Pergamon

European Journal of Cancer Vol. 32A, No. 12, pp. 2148–2154, 1996
Published by Elsevier Science Ltd
Printed in Great Britain
0959-8049/96 $15.00 + 0.00

PII: S0959-8049(96)00266-3

Original Paper

The Cellular Interaction of 5-Fluorouracil and Cisplatin in a Human Colon Carcinoma Cell Line

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The combination of 5-fluorouracil (5-FU) and cisplatin (CDDP) has been shown to have synergistic cytotoxicity in human tumours, but the biochemical mechanism for this interaction remains unclear. Therefore, the aim of this study was to investigate the interaction of 5-FU and CDDP in a human colon carcinoma cell line, NCI H548. A 24 h exposure to 5-FU resulted in a 5-FU IC_{50} value of 24.2 ± 4.5 μM, Dm 22.6 μM; exposure to CDDP for 2 h resulted in a IC_{50} value of 20.8 ± 8.0 μM, Dm 21.9 μM. When cells were exposed simultaneously to 5-FU for 24 h and CDDP for the initial 2 h, or when cells were treated with CDDP for 2 h followed by various concentrations of 5-FU for 24 h, no greater than additive cytotoxicity was observed. In contrast, when cells were treated with 5-FU for 24 h prior to CDDP for 2 h, a greater than additive interaction was noted (5-FU IC_{50} 1.2 ± 0.6 μM, Dm 1.3 μM, CI 0.45). Thymidine 10 μM partially reversed the growth inhibitory effects of the 5-FU/CDDP combination. Using both immunological and biochemical assays, no notable differences in the total amount of TS enzyme or the fraction of bound TS enzyme could be detected in the absence or presence of CDDP. No notable differences could be detected in intracellular reduced folate pools, FdUMP or FUTP pools, or 5-FU incorporation into RNA or DNA with the addition of CDDP to 5-FU. DNA fragmentation studies revealed that the combination of 5-FU followed by CDDP demonstrated a greater degree of single-stranded DNA fragments in parental ($P = 0.024$) and newly synthesised DNA ($P = 0.025$) compared with the administration of CDDP prior to 5-FU or either drug alone. The increase in smaller DNA fragment size was reversed with the addition of thymidine (10 μM). These findings suggest that the interaction of 5-FU and CDDP is associated with a greater degree of fragmentation of both nascent and parental DNA. Published by Elsevier Science Ltd

Key words: 5-fluorouracil, cisplatin, human colon carcinoma

Eur J Cancer, Vol. 32A, No. 12, pp. 2148–2154, 1996

INTRODUCTION

CISPLATIN (CDDP) and 5-fluorouracil (5-FU) are important drugs in the treatment of a variety of cancers including ovarian, colorectal, lung, and head and neck cancers [1–4]. The combination of 5-FU and CDDP has been shown to have synergistic cytotoxicity in both human and murine tumours, and has demonstrated good therapeutic activity, especially in patients with head and neck cancer [4, 5]. Despite the widespread use of these drugs in combination, the biochemical mechanisms responsible for the interaction and the optimal method of administration of 5-FU and CDDP remains unclear.

One of the principal mechanisms of action of 5-FU is inhibition of the enzyme thymidylate synthase (TS). TS catalyses the methylation of deoxyuridine (dUMP) to deoxythymidine monophosphate (dTMP) which is essential for DNA synthesis. Fluorodeoxyuridine monophosphate (FdUMP), which is the active intracellular metabolite of 5-FU, forms a covalent inhibitory complex with TS in the presence of the folate cofactor, 5-10-methylene tetrahydrofolate [6, 7]. Preclinical laboratory studies suggest that the formation of TS ternary complex (TS-FdUMP-

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Received 30 Aug. 1995; revised 7 May 1996; accepted 13 May 1996.

