Schedule-dependent Antagonism of Gemcitabine and Cisplatin in Human Anaplastic Thyroid Cancer Cell Lines

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ABSTRACT

Anaplastic thyroid carcinoma (ATC) affects primarily elderly patients, with a median survival of 4–12 months after diagnosis. Presently, under clinical investigation the combination of cisplatin (CDDP) and gemcitabine (GEM) has promising activity in several of human tumor types. To develop new approaches for therapy of ATC, we evaluated the antineoplastic activity of GEM and CDDP alone (1-h and 24-h drug exposure) or in combination in the ATC cell lines SW1736, 8505C, C643, and HTh74. IC_{50} values were determined by the sulforhodamine B assay, activity was evaluated by the relative antitumor activity (RAA) and drug interaction assessed by isobologram analysis. GEM seemed to be active in ATC, with RAA ranging from 12–114 and CDDP only modestly active (RAA, 0.24–1.4). In four different drug schedules tested, the drug combination was additive when GEM preceded CDDP exposure (combination index, ~1), whereas when CDDP preceded GEM exposure the combination was significantly antagonistic (combination index, >1). In SW1736 and 8505C cells, we observed a strong S phase arrest and DNA synthesis inhibition 24 h after a 1-h exposure to an IC_{50} of CDDP. On the basis of molecular drug targets, cell cycle arrest points, and DNA synthesis inhibition, a model was developed to explain the interaction observed for the combination of GEM and CDDP.

In conclusion, GEM shows promising cytostatic activity in ATC. Interaction of GEM and CDDP was schedule and dose dependent, favoring a regime in which GEM is followed by CDDP. Additionally, our model system suggests that DNA-synthesis inhibition and S phase arrest may be important determinants for the drug interaction between GEM and CDDP.

INTRODUCTION

ATC[3] is an aggressive and usually rapidly fatal tumor with median survival after diagnosis of 4–12 months (1, 2). By combining surgery with radiotherapy or radiochemotherapy, better local tumor control has been achieved over the past years, thereby improving the quality of life (3). Consequently, distant metastases have gained importance as a cause of death (2, 3) and have led to the addition of systemic chemotherapy as further treatment modality. Doxorubicin alone or in combination with CDDP has shown some clinical activity but is associated with relatively high toxicity in the mostly elderly patients (4). Therefore, new therapeutic approaches with mild toxicities are required to improve the clinical outcome of this disease.

GEM is a novel nucleoside analogue that has significant single-agent activity in various solid malignancies, such as NSCLC, pancreatic cancer, and head and neck cancer (5–7). GEM is well tolerated with leucopenia and thrombocytopenia as dose-limiting toxicities. Due to its overall favorable side effect profile, GEM is an excellent candidate for combination therapies with other anticancer agents.

GEM itself is inactive and requires metabolic activation after entering the cell. Activation includes phosphorylation to dFdC monophosphate by deoxycytidine kinase and, finally, to dFdCTP. dFdCTP is subsequently incorporated into DNA by DNA-polymerase, which terminates DNA-polymerization after the addition of one further deoxynucleotide. Because DNA exonuclease is unable to excise dFdCTP, this mechanism is referred to as "masked chain termination" (7–11). dFdC diphosphate is a potent inhibitor of ribonucleotide reductase, which results in depletion of deoxynucleotide pools required for DNA synthesis and repair (7, 12).

CDDP is among the most widely used anticancer drugs, with a broad spectrum of activity. Common toxicities are nephro- and neurotoxicity, ototoxicity, severe nausea, and vomiting. Intracellular CDDP undergoes hydrolysis to form active products that form DNA intra- and interstrand cross-links, in particular, intrastrand cross-links between adjacent guanines (13, 14). Thus, one mechanism of resistance to CDDP may be the removal of CDDP-induced DNA lesions (13). Furthermore, recent studies have demonstrated a correlation between the failure of DNA-mismatch repair proteins to recognize the platinum adduct and low-level resistance to CDDP. This suggests a role for the DNA-mismatch repair system in generating signals that contribute to the generation of apoptotic activity (15).

