c-myc Antisense Oligodeoxynucleotides Enhance the Efficacy of Cisplatin in Melanoma Chemotherapy in Vitro and in Nude Mice

Gennaro Citro, Igea D’Agnano, Carlo Leonetti, Roberto Perini, Barbara Bucci, Gerald Zon, Bruno Calabretta, and Gabriella Zupi

Laboratory of Experimental Chemotherapy, Regina Elena Cancer Institute, Centro Ricerca Sperimentale. 00158 Rome, Italy. G. C., I. D., C. L., R. P., B. B., G. Z.; Biomedical Technology Institute, Consiglio Nazionale delle Ricerche. 00161 Rome, Italy [I D.]; Lynx Therapeutics, Hayward, California 94545 [G. Z.]; and Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107-6799 [B. C.]

ABSTRACT

This study was designed to assess the efficacy of a new antineoplastic therapeutic strategy that relies on the use of a c-myc antisense 15-mer phosphorothioate oligodeoxynucleotide (S)ODN, in combination with cisplatin (cis-diamminedichloroplatinum; DDP), which is currently used in the clinical management of melanoma patients. Proliferation and colony formation of melanoma cells were both inhibited by the DDP/c-myc antisense [S]ODN combination to a greater extent than that observed with either agent alone. Inhibition was most effective when DDP was followed by c-myc antisense [S]ODNs. Cell cycle flow cytometric analysis of cells exposed to the two agents either alone or in combination demonstrated that (a) c-myc antisense [S]ODNs induced an accumulation of cells in S phase and apoptosis in a fraction of the cells, detectable at day 5 after the beginning of treatment; (b) DDP induced a block in G2-M phase detectable at day 1, which was partially recovered, and apoptosis similar in extent to that induced by c-myc antisense [S]ODNs; and (c) DDP and c-myc antisense [S]ODNs together induced arrest in G2-M phase, which was maximum at day 3, i.e., delayed as compared to the block induced by DDP. The combination induced a higher percentage of apoptosis, evident at day 3 from the start of treatment, that correlated with a marked reduction in Bel-2 expression. Mice bearing human melanoma xenografts and treated sequentially with DDP and c-myc antisense [S]ODNs showed a higher inhibition of tumor growth, reduction in the number of lung metastases, and increase in life span compared with those treated with either agent alone. Together, these data lend support to the development of anticancer therapies involving oncogene-targeted antisense ODNs and conventional antineoplastic drugs.

INTRODUCTION

Recent advances in delineating the genetic and molecular bases of cancer have raised interest in the possibility of correcting the genetic alterations responsible for the phenotype of neoplastic cells (1, 2). The identification of activated oncogenes and inactivated tumor suppressor genes as the fundamental genetic difference between tumor and normal cells has made it possible to consider these genes as potential targets for antitumor therapy.

An attractive approach to controlling gene expression is represented by the “antisense” strategy, which, by restoring normal regulatory functions in cancer cells, could render them more susceptible to standard chemotherapy. Systemic administration of exogenous antisense oligodeoxynucleotides complementary to oncogene-encoded mRNAs inhibits gene expression by forming RNA-DNA duplexes, thereby reducing the activity of the targeted gene products (3, 4). Antisense oligodeoxynucleotides have been reported to inhibit a variety of oncogenes, including ras, bcr/abl, bel-2, c-myc, and c-myc in tumors of different histotypes (5–7).

We reported previously that c-myc antisense [S]ODNs3 are effective inhibitors of human melanoma cell growth in vitro and in vivo (6). Mice bearing human melanoma xenografts and treated with c-myc antisense [S]ODNs showed a marked inhibition of tumor growth, a reduction in the number of lung metastases, and an increased life span. This antitumor activity was associated with an early down-regulation of c-Myc expression, followed by inhibition of cell proliferation and induction of apoptosis. However, despite the significantly longer survival time, all treated animals died of their disease, indicating that inhibiting the expression of a single dysregulated oncogene is insufficient to completely suppress tumor cell proliferation. Therefore, the use of ODNs in combination with antineoplastic drugs might result in more complete control of cancer growth (8, 9).