CH_2H_4PteGlu) may be a critical step for the cytotoxicity of the fluoropyrimidines. The stability and amount of ternary complex formed are dependent on the concentration of the reduced folate 5,10-methylene tetrahydrofolate [8–10]. In addition to TS inhibition, 5-FU has direct effects on RNA and DNA through incorporation in the form of 5-fluorouridine triphosphate (FUTP) and fluorodeoxyuridine triphosphate (FdUTP), respectively. The cytotoxic effect of CDDP results from DNA-interstrand and intrastrand adducts and their subsequent excision [11].

Previous studies have demonstrated that the combination of CDDP followed by 5-FU is synergistic in human ovarian and head and neck cancer cell lines, as well as Yoshida sarcoma cells transplanted into rodents [12–14]. Scanlon and coworkers have suggested that this interaction can be attributed to increased intracellular levels of reduced folates, resulting in enhanced ternary complex formation with TS inhibition [12]. Furthermore, Shirasaha and coworkers [14] have suggested that CDDP enhances 5-FU cytotoxicity by inhibiting intracellular methionine metabolism, resulting in increased intracellular reduced folate pools and increased TS inhibition. Other *in vitro* and *in vivo* studies [15–19] have suggested that the reverse sequence of 5-FU followed by CDDP is associated with a greater than additive interaction in human squamous head and neck carcinoma cell lines, mice bearing transplantable L1210 leukaemia cells as well as primary colon tumours in mice. The goal of the present study was to investigate the interaction of 5-FU and CDDP in a human colon carcinoma cell line in an effort to understand further the sequence dependency and the biochemical mechanism that may account for the interaction between these two important chemotherapeutic agents.

MATERIALS AND METHODS

Chemicals

Dextran (clinical grade), bovine albumin fraction V, 5-FU, and acid-washed activated charcoal were purchased from Sigma Chemical Co. (St Louis, Missouri, U.S.A.). [6-^{3}H]5-FdUMP (spec. activity 18 Ci/mmol), [6-^{3}H]5-FU (spec. activity 20 Ci/mmol), [2-^{14}C] (thymidine spec. activity 56 Ci/mmol) were obtained from Moravek Biochemicals (Brea, California, U.S.A.). All other chemicals were obtained from Sigma Chemical Co.

Cell culture

The characteristics of the human colon cancer cell line NCI H548 have been previously described [20]. The cells were maintained in RPMI-1640 (Biofluids Inc., Rockville, Maryland, U.S.A.) with 10% dialysed fetal calf serum (Gibco, Inc., Grand Island, New York, U.S.A.) plus 2 mM glutamine and grown in 75 cm^2 plastic culture flasks (Falcon Labware, Oxnard, California, U.S.A.) in a humidified incubator with 5% CO_2 at 37°.

In vitro *growth inhibition studies*

An equal number of NCI H548 cells (2.5×10^5 cells) were plated on to 25 cm^2 flasks (Falcon Labware) and incubated at 37°C. After 24 h, various concentrations of 5-FU or CDDP were added to each flask. Sterile phosphate buffered saline (PBS) was added to control flasks. After various timed exposures, the medium was removed from the tissue culture cells and the cells were washed three times in PBS. Five days after plating, the cells were trypsinised and counted using a coulter counter (Coulter Electronics, Hialeah, Florida, U.S.A.). The IC_{50} values were determined using a curve of cell number versus log of drug concentration. Various sequence schedules of 5-FU and CDDP were studied to determine the optimal timing and sequence of 5-FU and CDDP in this cell line including simultaneous addition of both drugs, 5-FU added for various time intervals prior to CDDP, and CDDP added for various time intervals prior to 5-FU. Dose–response interactions between 5-FU and CDDP were analysed using the method of Chou and Talalay [21]. Median dose values were determined from the lots of median effects: log(fraction affected/fraction unaffected). The combination index was used to signify antagonism (> 1), additivity (= 1) and greater than additivity (< 1) for the drug combinations. Once the optimal sequence and timing of 5-FU and CDDP were determined, these conditions were used in all further experiments. In thymidine reversal experiments, thymidine (10 μM) was added at the same time as 5-FU.

