ABSTRACT

8-Chloro-cAMP (8-Cl-cAMP) is a novel agent that is able to inhibit the growth of a wide variety of cancer cell types in vitro and in vivo and, at doses devoid of toxicity, to achieve plasma concentrations in cancer patients in a range effective for cancer cell growth inhibition. In this study, we have demonstrated that 8-Cl-cAMP, at a dose causing mild or no growth inhibition, synergistically increased the growth-inhibitory effect of paclitaxel or cisplatin in a wide series of cell lines including human breast, lung, ovary, colon, and head carcinomas and melanoma. A similar effect was also observed with another taxane, docetaxel, and with the platinum-derivative carboplatin. 8-Cl-cAMP also markedly enhanced apoptotic cell death induced by each cytotoxic drug. A cooperative antitumor effect was also observed in vivo, because treatment with paclitaxel followed by 8-Cl-cAMP markedly inhibited the growth of GEO human colon cancer xenografts as compared to paclitaxel alone without signs of toxicity. These data demonstrate that 8-Cl-cAMP synergistically increases the anti proliferative activity of taxanes and platinum-derived compounds and provide a rationale to use 8-Cl-cAMP in combination with taxanes and platinum-derived compounds.

INTRODUCTION

Medical treatment of cancer is mainly based on the use of cytotoxic drugs acting on intracellular targets that are generally common to both cancer and normal cells. Improvement in the efficacy of treatment has been obtained by combining two or more drugs and/or modifying their dosage and schedule of administration. However, the therapeutic benefits derived from such regimens seem to have reached a plateau charges. This article must therefore be hereby marked advertisement in accordance with 8-Cl-cAMP3 is a site-specific cAMP analogue that selectively down-regulates PKAI, a signaling protein that is directly involved in cell proliferation and neoplastic transformation and mediates the mitogenic effects of different oncogenes and growth factors (1—6). 8-Cl-cAMP inhibits the growth of a wide variety of cancer cell types in vitro and in vivo (1, 7—9) and can revert the multidrug-resistant phenotype of several cancer cell lines (10, 11). In Phase I studies conducted in cancer patients, 8-Cl-cAMP was safely administered and achieved plasma concentrations in a range previously shown effective for cancer cell growth inhibition (12, 13). We have also shown that

8-Cl-cAMP, in combination with a monoclonal antibody blocking the EGFR, synergistically inhibits the growth of different human cancer cell lines in vitro and in vivo (14, 15).

We have recently shown in two human colon cancer cell lines that 8-Cl-cAMP inhibits the growth of two colon cancer cell lines in vitro additive growth-inhibitory effect with different cytotoxic drugs acting by different mechanisms, with more effective results when administered after cisplatin or paclitaxel (16).

In the present study, we have investigated whether 8-Cl-cAMP has any cooperative effect with taxanes or platinum-derived compounds in a wide variety of human cancer cell lines in vitro and in nude mice bearing human GEO colon cancer xenografts.

MATERIALS AND METHODS

Materials. LS 174T and GEO human colon cancer; MDA-MB-231 human breast cancer; IGROV-1, CAOV-3, OVCAR-3, OVCAR-4, and OVCAR-8 human ovarian cancer; A-549 human lung adenocarcinoma; KB oral squamous cancer; and GLL-19 melanoma cell lines were purchased from the American Type Culture Collection (Rockville, MD). Paclitaxel and carboplatin were a gift of Bristol-Myers-Squibb (Rome, Italy), cisplatin was purchased from Sigma Chemicals (Milan, Italy), and docetaxel was a kind gift of Rhone-Poulenc Rorer (Origgio, Italy). All drugs were diluted in appropriate solvents and used as 100× concentrated stock. 8-Cl-cAMP was purchased from BioLog Life Science Institute (Bremen, Germany).