To determine whether an antisense strategy might enhance the efficacy of antitumor therapy, we investigated the effects of c-myc antisense [S]ODNs in combination with cisplatin (DDP) on human melanoma cells constitutively expressing the c-myc oncogene. DDP was chosen based on clinical studies showing that, in combination with other drugs, it improves the therapeutic efficacy in metastatic melanoma. In addition, other authors have demonstrated that the combination of DDP and c-myc antisense [S]ODNs determines a synergistic inhibitory effect on bladder tumor cells in vitro (10). However, no studies have been reported on the efficacy of such therapy in experimental models in vivo.

In the present study, we show that the combination of DDP and c-myc antisense [S]ODNs exerts significantly greater antiproliferative and antitumor effects than either agent used alone on human melanoma cells in vitro or xenografted to mice. Cell cycle perturbations and apoptosis might underlie the enhanced efficacy of this combination.

MATERIALS AND METHODS

Tumor Cell Lines and Tumor Implants. Human melanoma cell line M14 was maintained as a monolayer culture in RPMI supplemented with 10% heat-inactivated FCS, antibiotics, and l-glutamine at 37°C in a 5% CO2–95% air atmosphere in a humidified incubator. Human primary melanoma NG was obtained and transplanted as described (6). CD-1 male nude (nu/nu) mice, 6–8 weeks old, were injected in the hind leg muscles with a cell suspension of 2.5 × 106 NG cells. A tumor mass of ∼70 mg was evident in all mice on day 4 after implant. All experiments were carried out between the fifth and eighth passages of the NG tumor in nude mice.

Drug and [S]ODNs. Clinical grade DDP (Protop Platamine) was obtained from Pharmacia (Milan, Italy). DDP dilutions were freshly prepared before each experiment.

A 15-mer antisense [S]ODN (5'-AAGCTGAGGAGGCT-3') complementary to the translation initiation region of c-myc mRNA (Lynx Therapeutics Inc., Hayward, CA) was used. The scrambled sequence [S]ODN containing the

Received 7/2/97; accepted 11/10/97.

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3 The abbreviations used are: [S]ODN, phosphorothioate oligodeoxynucleotide; DDP, cis-diamminedichloroplatinum; EBSS, Earle’s balanced salt solution; TWI, tumor weight inhibition.

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G-quartet motif (5'-AACGATACGGGTTG-3') was used as control. [SJ]ODNs were resuspended at 5 mg/ml in sterile EBSS.

**In Vitro** [SJ]ODNs and DDP Treatment. M14 cells were seeded in 60-mm Petri dishes at a density of 3 x 10^5 cells per dish. After 24 h, [SJ]ODNs were added at concentrations of 100 μg/ml on day 1 and 50 μg/ml on days 2–4. Cells were exposed to DDP at a dose of 1 μg/ml on day 1 or day 5 of growth. Equimolar amounts of c-myc scrambled [SJ]ODNs were used in control experiments. In combination experiments, cells were pre- or posttreated with DDP for 2 h and exposed to c-myc antisense [SJ]ODNs for 4 days. For each combination, parallel analyses with each agent alone were performed and processed by changing the medium at the same time. Antiproliferative effects were evaluated daily based on cell number and viability as assessed in a Coulter counter (model ZM; Kontron Instruments, Lunen, United Kingdom) and by trypan blue dye exclusion, respectively.

To evaluate cell colony-forming ability, aliquots of cell suspension from each sample were seeded into 60-mm Petri dishes with RPMI 1640 and incubated at 37°C in a 5% CO2-95% air atmosphere for 10–12 days. Colonies were stained with 2% methylene blue in 95% ethanol and counted (1 colony ≥50 cells). Surviving fractions were calculated as the ratio of absolute survival of the treated sample/absolute survival of the control sample. All experiments were repeated four times in triplicate.