Because of their complementary mechanisms of action and

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[3] The abbreviations used are: ATC, anaplastic thyroid carcinoma; CDDP, cisplatin; GEM, gemcitabine; NSCLC, non-small cell lung cancer; dFdC, difluorodeoxycytidine; dFdCTP, dFdC triphosphate; SRB, sulforhodamin B; FACS, fluorescence-activated cell-sorting; CI, combination index; RAA, relative antitumor activity.

Table 1 IC_{50} values of GEM and CDDP in human ATC cell lines

Drug (exposure time)		IC_{50} (μM)[a]			
		SW1736	C643	8505C	HTh74
GEM	(1 h)	0.35 ± 0.04	0.8 ± 0.1	1.56 ± 0.33	3.4 ± 2.42
	(24 h)	0.15 ± 0.027	0.16 ± 0.023	0.017 ± 0.0028	0.51 ± 0.11
CDDP	(1 h)	41.8 ± 10.3	7 ± 5.48	31 ± 9.76	12.3 ± 2.52
	(24 h)	2.23 ± 0.45	0.28 ± 0.12	1.3 ± 0.27	0.8 ± 0.1

[a] IC_{50} values (μM) represent the mean of three to five independent experiments ± SD.

the nonoverlapping side effects, GEM and CDDP are attractive candidates for drug combinations. Recent *in vitro* and *in vivo* studies reported a schedule-dependent interaction ranging from antagonism to synergy between CDDP and GEM (16–20). Current clinical trials in NSCLC and bladder cancer combining CDDP with GEM demonstrate synergistic activity with only moderate side effects (6). However, a conclusive model for the combination of GEM and CDDP based on the molecular targets of CDDP and GEM has not yet been presented.

With respect to survival time, no major progress has been achieved in the treatment of ATC during the past decade. The necessity of new active chemotherapy regimes prompted us to analyze the activity of GEM alone or in combination with CDDP in ATC cell lines. With emphasis on drug action points, cell cycle arrest points, and DNA-synthesis inhibition, we intended to elucidate possible mechanisms for the observed antagonistic and additive effects in the combination between CDDP and GEM. These were summarized in a model.

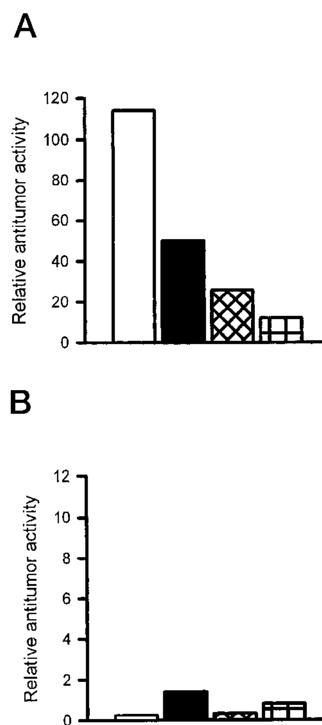

Fig. 1 RAA of GEM (*A*) and CDDP (*B*) in human ATC cell lines SW1736 (□), C643 (■), 8505C (▨), and HTh74 (▦). RAA is defined as ratio of clinical achievable peak plasma levels and *in vitro* IC_{50} values as determined by SRB assay. A RAA value ≪1 indicates possible clinical activity.

MATERIALS AND METHODS

Drugs and Chemicals. GEM and CDDP were generous gifts from Lilly (Bad Hamburg, Germany) and Bristol-Myers (Munich, Germany), respectively. GEM was dissolved in water to a concentration of 2 mM, and CDDP was dissolved in *N*,*N*-dimethylformamide to a concentration of 20 mM and stored at −20°C and 4°C, respectively.

SRB and propidium iodide were purchased from Sigma Chemical Company (Munich, Germany). [^{3}H]-thymidine was obtained from Amersham (Germany) and stored at −20°C.