Cell Growth. LS 174T, MDA-MB-231, IGROV-1, CAOV-3, A-549, KB, and GLL-19 cell lines were maintained in DMEM, OVCAR-3, OVCAR-4, and OVCAR-8 cells were maintained in a 1:1 (v:v) mixture of DMEM and Ham’s F-12 medium. GEO cells were grown in McCoy’s medium. All media were purchased from Flow Laboratories (Irvine, Scotland). The media were supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), 5 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml; Flow Laboratories) in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

For cell growth experiments in soft agar, 5×104 cells/well were seeded in 24 multiwell cluster dishes as described previously (5, 16). Cells were treated with different concentrations of the indicated cytotoxic drug (day 1) or 8-Cl-cAMP or with the cytotoxic drug (day 1) followed by 8-Cl-cAMP (twice, on days 2 and 4). Two weeks after the last treatment, the cells were stained with nitro blue tetrazolium (Sigma), and colonies larger than 0.05 mm were counted (5, 16).

Flow Cytometric Analysis of Cell Cycle by PI Staining. Cells seeded in monolayer in 6-well dish clusters were treated with the indicated cytotoxic drug. After 24 h, 8-Cl-cAMP was added, and the treatment was repeated 48 h thereafter. After 4 days, cells were harvested, fixed in 70% ethanol, and stained with a PI solution (Sigma), and their DNA content was analyzed in duplicate by a FACSScan flow cytometer (Becton Dickinson, Mountain View, CA) coupled with a Hewlett Packard computer, as described previously (5). Cell cycle data analysis was performed by the CELL-FIT program (Becton Dickinson; Ref. 5).

Apoptosis. Analysis of apoptotic cell death was performed by different techniques. To study the DNA fragmentation into nucleosome ladders, both adherent and detached cells were harvested and lysed, and the DNA was extracted and subjected to agarose gel electrophoresis as described previously (17). Flow cytometric analysis of apoptotic cell death was performed on a cell pellet fixed in 70% ethanol, washed in PBS, and mixed with Rnase (Sigma) and PI solution, following the method described previously (18). DNA content was analyzed by a FACSscan flow cytometer (Becton Dickinson) coupled with a Hewlett Packard computer.

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2 To whom requests for reprints should be addressed, at Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli Federico II, 80131 Naples, Italy. Phone: 39-81-7462061; Fax: 39-81-7462066.

3 The abbreviations used are: 8-Cl-cAMP, 8-chloro-cAMP; PKAI, type I protein kinase A; EGF-R, epidermal growth factor receptor; PI, propidium iodide.
GEO Xenografts in Nude Mice. Female 5–6-week-old BALB/c athymic (nu+/nu+) mice were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and mice were maintained in accordance with the institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimatized to the University of Naples Medical School Animal Facility for 1 week before they were injected with cancer cells. Mice were injected s.c. with 10⁶ GEO cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products). After 7 days, when well-established tumors of approximately 0.4 cm³ in diameter were detected, seven mice/group were treated i.p. with either paclitaxel alone (400 μg/dose, as a single injection; Ref. 19), 8-Cl-cAMP alone (1 mg/dose, twice a week for 2 weeks; Ref. 15), or both drugs at the same doses in a sequential schedule, with 8-Cl-cAMP administered starting 24 h after the cytotoxic drug, twice weekly for 2 weeks. The 2-week cycle of sequential treatment was repeated a second time, and tumor size was measured up to 84 days from tumor cell injection. Tumor size was measured using the formula \( \pi r^2 \times \text{diameter} \times \text{diameter}^2 \), as reported previously (20, 21).

**RESULTS**

8-Cl-cAMP at doses ranging between 0.5 and 10 μM determines a dose-dependent inhibition of colony formation of CAOV-3 and OVCAR-3 human ovarian cancer cells grown in soft agar with an IC₅₀ of approximately 5 μM. In both cell lines, 1 μM 8-Cl-cAMP determined a growth inhibition of less than 10% (Fig. 1). Therefore, we selected this low dose to study whether any cooperative growth-inhibitory effect occurred between 8-Cl-cAMP and paclitaxel or cisplatin in the two cell lines of ovarian cancer, a disease in which both cytotoxic drugs are active. CAOV-3 and OVCAR-3 cells were first treated with different doses of the cytotoxic drug for 24 h, and then 1 μM 8-Cl-cAMP was added on days 2 and 4. A synergistic growth inhibition was observed in both cell lines at all doses of paclitaxel or cisplatin tested. Fig. 1, A and C, shows the effect of the addition of 8-Cl-cAMP to CAOV-3 and OVCAR-3 cells pretreated with either paclitaxel (at doses of 0.5, 1, 5, and 10 nm) or cisplatin (at doses of 0.5, 1, 2.5, and 10 μg/ml), respectively. To study whether this cooperative effect also occurs with other related drugs, we tested the effect of 8-Cl-cAMP after treatment with either the taxane docetaxel or the platinum derivative carboplatin. In both CAOV-3 and OVCAR-3 cells, 8-Cl-cAMP caused a synergistic inhibition of growth when added after docetaxel or carboplatin, as compared to each agent alone (Fig. 1, B and D).