**Western Blotting.** Protein isolation and Western blot analyses were as described (6). Briefly, proteins (30 μg) were separated by 10% SDS-PAGE, transferred to nitrocellulose filters, and reacted with monoclonal antibodies specific for human p62 c-Myc (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA), p53 (clone Ab2; Oncogene Science, Manhasset, NY), and bcl-2 (clone 124; DAKO A/S, Glostrup, Denmark). After stripping, filters were incubated with an anthuman β-actin antibody (clone JLA 20; Oncogene Science), and reactivity was detected by enhanced chemiluminescence (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom) according to the manufacturer’s instructions. c-Myc, p53, and bcl-2 levels were quantified by scanning densitometry (Bio-Rad G700) of the autoradiography films and normalized to β-actin levels.

**Propidium Iodide Staining and Flow Cytometry.** Cell cycle analysis of M14 cells exposed to DDP and [SJ]ODNs, either separately or in combination, was performed by flow cytometry as described (8) during treatment and after treatment (0.5, 1, 2, 3, 4, 5, and 6 days after treatment). Apoptosis was evaluated as described (6) at days 3, 5, and 6, and an apoptotic index was calculated by dividing the percentage of apoptotic cells in treated samples by that in the control cells as determined by flow cytometry. Morphology of untreated and treated cells was analyzed in May-Grünwald/Giemsa-stained cytopsin.

**In Vivo Treatments.** CD-1 male nude mice (6–8 weeks old and 22–24 g in body weight) were obtained from Charles River Laboratories (Calco, Italy). Animals were kept under pathogen-free conditions and given acidified, autoclaved water and γ-irradiated commercial diet ad libitum. All manipulations were performed under sterile conditions in a laminar air-flow hood. All procedures involving mice and their care were in accord with institutional guidelines in compliance with national and international laws and policies (European Economic Community Council Directive 86/109, OJL 358, Dec. 1, 1987, and with the NIH Guide for the Care and Use of Laboratory Animals (11). Each experimental group included at least 10 mice, with a maximum of 18 animals.

**[SJ]ODNs were injected i.v. in alternating doses of 1 and 0.5 mg/day for 8 consecutive days. This schedule was chosen based on previous studies (6).** DDP was administered i.p. at 10 mg/kg (10% of the lethal dose; LD_{50}) in three consecutive daily injections (3.3 mg/kg). Preliminary experiments demonstrated that this scheduling was less toxic than the single injection in terms of body weight loss and drug deaths. Three cycles at 7-day intervals were administered according to the following schedules. In schedule A, DDP (3.3 mg/kg/day) was administered at days 4–6, 22–24, and 40–42, and in schedule B, DDP (3.3 mg/kg/day) at days 7–9, 25–27, and 43–45 was administered. In schedules C (c-myc antisense) and D (scrambled [SJ]ODNs), the cycles were as follows: 1 mg/mouse/day at days 7, 9, 11, and 13; 0.5 mg/mouse/day at days 8, 10, 12, and 14; 1 mg/mouse/day at days 25, 27, 29, and 31; and 0.5 mg/mouse/day at days 43, 45, 47, and 49; and 0.5 mg/mouse/day at days 44, 46, 48, and 50. In the combination experiments, schedules A plus C (schedule E) and schedules A plus D (schedule F) were administered (see Table 1). As a control, tumor-bearing mice treated with EBSS diluent were used.

The tumor weight was calculated from caliper measurements according to the method of Geran et al. (12). To evaluate the metastatic potential of NG melanoma, tumor-bearing mice were killed 28 days after tumor cell implantation, their lungs were removed and fixed in Bouin’s solution to distinguish tumor nodules from lung tissue, and the number of metastases was determined under a dissecting microscope. Tumor growth was assessed as the basis of apparent drug-related deaths and net body weight loss. Death in a treated mouse was presumed to be treatment related if the mouse died within 7 days after the last treatment. Net body weight loss was calculated as a percentage of the mean body weight of untreated mice.