Cell Lines and Culture. Human ATC cell lines SW1736, 8505C, C643, and HTh74 (21) were grown as monolayers of up to 80% confluence in RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin at 37°C, 5% CO_2, and humidified air.

Cytotoxicity Assay. For assessment of cytotoxic effects, the total protein SRB assay was used as described previously (22). In brief, 3000 cells/well for SW1736, 8505C, and C643 and 6500 cells/well for HTh74 were seeded in 96-well plates. After 24 h, exponentially growing cells were exposed to serial dilutions of drugs for the times indicated, washed thoroughly, and further grown in drug-free media. After 120 h, total assay time media was removed and cells were fixed with 10% trichloric acid and processed according to the published SRB assay protocol. Absorbance was measured at 570 nm using a 96-well plate reader (Rainbow; SLT, Germany.)

DNA Synthesis Inhibition Assay. To measure DNA synthesis inhibition, exponentially growing cells were treated with an 50% inhibitory concentration (IC_{50}) of CDDP for 1 h, washed, and cultured in drug-free media for an additional 23 h. Then, cells were exposed to 1 μCi/ml [^{3}H]-thymidine (5 Ci/mM) for 20 min, washed thoroughly with cold PBS, and harvested by trypsinization. Following a washing step with ice-cold HBSS, cells were counted and equal numbers of cells per sample were precipitated twice with 1 ml of 10% trichloric acid. Cells were pelleted, the precipitate was then dissolved in 0.4 M NaOH, and radioactivity was determined by liquid scintillation counting.

Cell Cycle Analysis. Exponentially growing cells were treated with the IC_{50} of CDDP for 1 h and with the IC_{50} of GEM for 1 h or 24 h, respectively. They were harvested by trypsinization either immediately (24 h exposure) or at 23 h posttreatment incubation (1 h exposure). After washing with HBSS, 10^6 cells/ml were stained on ice with propidium iodide and Krishan

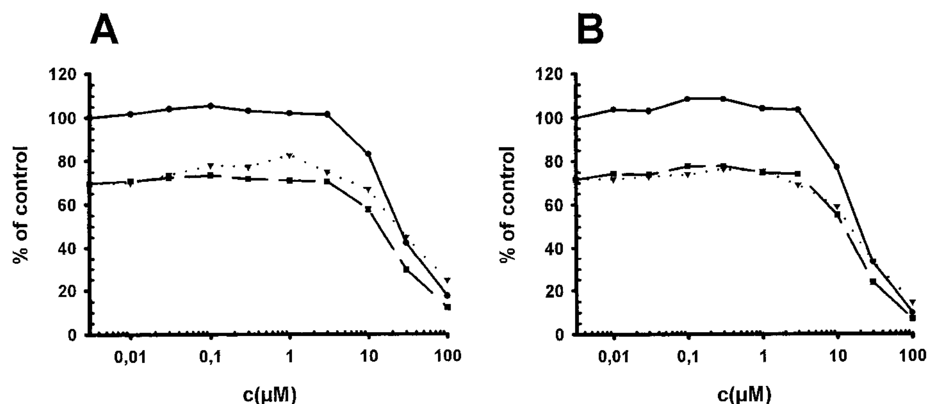

Fig. 2 Representative growth inhibition curve of the cell line SW1736 (*A*) and 8505C (*B*). Cells were exposed to various concentrations of CDDP alone for 1 h (●) or followed by 60% of IC_{50} of GEM for 23 h (▼). After drug exposure, cells were washed thoroughly and then cultured in fresh growth media for a total assay time of 120 h. From toxicity data of GEM and CDDP alone, an expected curve was calculated (■). All growth inhibition assays were performed at least three times, and variation between experiments was <18%.