Western blot analysis

Western blot analysis using monoclonal antibody TS106 was accomplished as previously described [22]. Briefly, equal amounts of cytosol (300 μg) were resolved on 15% polyacrylamide gel electrophoresis with 15% acrylamide according to the method of Laemmli [23]. Gels were then electrotransferred on to nitrocellulose membranes (Schleicher and Schull, Keene, New Hampshire, U.S.A.). Membranes were treated with blocking solution, washed, and reacted with TS106 antibody. Blots were then overlaid with goat-antimouse secondary antibody (10 μg/ml) conjugated with horseradish peroxidase (BioRad). Protein bands representing complex and free TS were detected using 3′,5′,-tetramethylbenzidine (TMB) colorimetric method. TS protein detected on the blots was quantitated by scanning densitometry using a HP Scan Jet digital imager coupled with Image analysis software (v. 1.52 Wayne Rasband, NIMH).

Thymidylate synthase FdUMP binding assay

After various drug exposures, cells were twice washed with PBS, harvested and resuspended in 1 ml of 0.1 M KH_2PO_4 pH 7.4. Cell lysis was carried out by sonication using 2–3 sec bursts from a Branson sonicator. The cellular extract was centrifuged at 5000*g* for 30 min and the supernatants collected and assayed as previously described [24–26]. The formation of TS ternary complex and the effect of CDDP on this process were determined by measuring the total FdUMP binding sites or TS total (TS_T) and unoccupied binding sites (TS_F) as previously described [24]. The differences between TS_T and TS_F represented the amount of ternary complex formation or TS bound (TS_B).

The assay to determine TS_F was performed in a total volume of 200 μl containing 50 μl of cell lysate, 75 μM CH_2H_4PteGlu, 3 pmol [6-^{3}H]FdUMP, 100 μM 2-mercaptoethanol and 100 μM KH_2PO_4 pH 7.4. Samples were incubated at 37° for 30 min and subsequently 1 ml of an albumin-coated charcoal slurry pH 7.2 (prepared by mixing 10 g of acid-washed activated charcoal with 2.5 g of bovine albumin, 0.25 g of dextran and 100 ml of ice cold water)

was added. The mixture was vortexed, allowed to stand at room temperature for 10 min, and then centrifuged for 30 min at 3000*g*. The residual radioactivity representing enzyme bound FdUMP in the supernatant was counted by liquid scintography [22]. Under these conditions, the exchange rate for the 30 min incubation is $7 \pm 1\%$. Thus, 7% of the total TS is used to correct the measured amount of free TS enzyme.

The TS_T was determined by allowing the TS_B present in the cytosol to exchange with the [6-^{3}H]FdUMP in ammonium bicarbonate buffer 0.6 M NH_4CO_3 pH 8.0 over a 3 h period. TS_T was assayed in a total volume of 200 µl containing 50 µl of cell lysate, 75 µM $CH_2H_4PteGlu$, 3 pmol [6-^{3}H]FdUMP and 0.6 M NH_4CO_3 buffer. Protein levels were determined by the method of Bradford [27].

Incorporation of 5-FU into nucleotide pools and into RNA and DNA

Following exposure to [^{3}H]5-FU, cells were washed three times with ice-cold PBS and fractionated for cold acid soluble and insoluble, RNA, and DNA fractions as previously described [28, 29]. The cold acid soluble fraction was fractionated by high performance liquid chromatography (HPLC) [30]. The fluoropyrimidine metabolites were detected by their retention times with respect to standard compounds and quantitated using an in-line scintillation counter (Flow/One Beta, Radiomatic Inc., Tampa, Florida, U.S.A.). Cellular RNA was hydrolysed in 0.2 M NaOH and DNA was hydrolysed in 1 M perchloric acid. The amount of tritium in the hydrolysates was counted by liquid scintigraphy.

Measurement of folate pools

NCI H548 cells were incubated with 50 nM [^{3}H]-labelled leucovorin (spec. activity 1 Ci/ml) for 24 h following which cells were washed twice with PBS and then exposed to CDDP 5 µM for 2 h. The cells were then harvested and the intracellular folate pools extracted, separated and quantitated as previously described [31].