Therapeutic efficacy of treatments was evaluated using the following end points: (a) percentage of TWI calculated as 1 − (mean tumor weight of treated mice/mean tumor weight of controls) × 100; (b) tumor growth delay, evaluated as T − C, where T and C are the median times for treated and control tumors, respectively, to reach the same size (i.e., 700 mg); (c) percentage of increase in the number of lung metastases; and (d) percentage of increase in life span calculated as [1 − survival time (in days) of treated mice/median survival time (in days) of control mice] × 100.

**Statistical Analysis.** Results were analyzed by the Mann-Whitney non-parametric test. Differences were considered significant at P < 0.05 (two-sided). Linear regression analysis was performed on in vivo tumor growth curves, and the slopes were compared to evaluate the statistical significance of the differences.

## RESULTS

**Effects of c-myc Antisense [SJ]ODNs and DDP on M14 Cells.** Fig. 1 shows the proliferation of M14 melanoma cells treated with c-myc antisense [SJ]ODNs and DDP used alone or in combination. Only the scrambled [SJ]ODN sequence containing the G-quartet motif was used as a control, because sense, scrambled, and scrambled G-quartet sequences were all completely ineffective on M14 and NG melanoma cells both in vitro and in vivo (6). The scrambled [SJ]ODN control sequence did not exert any antiproliferative effect either alone or in combination with DDP. Proliferation of cells exposed to DDP for

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**Table 1: Therapeutic efficacy of c-myc [SJ]ODNs and DDP in NG melanoma-bearing mice**

<table>
<thead>
<tr>
<th>Schedulea</th>
<th>TWI at first cycle, %</th>
<th>TWI at second cycle, %</th>
<th>TWI at third cycle, %</th>
<th>T − Cb</th>
<th>Toxic deaths</th>
<th>Metastasis reduction, %c</th>
<th>Increase in life span, %d</th>
</tr>
</thead>
<tbody>
<tr>
<td>A DDP (day 4)</td>
<td>61</td>
<td>60</td>
<td>52</td>
<td>10</td>
<td>0 of 16</td>
<td>8</td>
<td>35</td>
</tr>
<tr>
<td>B DDP (day 7)</td>
<td>52</td>
<td>51</td>
<td>46</td>
<td>7.5</td>
<td>0 of 10</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>C antisense</td>
<td>45</td>
<td>50</td>
<td>50</td>
<td>7</td>
<td>0 of 10</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td>D scrambled</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0 of 10</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>E DDP (Antisense)</td>
<td>87</td>
<td>92</td>
<td>93</td>
<td>36</td>
<td>0 of 18</td>
<td>52</td>
<td>75</td>
</tr>
<tr>
<td>F DDP (Scrambled)</td>
<td>63</td>
<td>62</td>
<td>65</td>
<td>45</td>
<td>8</td>
<td>1 of 10</td>
<td>8</td>
</tr>
</tbody>
</table>

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*Three cycles at 7-day intervals were administered according to the following schedules: A, DDP 3.3 mg/kg/day at days 4–6, 22–24, and 40–42; B, DDP 3.3 mg/kg/day at days 7–9, 25–27, and 43–45; C, c-myc antisense; and D, scrambled [SJ]ODNs 1 mg/mouse/day at days 7, 9, 11, and 13 and 0.5 mg/mouse/day at days 8, 10, 12, and 14; E, [SJ]ODNs 1 mg/mouse/day at days 25, 27, 29, and 31 and 0.5 mg/mouse/day at days 26, 28, 30, and 32; and F, scrambled [SJ]ODNs 1 mg/mouse/day at days 43, 45, 47, and 49, and 0.5 mg/mouse/day at days 44, 46, 48, and 50. In the combination experiments, schedule A + C (schedule E) and schedule A + D (schedule F) were administered. Tumor-bearing mice treated with EBSS diluent were used as a control.

*14 Tumor growth delay (median times in days for treated and control tumors to reach the size of 700 mg).

*15 The median number of metastases in the untreated group was 29 (15–35).