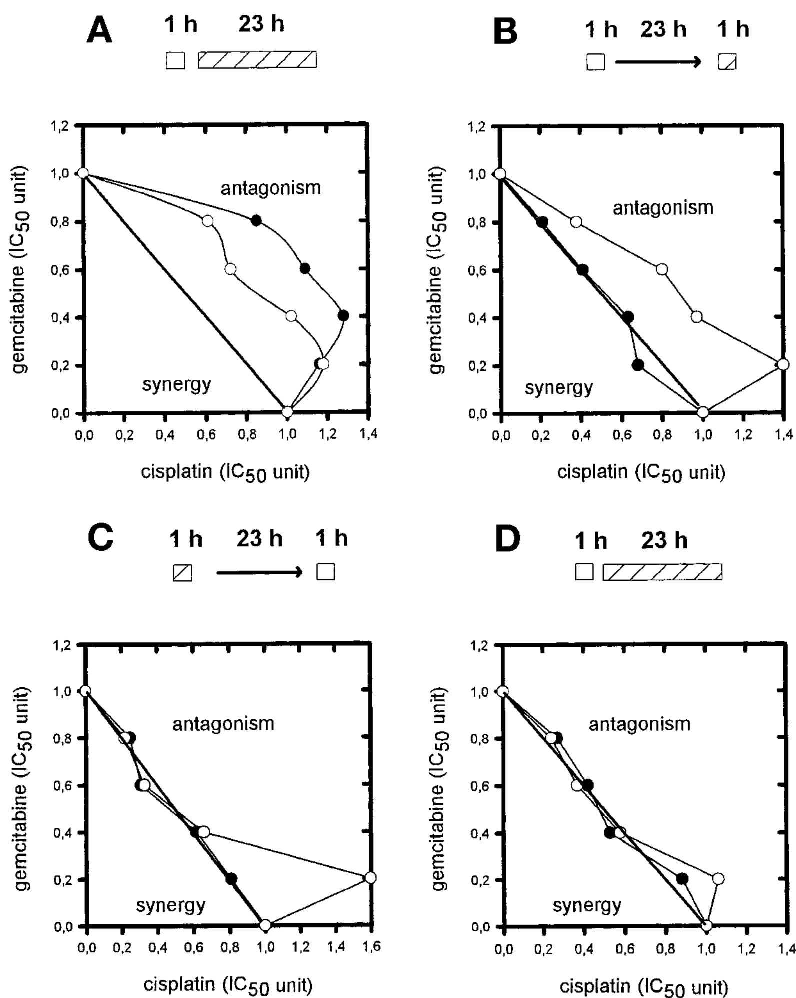

Fig. 3 Isobologram analysis (50% isodose) of combinations of GEM (▨) and CDDP (□) in cell lines SW1736 (●) and 8505C (○). Four different schedules were evaluated for drug interaction by classical isobologram analysis. Cells were either pretreated with GEM for 1 h or 23 h, followed by 1-h CDDP, or pretreated with CDDP, followed by GEM for 1 h or 23 h. Analysis is based on at least three independent experiments.

buffer for 1 h. Cells were then centrifuged at 2000 *rpm* and resuspended in 1 ml of the same buffer. FACS was performed on Becton Dickinson Facscalibur, and quantification of cell cycle compartments was carried out by Mod Fit 2 (Becton Dickinson).

Data Analysis. Dose-response curves were created by Sigma Plot (Jandel Scientific, San Rafael, CA), and IC_{50} values were determined graphically from those plots. Potential clinical activity was estimated by RAA, which was defined as ratio of peak plasma level and *in vitro* IC_{50} value (23). Drug interaction