Standard alkaline elution

Exponentially growing NCI H548 cells were pulsed with [^{3}H]thymidine for 24 h and chased for 4 h. These thymidine labelled cells were exposed to various combinations of 5-FU and CDDP as described previously. Equal numbers of cells were loaded onto Nucleopore^{TM} filters held in an alkaline elution funnel (Millipore) then lysed in the dark with 5 ml of buffer containing 2 M NaCl with 0.3% sarcosyl detergent pH 7.0 and 20 mM EDTA pH 10.0 as previously described [32]. DNA fractions were collected at 90 min intervals and neutralised with 250 µl of glacial acetic acid and the [^{3}H]-labelled DNA fragments counted by liquid scintography. After correction for background counts, the data were expressed as percentage of the total counts retained on the filter.

pH step alkaline elution of nascent DNA

Exponentially growing cells were exposed to 5-FU and CDDP as described. Cells were pulsed with [^{3}H]thymidine (10 µCi) for the final 2 h of drug exposure. The cells were then harvested, deposited on a Nucleopore^{TM} filter, and lysed as described above. Successive pH step alkaline elutions were performed at pH 11.0, 11.3, 11.5 and 12.1 as previously described [33]. The filter and elution fractions were neutralised with 150 µl of glacial acetic acid and the [^{3}H]-labelled DNA fragments counted by liquid scintography. The data were corrected for background counts, and were expressed as the percentage of the total counts eluting with pH steps 11.0–11.5.

Statistics

All quantitative data were compared using the Student *t*-test. A P value of < 0.05 was considered significant.

RESULTS

Effect of CDDP and 5-FU on growth inhibition

The growth inhibitory effect of various schedules and concentrations of 5-FU and CDDP were initially determined. When H548 cells were exposed to 5-FU for 24 h followed by a 72 h drug-free incubation, an IC_{50} value of 24.2 ± 4.5 µM and Dm of 22.6 for 5-FU was noted (Figure 1). A 2 h exposure of cells to CDDP resulted in a IC_{50} value of 20.8 ± 8.0 µM and a Dm of 21.9 (data not shown). A variety of timed exposures and schedules of 5-FU plus cisplatin were next tested for their combined effects on cell growth using the median effect analysis programme. CDDP 5 µM, which was minimally growth inhibitory when given alone (20% inhibition), enhanced the growth inhibition in H548 cells associated with 5-FU. The degree of enhanced growth inhibition associated with this combination was schedule dependent. When cells were exposed simultaneously to 5-FU for 24 h and CDDP 5 µM for the initial 2 h, the IC_{50} decreased 2-fold to 12.6 ± 3.5 µM, with a Dm of 12.3 and a CI of 1.3 (data not shown). Similarly, when cells were treated with CDDP 5 µM for 2 h followed by 5-FU for 24 h, the 5-FU IC_{50} value decreased to 12.1 ± 3.4 µM, with a Dm of 12.7 and a CI of 1.2 (Figure 1). In contrast, when cells were treated with 5-FU for 24 h followed by CDDP for 2 h, the 5-FU IC_{50} value

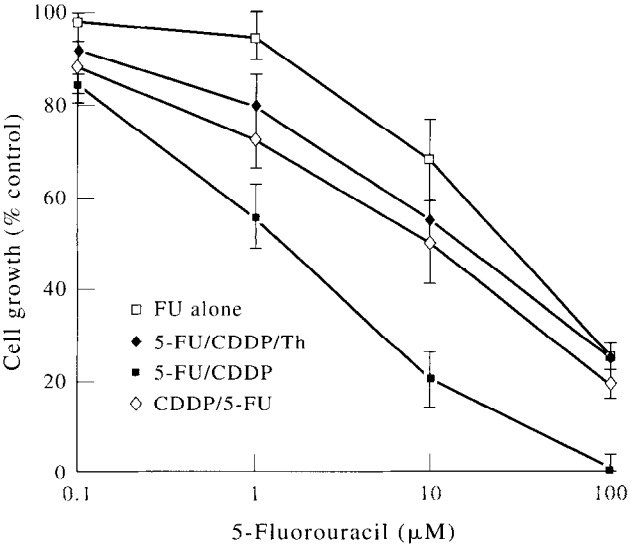

Figure 1. 5-FU/CDDP growth inhibition curves. 5-FU alone for 24 h —□—; 5-FU for 24 h followed by CDDP 5 µM for 2 h —■—; CDDP 5 µM for 2 h followed by 5-FU for 24 h —◇—; 5-FU + thymidine 10 µM for 24 h and CDDP 5 µM —◆—). These results represent the mean ± the SE of at least five separate experiments.