*16 All end point values were significantly different (P < 0.0001) among schedule (E) and the other schedules.

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Fig. 1. Effect of c-myc antisense [S]ODNs and DDP alone or in combination on M14 melanoma cell proliferation. Cells were treated beginning 1 day after cell seeding with the following schedules: A, scrambled (X) or c-myc antisense (C) [S]ODNs for 4 days (100 μg/ml on the 1st day and 50 μg/ml on days 2–4), and DDP (•) for 2 h (1 μg/ml) and DDP for 2 h followed by scrambled (A) or c-myc antisense (C) [S]ODNs for 4 days at the same concentrations. Statistical analysis: □—□ curve versus □—□ curve, P = 0.004; □—□ curve versus •—• curve, P = 0.04; B, symbols and concentrations of the two agents are the same as in A. DDP was administered for 2 h at day 3 after cell seeding. In the combination, DDP followed [S]ODN exposure. Statistical analysis: □—□ curve versus □—□ curve, P = 0.026; curve □—□ curve versus •—• curve, P = 0.0022. Data in A and B are representative of four experiments with similar results; values are means (bars, SE) of triplicate samples within the same experiment. When not shown, the SE is smaller than the symbols.

Fig. 2. p62 c-Myc protein levels in M14 cells treated with scrambled [S]ODNs (S), c-myc antisense [S]ODNs (AS), DDP (DDP), and DDP followed by c-myc antisense [S]ODNs (DDP/AS; see Fig. 1 legend); C, untreated cells. Analysis was performed at day 2 from the beginning of treatments.

2 h followed by c-myc antisense [S]ODN treatment was inhibited to a greater extent than that observed after treatment with either agent alone (Fig. 1A). This effect was already evident 2 days after the start of treatment (day 3 of growth), and cell number decreased further between days 6–10 of culture (more than 90% growth inhibition with respect to control cells). DDP induced a maximal accumulation of cell proliferation of about 65%, and the antisense [S]ODNs determined a reduction in cell number evident from day 4 of growth, with a maximum inhibition of 55% as compared to control cells. In cells treated first with c-myc antisense [S]ODNs for 4 days followed by DDP for 2 h, cell number was also markedly reduced (75%), especially compared to the effect of DDP, which reduced cell number by about 40% (Fig. 1B). As expected, DDP was less effective when administered in the plateau growth phase than during exponential growth.

All subsequent experiments were performed using the DDP followed by the c-myc antisense [S]ODN protocol, which is the most effective.

Immunoblot analysis of c-Myc protein levels in lysates from M14 cells exposed to the different treatments to confirm the sequence-specific effect of [S]ODNs (Fig. 2) demonstrated that c-myc antisense [S]ODN treatment reduced c-Myc protein levels by about 40% in cells treated with antisense [S]ODNs alone or in combination with DDP, as compared to control cells. By contrast, c-Myc protein levels detected in lysates from both scrambled [S]ODN- and DDP-treated cells were superimposable to levels of control cells.

Comparison of survival curves of M14 cells exposed to DDP followed by c-myc antisense [S]ODNs and to each compound used alone (Fig. 3) revealed a constant inhibition of colony formation elicited by antisense [S]ODNs alone starting from day 4 of culture, with a surviving fraction of about 50%, whereas DDP administered alone induced a decrease in cell survival of about 85% observed at day 4 of growth, with a partial recovery within the following days to about 50%. The survival curve in cells exposed to DDP followed by c-myc antisense [S]ODNs was biphasic: after an exponential decrease in cell survival similar to that obtained from administering DDP alone, a plateau phase was observed, indicating that the cells are unable to recover from the DDP-induced damage.