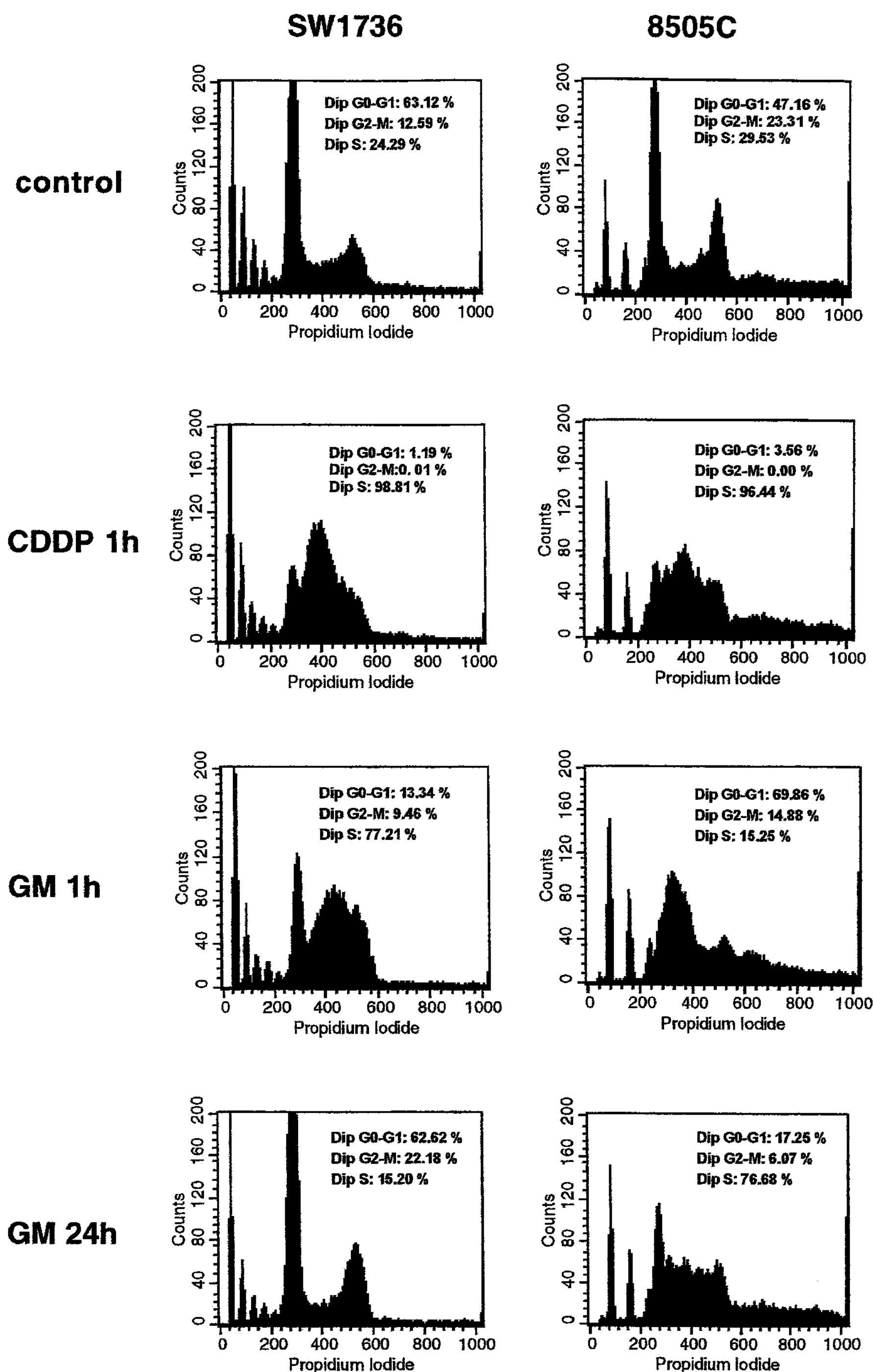

Fig. 4 Analysis of cell cycle perturbation following treatment of cell lines SW1736 and 8505C with CDDP or GEM. Cells were exposed to an IC_{50} of CDDP or GEM for 1 h or 1 h and 24 h, respectively. For the 1-h exposure schedule, cells were washed free of drug and grown in drug-free media for an additional 23 h before trypsinization. For the 24-h continuous exposure schedule, cells were harvested by trypsinization immediately after a 24-h exposure time. Analysis was carried out by FACS. Cell cycle distribution was assessed by mod fit, and results were expressed in percentages. Experiments were performed three times, and representative DNA histograms were shown.

was assessed using classical isobologram analysis (24). The CI was defined according to the following equation: CI = (dose of GEM/IC_{50} GEM) + (dose CDDP/IC_{50} CDDP). A CI <1 was considered synergistic, CI = 1 additive, and a CI >1 antagonistic. To illustrate drug interaction in representative growth curves, the expected growth curve was calculated as described recently (25). Unless otherwise stated, all experiments were performed at least three times and statistical significance assessed using the unpaired two-tailed Student's t test; differences were considered significant at $P < 0.05$.