decreased 18-fold to 1.2 ± 0.6 μM with a Dm of 1.3 and a CI of 0.45 (Figure 1). Increasing the duration of CDDP exposure beyond 2 h did not result in a further increase in cytotoxicity (data not shown).

To determine if thymidine depletion played a role in the enhanced interaction between 5-FU and CDDP, the effect of adding exogenous thymidine to this combination was examined. Thymidine 10 μM decreased the growth inhibitory effects of the combination of 5-FU/CDDP and increased the IC_{50} value to 13.3 ± 4.8 μM with a Dm of 13.1 and a CI of 1.6 (Figure 1).

Effect of CDDP on 5-FU associated thymidylate synthase inhibition

The effect of 5-FU and CDDP on the expression of TS and the formation of TS ternary complex using both enzymatic and Western blot methods was investigated. The TS level in NCI H548 cells increased 3.1-fold over control levels when cells were treated with 5-FU (1 μM) alone for 24 h (Table 1). Increases of 3.0- and 3.3-fold TS levels were apparent when cells were exposed to 1 μM 5-FU for 24 h followed by 5 μM CDDP for 2 h or the reverse sequence, respectively (Table 1). The TS ternary complex (TS_B) and free TS (TS_F) levels were measured when cells were treated with 5-FU in the presence and absence of CDDP 5 μM. The addition of CDDP did not alter ternary complex formation nor was any effect noted in the overall amount of free TS (Table 1, Figure 2).

Effects of CDDP on the intracellular folate pools

NCI H548 cells were incubated for 24 h with 50 nM [^{3}H]leucovorin and then exposed to CDDP 5 μM for 2 h. It has previously been demonstrated that the intracellular folate pools reach an equilibrium with the radiolabelled leucovorin within 24 h [28]. There was no notable difference in the total intracellular folate pools between CDDP-treated (11.1 ± 6.3 pmol/mg protein) and untreated control cells (13.7 ± 4.7 pmol/mg protein). Moreover, no major differences in the 10-formyltetrahydrofolate pools (2.7 ± 1.4 versus 3.2 ± 0.8), 5-methyltetrahydrofolate pools (4.3 ± 2.2 versus 3.8 ± 1.0), or tetrahydrofolate pools (4.0 ± 2.6 versus 4.4 ± 2.1) were noted between CDDP treated and control cells. The 5,10 methylenetetrahydrofolate pools were below the limits of detection in these cells (< 0.5 pmol/mg).

Table 1. Thymidylate synthase activity in human colon carcinoma cells NCI H548 cells after exposure to 5-FU or the combination of 5-FU and CDDP

Treatment	TS binding pmol/mg protein TS total	TS free
Control	0.7 ± 0.3	0.7 ± 0.3
5-FU 1 μM	2.2 ± 0.7	0.3 ± 0.2
5-FU 1 μM followed by CDDP 5 μM	2.1 ± 0.5	0.3 ± 0.1
CDDP 5 μM followed by 5-FU 1 μM	2.3 ± 0.6	0.5 ± 0.3

NCI H548 cells in the log phase of growth were exposed to the combination of 5-FU 1 μM for 24 h followed by CDDP 5 μ for 2 h, or CDDP 5 μM for 2 h followed by 5-FU 1 μM for 24 h. Control cells were exposed to 5-FU 1 μM for 24 h, the diluent, sterile PBS was added in place of CDDP. Total free and bound TS protein was analysed as outlined in Materials and Methods. These results are the mean ± SE of at least five separate experiments.