Cell Cycle Perturbations and Apoptosis in M14 Cells. To elucidate the mechanisms by which the DDP plus c-myc antisense [S]ODN combination determines a significant inhibition of cell proliferation and survival, we analyzed cell cycle distribution of treated and untreated M14 cells. Histograms of DNA content (Fig. 4) revealed that antisense treatment induced an accumulation of cells in S phase, evident at day 3 after the start of treatment and concomitant with a decrease in the percentage of G1 cells. This result is consistent with a prolonged staying of the antisense-treated cells in S phase as evaluated by bromodeoxyuridine incorporation (not shown). DDP treatment induced G2-M-phase accumulation detectable at 12 h after treatment and partially recovered within the following 3 days. However, DDP followed by c-myc antisense [S]ODNs produced significantly greater G2-M-phase accumulation still markedly evident at day 3 from the beginning of treatment than that induced by DDP alone. At day 6, cells exposed to either DDP or c-myc antisense [S]ODNs showed a sub-G1 peak, typical of apoptotic cells, with a concomitant loss of cells from the G2-M and S phases, respectively. The cell fraction in the sub-G1 peak increases after exposure to the DDP plus...
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Fig. 4. Effect of DDP and [S]ODNs alone or in combination on the M14 cell cycle. Cell cycle analysis after propidium iodide staining was performed by flow cytometry at days 0.5 (a), 3 (b), and 6 (c) after the start of treatments. Untreated cells (C) and cells treated with scrambled [S]ODNs (S), c-myc antisense [S]ODNs (AS), DDP (DDP), and DDP followed by c-myc antisense [S]ODNs (DDP/AS) were analyzed.

Fig. 5. Effect of DDP (■), DDP followed by c-myc antisense [S]ODN combination (▲), and c-myc antisense [S]ODNs (●) on G2-M phase distribution in M14 cells. The percentage of change in the G2-M phase fraction was calculated by dividing the percentage of treated cells in G2-M phase by that of untreated cells at the indicated time after the start of treatments.

The c-myc [S]ODN combination, concomitant with a depletion of cells from both S and G2-M phase. The scrambled [S]ODN sequence did not affect cell cycle phase distribution. Daily analysis to evaluate progression through the cell cycle during and immediately after the treatment (Fig. 5) revealed no detectable changes in the percentage of cells in G2-M phase after exposure to c-myc antisense [S]ODNs alone compared to untreated cells. Consistent with the data in Fig. 4, the DDP plus c-myc antisense [S]ODN combination induced an arrest in G2-M phase, reaching a maximum value with a delay of 2 days compared to the block induced by DDP. Apparently, the block induced by DDP alone is completely recovered at day 5, whereas the DDP plus c-myc antisense [S]ODN combination prevents the cells from progressing through the cell cycle. These results suggest that the DDP reversible G2-M phase arrest becomes irreversible on DDP plus c-myc antisense [S]ODN treatment.

On the basis of DNA histograms at day 6 from the beginning of treatment (Fig. 4) and based on previous data indicating the induction of apoptosis by c-myc antisense [S]ODNs and by DDP (6, 13, 14), we analyzed apoptosis in M14 cells (Fig. 6A).

Apoptosis was more extensive in M14 cells exposed to the DDP plus c-myc antisense [S]ODNs than after treatment with either agent alone. DDP plus c-myc antisense [S]ODN-induced apoptosis was evident at day 3 after treatment with an apoptotic index ranging from 13 to 20 up to day 6 from the beginning of treatment, whereas DDP and c-myc antisense [S]ODNs alone induced a lower level of apoptosis even at day 6 (maximum apoptotic index, 10; Fig. 6A). Apoptosis was confirmed by morphological analysis of untreated and treated cells (data not shown). These data are in agreement with the Western blot analysis of p53 and Bcl-2 proteins associated with the apoptotic program (Fig. 6B). Densitometric analysis of the blots indicated that...
c-myc antisense [S]ODNs, DDP, and the combination of the agents all induced an increase in cellular levels of p53 (~2-fold) evaluated at either day 2 or day 3. Bcl-2 levels decreased slightly at day 2 in all treated cells; at day 3, DDP and the c-myc antisense [S]ODNs each determined a 50% reduction in Bcl-2 expression, whereas the combination reduced Bcl-2 expression by ~75%.

