. 4A) and resulted in tumor regressions of 38%. When CGP 69846A and CPT were used in combination, a superadditive antitumor activity was found with a complete tumor response approximately 20 days following initiation of treatment (Fig. 4A).

Human Colon Carcinomas. The antitumor activity of CGP 69846A was studied as a single agent and in combination with CPT against HCT 116 human colon carcinomas. CGP 69846A was more effective than CPT as a single agent in this tumor (Fig. 4B). Additive antitumor activity was observed when CPT and CGP 69846A were given in combination (Fig. 4B); however, no complete inhibition of tumor growth was seen with this combination.

Effect of a Control ODN in Combination with MIT-C and CPT against NCI-H69 Human Small Cell Lung Carcinomas. A mismatched control phosphorothioate ODN was synthesized (see “Materials and Methods” for sequence), and the antitumor effect was studied in combination with MIT-C or CPT against NCI-H69 human small cell lung carcinomas. The control ODN (6 mg/kg i.v. daily) as a single agent did not influence the growth of NCI-H69 tumor in nude mice (Fig. 5). When the control ODN and MIT-C were given in combination, the antitumor activity of mitomycin as a single agent was slightly inhibited (Fig. 5A). The same effect, i.e., slight inhibition of antitumor activity was seen when the control ODN was given in combination with CPT against NCI-H69 human small cell lung carcinomas (Fig. 5B).

DISCUSSION

CGP 69846A has previously been shown to be a potent antitumor compound as a single agent against a variety of human tumors transplanted into nude mice (10). The antitumor activity of CGP 69846A in combination with standard chemotherapeutic agents against a panel of human tumors transplanted into nude mice was investigated in this study. In none of the combinations studied were antagonistic effects observed in vivo between CGP 69846A and traditional anticancer agents, which is an important observation with relevance to the planning of combination studies of CGP 69846A with chemotherapeutic agents in Phase II clinical trials in humans. Binding of phosphorothioate ODNs to actinomycin D and neutralization of actinomycin D effects has been described in cell culture exper-
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Taken together, the hypothesis of apoptosis induction may, although this hypothesis has not been addressed experimentally in the present study. It has been shown recently that inhibitors of protein kinase C enhance chemotherapy-induced apoptosis in cancer cells by interaction with the anti-apoptotic protein bcl-2 and phosphorylation of the proapoptotic protein Bad (16, 17). It is, therefore, likely that the inhibition of C-raf expression in tumors by CPG 69846A induces apoptosis on its own and potentiates the chemotherapy-induced apoptosis. Taken together, the hypothesis of apoptosis induction may, therefore, explain the synergistic antitumor effects of CPG 69846A in combination with chemotherapeutic agents in vivo.

The lack of additive or synergistic antitumor activity of MIT-C or CPT in combination with a mismatched phosphorothioate control oligonucleotide excluded the possibility that the observed synergistic effects are due to a nonsequence-dependent phosphorothioate effect and supports an antisense mechanism of action. For the experiment with the control ODN in combination with CPT and MIT-C, NCI-H69 human small cell carcinomas were used because in this cell line, impressive superadditive antitumor effects were observed with CPG 69846A in combination with MIT-C or CPT with tumor cures. The molecular mechanism of action that leads to the superadditive and synergistic antitumor activity of CPG 69846A in combination with MIT-C or CPT with tumor cures is still unclear. A potential hypothesis to explain the enhancing effect of CPG 69846A on chemotherapy may be the potentiation of chemotherapy-induced apoptosis, although this hypothesis has not been addressed experimentally in the present study. It has been shown recently that inhibitors of protein kinase C enhances the effectiveness of CPG 69846A on chemotherapy, and therefore, the hypothesis of apoptosis induction may, therefore, explain the synergistic antitumor effects of CPG 69846A in combination with chemotherapeutic agents in vivo.

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its antitumor activities and its chemotherapy-potentiating effects by a sequence-specific inhibition of C-raf expression, presumably through an antisense mechanism of action. Demonstration of specificity is crucial for the proof of concept and is especially important for the class of phosphorothioate ODNs because it has been shown that phosphorothioates can exert their effects on cultured cells and in vivo by nonsequence-dependent mechanisms of action (19–23). CGP 69846A is presently in development for the treatment of human tumors and is currently in Phase I clinical trials. It is planned to administer CGP 69846A in Phase I clinical trials as a single agent and in combination with standard chemotherapeutic agents. The demonstration of additive and synergistic antitumor effects of CGP 69846A in combination with chemotherapeutic agents warrants the hope that this compound may increase either the response rate of resistant tumors or the overall effect of chemotherapeutic agents for a variety of different cancers without increasing the toxic side effects. Various cancers such as prostate cancers, melanomas, and non-small cell lung cancers that do not respond effectively to standard chemotherapies may become susceptible when treated with cytoxins in combination with CGP 69846A. It is hoped that this novel anticancer agent that exerts its effects by a totally novel mechanism of action helps to improve the treatment of human cancers. The improvement of cancer therapy would be a highly important progress and would certainly help to establish this novel signal transduction inhibitor as a therapeutic agent for the therapy of cancer in the clinic.

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