To investigate whether the observed cooperative antiproliferative effect is specific for a cancer cell type or is a more general phenomenon, we performed similar experiments in a variety of human cancer cell types including human colon, breast, lung, ovary, and head carcinomas and melanoma. In all of these cell lines, the IC₅₀ of 8-Cl-cAMP ranged between 4 and 8 μM, and the dose of 1 μM produced no more than 10% growth inhibition. In all cell lines, a variable degree of synergism was observed. The synergism quotient,

**Fig. 1.** Synergistic effect of paclitaxel, cisplatin, docetaxel, carboplatin, and 8-Cl-cAMP on CAOV-3 (A and B) and OVCAR-3 (C and D) ovarian cancer cell growth. Cytotoxic drugs were used at the following doses: paclitaxel (A and C; a–d), 0.1, 1, 5, and 10 nm, respectively; cisplatin (A and C; e–h), 0.5, 1, 2.5, and 10 μg/ml, respectively; docetaxel (B and D; a–c), 0.05, 0.1, and 0.5 μM, respectively; carboplatin (B and D; d–f), 1.5, and 10 μg/ml, respectively. 8-Cl-cAMP was used at a dose of 1 μM. Data are expressed as a percentage of growth inhibition in reference to the growth of untreated control cells and, as indicated in the respective legends, represent the effect of 1 μM 8-Cl-cAMP alone, paclitaxel or docetaxel alone, and cisplatin or carboplatin alone. The growth inhibition caused by drugs and 8-Cl-cAMP in combination. Because the height of the stacked bars represents the sum of the individual agent effects (or the expected percentage of growth inhibition if the drugs caused an additive effect), the differences between the heights of the paired bars reflect the magnitude of the synergism of growth inhibition. The data represent means and SEs of triplicate determinations of at least two experiments. The SE of the stacked bar representing each individual agent was less than 15%.
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Figure 2. Effect of treatment with 1 nM paclitaxel (A) or 0.5 µg/ml cisplatin (B) followed by 1 µM 8-Cl-cAMP on the growth of various human cancer cell lines. Data are expressed as the percentage of growth inhibition caused by 1 nM paclitaxel, as determined in triplicate determinations of at least two experiments. SE of the stacked bar representing each individual agent was less than 15%.

The long-term control of tumor growth with the induction of a status of tumor dormancy is currently considered a major goal in cancer therapy. Inhibition of angiogenesis and growth factor production is an important tool to implement such a strategy (26, 27). For this purpose, and to improve tumor cell killing, several biological agents interfering with mitogenic signaling have been used in combination or after treatment with conventional cytotoxic drugs. Antibodies blocking either EGFR or erbB-2 in combination with doxorubicin (20) or cisplatin (21, 28, 29) have shown antitumor activity in different human cancer cell lines in vitro and in vivo. We have previously demonstrated that PKA acts downstream of several growth factor receptors and oncogenes, including EGFR, erbB-2, and ras, and that down-regulation of PKA by 8-Cl-cAMP inhibits the expression and function of these mitogenic proteins (1, 4—6, 23, 25). In nude mice bearing human GEO colon cancer xenografts, we have shown that 8-Cl-cAMP inhibits tumor angiogenesis and the secretion of growth factors of the epidermal growth factor family (25) and synergizes with antibodies blocking EGFR in inhibiting tumor growth (15). Recently, we have shown that in LS 174T and GEO human colon cancer cell lines, 8-Cl-cAMP has an in vitro additive growth-inhibitory effect with synergism quotients ranging between 1.5 and 6.5 was also observed when 1 µM 8-Cl-cAMP was added to doxorubicin or carboptatin in MDA-MB-231 breast cancer and GEO colon cancer cells (data not shown).