**In Vivo Antitumor Effect of DDP and c-myc Antisense [S]ODNs.** To evaluate the efficacy of DDP and c-myc antisense [S]ODNs in vivo, mice bearing NG melanoma were treated with the two agents, alone or in combination, at day 4 after cell injection, and tumor weight was monitored (Fig. 7). DDP and c-myc antisense [S]ODNs administered separately caused a significant decrease in tumor growth of ~50% after the end of the third treatment cycle, whereas the scrambled sequence did not affect tumor growth. DDP plus c-myc antisense [S]ODNs in combination, however, inhibited tumor growth by ~90% even after the first cycle of treatment, with complete tumor regression evident from day 10 up to day 18 of growth. The antitumoral effect elicited by the combination of DDP plus c-myc antisense [S]ODN treatment was significantly more potent (P < 0.0001) than that obtained using the two agents separately.

Table 1 summarizes the end points evaluated after the various treatments. DDP was administered at day 4 (schedule A) and at day 7 (schedule B) to compare the efficacy of DDP versus the c-myc antisense [S]ODNs administered at day 7 and versus the combination started at day 4. DDP given alone (schedule A) induced a significant inhibition of tumor weight (P < 0.0001 versus untreated mice) after the first (61%), second (60%), and third (52%) cycles of administration. A marked delay in tumor growth (10 days) was also observed (P < 0.0001 versus untreated mice) accompanied by a significant increase in survival (P < 0.0001 versus untreated mice). No significant reduction in the number of lung metastases was observed. As expected, DDP was less effective when administered at day 7, in a relatively late stage of tumor growth. Repeated cycles of c-myc antisense [S]ODNs (schedule C) started at day 7 also produced a significant inhibition of tumor weight and a growth delay of 7 days as compared to untreated mice (P < 0.0001). Moreover, c-myc antisense [S]ODN treatment caused a marked reduction in the number of lung metastases compared to DDP-treated and untreated mice, whereas treatment with c-myc scrambled [S]ODNs showed no antitumor activity. The combination of DDP and c-myc antisense [S]ODNs was significantly more effective in delaying (36 days) and in inhibiting (P < 0.0001) tumor growth (ranging from 87% after the first cycle to 93% at the end of treatment) than DDP or c-myc antisense [S]ODNs alone or DDP in combination with scrambled [S]ODNs. Also, the number of lung metastases was reduced significantly. Finally, median survival, which represents the most important end point in the evaluation of the efficacy of a new therapeutic regimen, was increased markedly (75%).

**DISCUSSION**

Melanoma is a highly malignant cancer, and despite some efficacy of many chemotherapeutic agents used alone or in combination (15, 16), no agent has made a significant impact on the survival of patients with metastatic melanoma.
In this study, we demonstrate that the use of antisense [S]ODNs in combination with a conventional anticancer drug such as DDP represents an attractive cancer treatment modality. The combination of DDP with antisense c-myc antisense [S]ODNs, independent of the order of administration, produced a greater inhibitory effect on cell proliferation of melanoma cells than did either agent given alone. However, administration of DDP followed by c-myc antisense [S]ODNs appeared to be more effective, because it produced a 90% cell growth inhibition compared to the 70% induced by the agents used in the reverse order. In turn, the inhibition of cell proliferation was accompanied by a decrease in colony-forming capacity, with only 10% of the population surviving the combined treatment. Analysis of the survival curves clearly indicated that the tumor cells partially recovered from the cytotoxic effect induced by DDP treatment; in fact, cell survival increased from 25 to 50%, whereas the addition of c-myc antisense [S]ODNs blocked the ability of the cells to recover from the DDP damage. Our findings are in accord with those of a previous study, in which c-myc antisense ODNs were shown to enhance the cytotoxic activity of DDP when used in combination in bladder tumor cells in vitro (10). In addition, combined treatments using other [S]ODNs and cytotoxic drugs proved more effective in inhibiting leukemia cell growth in severe combined immunodeficient mice than the agents used individually (17).