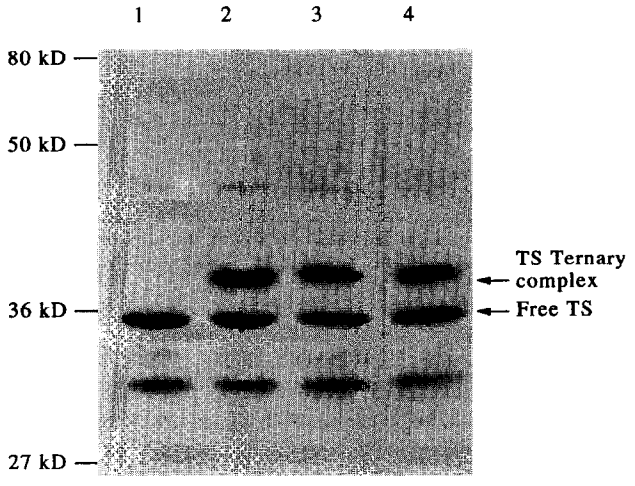

Figure 2. Western immunoblot cell lysates from (1) untreated control NCI H548 cells; (2) H548 cells treated with 5-FU (1 μM) for 24 h; (3) H548 cells treated with 5-FU (1 μM) for 24 h followed by CDDP (5 μM) for 2 h; or (4) H548 cells treated with CDDP (5 μM) for 2 h followed by 5-FU (1 μM) for 24 h. Western blotting was performed as outlined in Materials and Methods section.

Effect of CDDP on 5-FU nucleotide formation and incorporation into RNA and DNA

Since the level of FdUMP is an important determinant of ternary complex formation, the effect of CDDP on intracellular FdUMP levels was examined. When cells were treated for 24 h with 5-FU 1 μM followed by CDDP no significant difference in the FdUMP pools was noted compared with CDDP followed by 5-FU, or 5-FU alone (Table 2). No major difference in FUTP levels or 5-FU incorporation into RNA and DNA in the presence or absence of CDDP were noted (Table 2).

DNA fragmentation assays

Fixed pH step (pH 12.1) alkaline elution of prelabelled DNA was used to measure the induction of single-stranded parental DNA damage in cells treated with the combination of 5-FU and CDDP compared with either drug alone. The percentage of single-stranded DNA damage in cells treated with 5-FU followed by CDDP was significantly different from any other schedule or either drug alone over the first 10 h of the elution ($P = 0.024$) (Figure 3). The percentage of large molecular weight DNA retained on the filter was 67% in 5-FU 1 μM treated cells versus 66% in CDDP 5 μM treated cells and 74% in cells treated with the combination of CDDP 5 μM followed by 5-FU 1 μM. In contrast, only 55% of the parental DNA was retained on the filter when cells were treated with the combination of 5-FU 1 μM followed by CDDP 5 μM. This increase in single strand DNA breaks was reversed by the addition of thymidine 10 μM (Figure 3). Thus, cells treated with the combination of 5-FU plus CDDP had an 8–18% increase in the amount of single strand DNA damage compared with the reverse sequence or either drug alone.

pH step alkaline elution was also used to assess the effect of 5-FU and CDDP on newly synthesised DNA. The proportion of single-stranded DNA eluting in cells treated with 5-FU followed by CDDP was significantly greater than with either drug alone ($P = 0.025$). The proportion of single strand DNA eluting with the pH 11.0–11.5 fractions in con-

Table 2. The incorporation of 5-FU into nucleotide pools, RNA and DNA in NCI H548 cells exposed to 5-FU or the combination of 5-FU and CDDP

Drug	FdUMP pmol/mg protein	FUTP pmol/mg protein	RNA pmol/mg RNA	DNA fmol/mg DNA
5-FU 1 μM	0.03 ± 0.04	0.15 ± 0.02	0.30 ± 0.02	13 ± 0.1
5-FU 1 μM followed by CDDP 5 μM	0.02 ± 0.01	0.11 ± 0.01	0.27 ± 0.02	14 ± 0.3
CDDP 5 μM followed by 5-FU 1 μM	0.03 ± 0.01	0.17 ± 0.02	0.32 ± 0.04	19 ± 0.6