Cell cycle analysis in different cancer cell lines demonstrated that both classes of compounds increase the percentage of cells in G2-M phase and that the addition of 1 µM 8-Cl-cAMP, which does not affect cell cycle distribution, further increases accumulation in the G2-M phase (data not shown).

We then studied whether the cooperation between 8-Cl-cAMP and the cytotoxic drugs had any effect on the induction of programmed cell death. Analysis of apoptosis was performed by gel electrophoresis of DNA fragmentation and by flow cytometry. Fig. 3B shows the effect of paclitaxel and 8-Cl-cAMP on CAOV-3 cells. Whereas 1 µM 8-Cl-cAMP induced apoptosis, 5 nm paclitaxel caused apoptotic cell death in 14% of treated cells. Treatment with 8-Cl-cAMP after paclitaxel increased the percentage of cells entering programmed cell death to 41%, suggesting that the cooperative antiproliferative effect between 8-Cl-cAMP and the cytotoxic drug involves apoptosis. A similar cooperative effect in increasing the percentage of cells accumulating in the G2-M phase and entering apoptosis was also observed at the same doses in other human cancer cell lines (data not shown).

We next evaluated whether the cooperative effect observed in vitro could also be obtained in vivo. We have previously studied the effect of different novel biological agents including 8-Cl-cAMP in nude mice bearing GEO cell xenografts (15, 25). Therefore, we used this model to investigate the antitumor activity of 8-Cl-cAMP and paclitaxel combination. When large GEO tumors of approximately 0.4 cm3 were detected, seven mice/group were treated i.p. with paclitaxel at the maximum tolerated dose in mice (400 µg/dose; Ref. 19), 8-Cl-cAMP (1 mg/dose), or both drugs in a sequential schedule. The 2-week cycle of sequential treatment was repeated a second time, and tumor size was measured up to 84 days from tumor cell injection. As illustrated in Fig. 4, each agent alone had a moderate inhibitory effect on tumor growth. In fact, 54 days after tumor cell injection, 8-Cl-cAMP or paclitaxel determined a 25% tumor growth inhibition compared to that of controls (two-sided P < 0.05). When the two agents were used in combination, we observed a 59% reduction of tumor size that was statistically different compared to control mice (two-sided P < 0.005) or mice treated with only compound alone (two-sided P < 0.01). All control nontreated mice died within 65 days, whereas all mice treated with paclitaxel or 8-Cl-cAMP alone died within 72 days after GEO tumor cell injection. In contrast, five of seven mice treated with the sequential combination of paclitaxel and 8-Cl-cAMP were still alive, and tumor growth almost ceased up to 84 days. Moreover, no weight loss or other signs of acute or delayed toxicity were observed in these mice.

DISCUSSION

The long-term control of tumor growth with the induction of a status of tumor dormancy is currently considered a major goal in cancer therapy. Inhibition of angiogenesis and growth factor production is an important tool to implement such a strategy (26, 27). For this purpose, and to improve tumor cell killing, several biological agents interfering with mitogenic signaling have been used in combination or after treatment with conventional cytotoxic drugs. Antibodies blocking either EGFR or erbB-2 in combination with doxorubicin (20) or cisplatin (21, 28, 29) have shown antitumor activity in different human cancer cell lines in vitro and in vivo. We have previously demonstrated that PKA acts downstream of several growth factor receptors and oncogenes, including EGFR, erbB-2, and ras, and that down-regulation of PKA by 8-Cl-cAMP inhibits the expression and function of these mitogenic proteins (1, 4—6, 23, 25). In nude mice bearing human GEO colon cancer xenografts, we have shown that 8-Cl-cAMP inhibits tumor angiogenesis and the secretion of growth factors of the epidermal growth factor family (25) and synergizes with antibodies blocking EGFR in inhibiting tumor growth (15). Recently, we have shown that in LS 174T and GEO human colon cancer cell lines, 8-Cl-cAMP has an in vitro additive growth-inhibitory effect with several cytotoxic drugs acting by different mechanisms and was most effective with paclitaxel and cisplatin (16). We have also demonstrated that the sequence of treatment is important, because this
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Fig. 3. Flow cytometric analysis of cell cycle distribution (A) and apoptosis (B) in CAOV-3 ovarian cancer cells. Treatment with either paclitaxel, cisplatin, or 8-Cl-cAMP alone or in combination was performed as described in “Materials and Methods.”