The enhanced cytotoxic effect elicited by the combined treatment might rest in the different mechanisms of action of the two agents. In fact, cell cycle analysis after DDP treatment revealed the characteristic G2-M phase block after 1 day, although this block was in part circumvented, as evidenced by the cell cycle repopulation during the days following the treatment. Instead, the targeting of c-myc mRNA by the antisense [S]ODNs in melanoma cells constitutively expressing c-myc, although not arresting the cells in the G2/M phases (18, 19), did prevent cell progression through the S phase, suggesting that at least in melanoma cells c-myc plays a role in the S to G2-M transition. Additional studies are needed to assess the precise function of c-myc in melanoma cell cycle phases. On the other hand, the S-phase arrest observed after exposure of the cells to the c-myc antisense [S]ODNs is in agreement with the reported ability of ectopic c-myc to rescue S-phase arrest of interleukin 3-dependent hematopoietic cells stimulated by epidermal growth factor (20). A similar S-phase arrest induced by the c-myc antisense sequence, identical to that used herein, has been reported in a leukemia model (5). When the melanoma cells were treated with DDP followed by antisense [S]ODNs, accumulation in G2-M phase was greater than after treatment with each agent used separately. In addition, maximum G2-M accumulation was delayed by 2 days, probably due to the antisense-induced block in S phase in cells initially damaged by DDP. This block appears to be irreversible, because even at day 3 from the start of treatment, a fraction of the cells, which is unable to repopulate the cell cycle, died from apoptosis. The proportion of apoptotic cells increased dramatically during the following days (~75%), with a marked loss of cells with S and G2-M DNA content. However, DDP or c-myc antisense [S]ODNs alone can also induce apoptosis. In fact, a fraction of the cell population arrested in G2-M phase by DDP was apoptotic at day 6 from the start of treatment, concomitant with a loss of cells with G2-M DNA content. This is consistent with the reported apoptosis induced by DDP treatment in leukemia cells, as well as in solid tumors (14, 21). Furthermore, we showed previously that c-myc antisense [S]ODN treatment induces apoptosis in M14 cells (6), in association with a significant depletion of the S-phase compartment. Apoptosis after c-myc antisense treatment has also been described for BV173 leukemia cells (5). The increased proportion of apoptotic cells observed after treatment with DDP followed by c-myc antisense [S]ODNs is consistent with the marked decrease in levels of the antiapoptotic Bcl-2 protein after the combined treatment.

The effects of DDP/[S]ODN treatment were examined in nude mice bearing human melanoma (NG). This tumor spontaneously gives rise to reproducible lung metastases, providing a good model for evaluation of new pharmaceutical drugs. Using the same experimental model, we demonstrated previously that two repeated cycles of c-myc antisense [S]ODNs effectively inhibits melanoma growth. In the present study, we also demonstrate that c-myc antisense [S]ODNs can inhibit tumor growth at a relatively late stage when the tumor mass is ~180 mg. These in vivo effects were sequence specific, given that the scrambled [S]ODNs did not affect tumor growth. Our in vivo results suggest that the therapeutic index of c-myc antisense [S]ODNs is somewhat higher than that of repeated cycles of DDP, because, although tumor weight inhibition and lack of toxicity were similar in both treatments, c-myc antisense [S]ODNs also induced a significant decrease in the number of lung metastases. Antitumoral efficacy was enhanced when DDP was followed by the c-myc antisense [S]ODNs for three repeated cycles at 7-day intervals. In fact, tumor weight was significantly inhibited, and growth was delayed by 36 days. This last result is particularly impressive, considering that the life span of mice bearing melanoma is ~60 days. The significant increase in animal life span (75%), which exceeds that reported to date for other combination regimens with antineoplastic drugs or immunomodulatory therapy (22), suggests this combination as a promising therapeutic approach in melanoma treatment.

ACKNOWLEDGMENTS

We thank Antonio Candidor for his technical help. We thank Maria Letizia Polino and Simona Righi for their helpful assistance in preparing the manuscript.

REFERENCES


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