RESULTS

Drug Activity. *In vitro* activity of GEM and CDDP was evaluated in four human ATC cell lines (SW1736, 8505C, C643, and HTh74) for short-term (1 h) and long-term

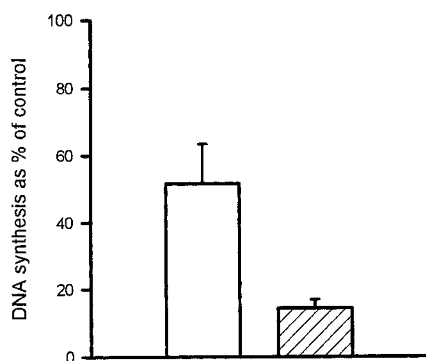

Fig. 5 DNA synthesis inhibition after treatment of SW1736 (□) and 8505C (▨) cell lines with an IC_{50} of CDDP. Cells were exposed to an IC_{50} of CDDP for 1 h and then grown for 23 h in drug-free media. Analysis for residual DNA synthesis was performed by ^{3}H-thymidine-incorporation assay. Experiments were conducted three times, and results were expressed as percentage of untreated control ± SD.

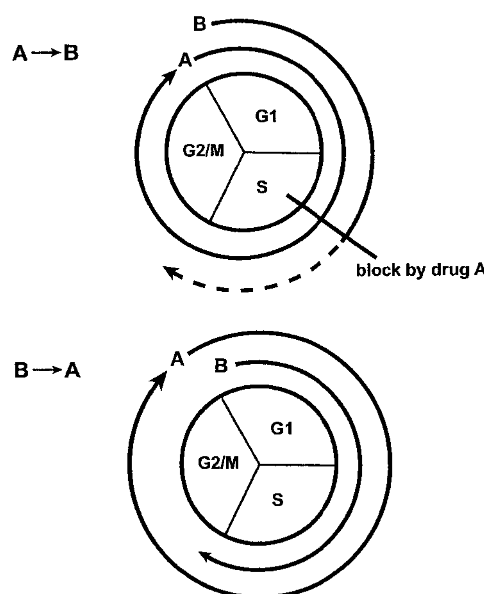

Fig. 6 Model for sequential drug treatment with CDDP (drug A) and GEM (drug B). The activity of GEM and CDDP in the different cell cycle compartments is illustrated by *circular arrows.* If CDDP is followed by GEM, CDDP induces a S phase arrest and abolishes the activity of GEM in S phase (*dashed line*). Hence, antagonism is expected. However, if GEM is followed by CDDP, GEM is incorporated into DNA and inhibits the removal of CDDP DNA-adducts. Thus, additivity to synergy is expected.

exposure (24 h). The individual IC_{50} values are summarized in Table 1.

In comparison, both drugs were more active at 24-h drug exposure with a more pronounced increase in activity for CDDP. In the four cell lines tested, drug response was heterogeneous for GEM and CDDP and no cross-sensitivity or resistance was detectable.

Considering a clinically achievable peak plasma concentration of 50 μM (26), RAA for GEM (1-h exposure) ranged from 12–114, indicating potentially high clinical activity (Fig. 1). However, RAA for CDDP (1-h exposure) ranged from 0.24–1.4, indicating only moderate activity for CDDP (Fig. 1), considering a clinically achievable peak plasma level of 10 μM (27). These findings are in agreement with previous clinical data on the activity of CDDP in ATC (4). For comparison, the RAA for doxorubicin, currently the most active chemotherapeutic agent in the treatment of ATC, ranged from 1.4–2.2 (data not shown).