Fig. 4. Antitumor activity of paclitaxel treatment followed by 8-Cl-cAMP on established GEO human colon carcinoma xenografts. Seven mice/group bearing GEO tumors (average tumor size, 0.4 cm³) were treated i.p. with either paclitaxel alone (○; 400 μg/dose), 8-Cl-cAMP alone (□; 1 mg/dose), or with a combination of paclitaxel (400 μg in a single dose) followed 24 h later by 8-Cl-cAMP (○; 1 mg/dose, twice weekly for 2 weeks), as described in “Materials and Methods.” Treated mice received two cycles of treatment. Student's t test was used to compare tumor sizes among different treatment groups at day 54 after tumor cell injection: paclitaxel versus control, two-sided P < 0.05; 8-Cl-cAMP versus control, two-sided P < 0.05; paclitaxel plus 8-Cl-cAMP versus control, two-sided P < 0.005; or versus either compound alone, two-sided P < 0.01.

Unpublished observations.

Unpublished observations.

Effect was obtained only when 8-Cl-cAMP was added after the cytotoxic drug (16).

In the present study, we investigated whether 8-Cl-cAMP has any cooperative effect with paclitaxel, cisplatin, or the related compounds docetaxel and carboplatin in a wide variety of human cancer cell lines including colon, breast, lung, ovary, and head carcinomas and melanoma. A variable degree of synergism was observed in all cell lines. Because our series included cancer types with extremely variable sensitivity to taxanes and platinum-derived compounds, it is remarkable that the synergistic interaction occurs only with these classes of drugs, regardless of the cancer cell type. These data suggest that such a combination affects specific targets common to different cancer cells.

We have demonstrated that the synergistic effect of 8-Cl-cAMP with taxanes and cisplatin is associated with a marked increase of apoptosis in the different cells tested.

The cooperative effect observed in vitro was reproduced in vivo with the GEO human colon cancer xenografts. In fact, at day 54 after tumor cell injection, sequential treatment with paclitaxel and 8-Cl-cAMP determined an inhibition of tumor growth that was statistically different from that of control mice or from that of mice treated with either compound alone. Moreover, the growth of GEO tumors was very slow and had not recovered by the end of the treatment (up to 84 days). Finally, whereas control untreated mice were all dead by day 65, 70% of the mice receiving the two agents in combination were still alive at day 84.

Phase I studies in cancer patients have demonstrated that 8-Cl-cAMP can achieve plasma concentrations in a range effective for cancer growth inhibition at doses devoid of toxicity. 8-Cl-cAMP is not toxic in nude mice at the dose used in this study (1, 15, 25) even if administered in combination with other agents, such as an antibody blocking the EGFR (15). We show here that 8-Cl-cAMP in combination with paclitaxel has a cooperative antiproliferative effect in nude mice. It must be remarked that although paclitaxel was used at the maximum tolerated dose in mice (19), no evidence of toxicity was observed. Preliminary data suggest that 8-Cl-cAMP can be safely administered in combination with cisplatin.4 The improved results obtained when 8-Cl-cAMP was administered as a sequential treatment rather than together with cytotoxic drugs (16) suggest that these agents provide a transient optimal condition for 8-Cl-cAMP to work.
It could be speculated that the accumulation of cells in G2-M phase facilitates the targeting by 8-Cl-cAMP of the PKAI peak occurring in these phases of the cell cycle (30), resulting in an increased commitment toward apoptosis. However, the molecular mechanism responsible for the described cooperative effect with taxanes and platinum-based compounds is still the object of investigation.

The ability of 8-Cl-cAMP to inhibit mitogenic signaling, which would allow its use as a long-term treatment after conventional chemotherapy, combined with the capacity to markedly potentiate the antitumor activity of specific cytotoxic agents makes this cAMP analogue a prototype of a new class of anticancer drugs. Because the doses of 8-Cl-cAMP and taxanes or platinum-derived compounds used in our study can be safely administered in vivo, our results provide a rationale for using such agents in combination in a clinical setting.

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Synergistic Inhibition of Growth and Induction of Apoptosis by 8-Chloro-cAMP and Paclitaxel or Cisplatin in Human Cancer Cells

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