Drug Combination. To investigate the schedule and dose-dependent drug interaction of GEM and CDDP, we chose the cell lines SW1736 and 8505C because of their better adherence in 96-well plates (cell loss ~20% at two washing steps as determined by wash kinetics; data not shown). Various concentrations of CDDP were combined with 20, 40, 60, and 80% of the individual IC_{50} concentrations of GEM. Four different schedules were tested, and representative growth curves are shown in Fig. 2. As assessed by the expected growth inhibition curve, the combination of various concentrations of CDDP, followed by 60% of IC_{50} of GEM for 24 h, was antagonistic at an IC_{50} concentration of CDDP (Fig. 2). Notably, this antagonism even increased with increasing concentrations of CDDP.

Drug interaction was schedule and dose dependent as further analyzed by the classical isobologram approach (Fig. 3). Pretreatment of cells with GEM (▨) for 1 h or 23 h, followed by a 1-h treatment with CDDP (□) was, in general, additive (Fig. 3, schedule *C* and *D*) with CI ~1, whereas the inverse schedule exhibited either additive (schedule *B* in cell line SW1736; CI, ~1) or significantly antagonistic (Fig. 2, schedule *A* and *B*) effects (CI, >1; $P < 0.05$).

Cell Cycle Analysis. Distinct cell cycle arrest points achieved by specific drugs might represent a critical parameter for drug interaction in sequential drug application. Therefore, the influence of GEM and CDDP on cell cycle progression was studied by FACS analysis. In accordance with schedules used in the growth inhibition experiments, SW1736 and 8505C cells were treated with the IC_{50} of CDDP for 1 h and maintained in drug-free media for 23 h before analysis. Both cell lines revealed a marked S phase accumulation of approximately 95% (Fig. 4). However, treatment with the IC_{50} of GEM for 1 h or 24 h gave differing results for both cell lines. A G_1/S phase (1-h exposure) and S phase (24-h exposure) accumulation was observed in 8505C, whereas a S phase (1-h exposure) and G_2 phase (24-h exposure) accumulation was detected in SW1736.

DNA Synthesis Inhibition: Because the incorporation of GEM into DNA depends on ongoing DNA synthesis, the presence of this synthesis itself may represent another critical parameter for drug interaction of CDDP and GEM.

To determine the degree of DNA synthesis inhibition by exposure of cell lines SW1736 and 8505C to CDDP, cells were exposed to the IC_{50} of CDDP for 1 h using the same schedule as for cell cycle analysis. CDDP significantly reduced DNA synthesis in cell line SW1736 to about 51% and in cell line 8505C to 14.2% of the untreated control (Fig. 5). Thus, DNA synthesis is clearly reduced in cells arrested in S phase (Fig. 4), which conceivably antagonizes the activity of GEM.

DISCUSSION

New active chemotherapy regimes for treatment of highly aggressive and rapidly fatal ATC are needed. This prompted us to analyze the activity of GEM alone or in combination with CDDP in four different ATC cell lines. When using the model of RAA (23, 28) CDDP displayed moderate activity in all tested cell lines, which is in agreement with previous clinical results (4). Moreover, RAA of GEM was high in all cell lines tested, indicating potential clinical activity of GEM in ATC. Because GEM seemed to be active in ATC and combinations of GEM and CDDP have already been successfully applied to other tumor types (5, 6), four different clinically relevant schedules were tested (Figs. 2 and 3). When CDDP exposure was followed by GEM (Fig. 2 and Fig. 3, schedule *A* and *B*) the drug interaction was significantly antagonistic, with the exception of schedule B in cell line SW1736. The inverse schedule (Fig. 3, *C* and *D*) exerted an additive effect for the drug combination with an antagonistic area in cell line 8505C when low GEM concentrations were combined with high CDDP doses. On the basis of our data, it seems that the combination of GEM and CDDP may exert activity in ATC. Furthermore, in a clinical setting, sequential treatment with GEM given on day 1 and CDDP on day 2 might be superior to the inverse schedule, because a significant antagonism was observed in our cell line model when CDDP was followed by GEM. In Lewis lung tumor-bearing mice, a clear schedule dependency for the combination of CDDP and GEM was recently described. GEM preceding CDDP treatment was considered the most effective schedule. However, when the treatment interval between the two drugs was increased up to 24 h, the toxicity became unacceptably high (29). Further support for the presented data comes from a recent Phase II study in NSCLC by Crino *et al.* (30) who observed a high response rate of 54% using a regime in which GEM preceded CDDP by 24 h.

Reviewing previous studies, it seems that interaction between CDDP and GEM is, to a large extent, dependent on dosage, schedule, and the model system used (17, 19). However, the mechanism of interaction between the two drugs has yet to be elucidated. Thus, we investigated whether cell cycle arrest and DNA synthesis inhibition may account for the observed effects of the drug combination. In both cell lines, CDDP induced a clear S phase arrest 24 h after drug exposure and GEM induced a G_1/S to S phase arrest. In addition, CDDP inhibited DNA synthesis to a substantial degree in both cell lines.

Although CDDP does not influence intracellular dFdCTP accumulation (8, 25), it influences the incorporation of dFdC into DNA and RNA in a cell line-dependent manner (25). Moreover, it was recently reported that dFdC increases cellular CDDP uptake and subsequent DNA-platination (16). CDDP forms intra- and interstrand cross-links independent of the cell cycle stage, thereby leading to S phase arrest and inhibition of DNA synthesis (14, 31). Notably, due to its dominant mechanism of action, dFdC is dependent on DNA synthesis to exert activity (10). Considering our results and previously published reports, we designed a model to explain the observed schedule-dependent interaction between CDDP and GEM (Fig. 6). CDDP was shown to induce S phase arrest and to substantially inhibit DNA synthesis in both cell lines. Therefore, if exposure to CDDP precedes GEM (Fig. 3, schedule *A* and *B*), the incorporation of GEM into DNA should be impaired and the combination should be antagonistic (Fig. 6, schedule *A*→*B*). However, given that GEM increases the intracellular accumulation of CDDP as well as to stabilize and increase the formation of CDDP-DNA adducts, the combination of GEM and CDDP should be additive to synergistic if exposure to GEM precedes CDDP (Fig. 3, schedule *C* and *D* and Fig. 6, schedule *B*→*A*). As summarized in Fig. 6, our data suggest that the parameters of DNA synthesis inhibition and S phase arrest, as well as drug target points within the cell cycle, played a role in determining drug interaction. These parameters may thereby provide a basis for the design of drug schedules.

Mainly synergistic interaction between CDDP and GEM has been described previously. However, these experiments have been performed with cell lines that are more sensitive toward GEM and CDDP when compared with SW1736 and 8505C. It has been suggested that the synergistic interaction between CDDP and GEM requires a certain degree of sensitivity of cell lines toward GEM or CDDP (8). Hence, it is feasible that further mechanisms might be involved in the interaction of CDDP and GEM. In this regard, it is likely that a functioning DNA mismatch repair system might be of some importance because it was recently reported that loss of DNA mismatch repair confers resistance to CDDP. It is hypothesized that the DNA mismatch repair proteins serve to detect DNA damage caused by CDDP and, by generating an injury signal, trigger apoptosis (15). Recently, Lin (32) reported a selection of GEM-resistant variants in mismatch repair-deficient cell lines by CDDP. This might imply that CDDP and GEM interact by modulating the DNA mismatch repair system, although the structural basis for this interaction is not yet defined.

In conclusion, GEM has potentially clinically relevant activity in ATC as a single agent. DNA synthesis inhibition and S phase arrest seemed to be important determinants for drug interaction of GEM and CDDP in our model system. Furthermore, the cytotoxic interaction of CDDP and GEM is dependent on the dose and sequence of treatment and favors a clinical regime in which GEM precedes CDDP.

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