NCI H548 cells in the logarithmic phase of growth were exposed to [^{3}H]5-FU 1 μM (spec. activity 40 mCi/mmol) for 24 h. CDDP 5 μM was added for 2 h either prior to or after [^{3}H]5-FU exposure. The results are the mean ± SE of at least five separate experiments.

trol cells and cells treated with 5-FU 1 μM was 7.7% (Figure 4). In cells treated with CDDP 5 μM alone, 17.3% of single strand DNA eluted; whereas in cells treated with 5-FU 1 μM followed by CDDP 5 μM this increased to 24.35% (Figure 4). When these cell were simultaneously exposed to thymidine 10 μM, the proportion of single strand DNA that eluted decreased to 4% (Figure 4). Greater effects on the proportion of single strand DNA eluting with the pH 11.0–11.5 fractions were also noted when higher doses of 5-FU 10 μM and CDDP 50 μM were used in combination, compared with similar doses of either drug alone (Figure 4).

DISCUSSION

In this study, we have examined the sequence dependency and the mechanism of interaction between 5-FU and CDDP in the NCI H548 human colon carcinoma cell line. The sequential administration of 5-FU followed by CDDP was more effective at inhibiting cell growth than either simultaneous administration of both drugs or the administration of CDDP prior to 5-FU. No more than additive cytotoxicity was seen with these latter schedules. Thymidine 10 μM reversed the growth inhibitory effects of the combination, suggesting that a DNA directed mechanism of action of 5-FU may play a significant role.

In an effort to understand the mechanism of the 5-FU/CDDP interaction, we assessed TS levels and the degree of TS ternary complex formation in the presence and absence of CDDP. No notable difference was seen in the amount of total TS, the degree of induction of TS, the amount of ternary complex formed or the amount of free TS protein in the presence or absence of CDDP. FdUMP levels and FUTP pools were similar in the presence or absence of CDDP, as was the incorporation of 5-FU anabolites into RNA and DNA; moreover, CDDP had no effect on intracellular folate pools in H548 cells. These data suggest that CDDP is not modulating the TS directed effects of 5-FU in NCI H548 cells.

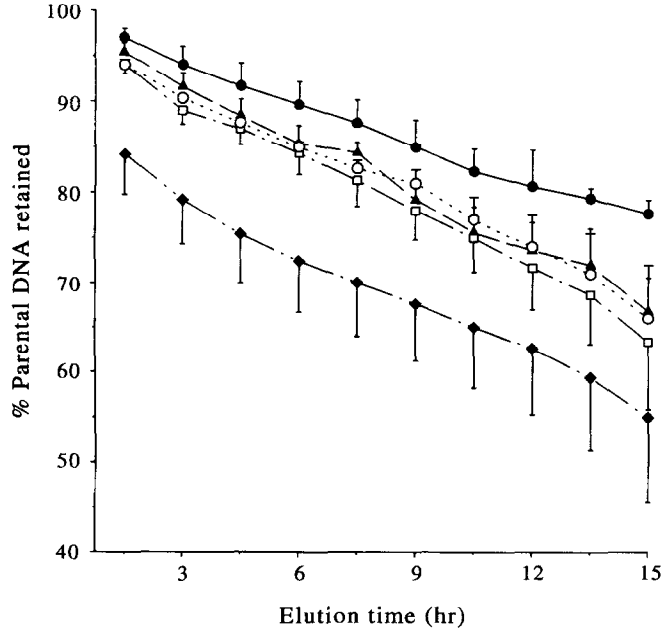

Figure 3. The percentage of total radiolabelled parental DNA retained on the filter expressed as a function of duration of pH 12.1 elution in cells exposed to 5-FU 1 μM for 24 h followed by CDDP 5 μM for 2 h —◆—; 5-FU alone —▲—; CDDP alone —□—; 5-FU 1 μM and thymidine 10 μM for 24 h followed by CDDP 5 μM for 2 h —○—. Control cells were exposed to PBS diluent in place of 5-FU or CDDP. These results represent the mean ± the SE of at least three separate experiments.

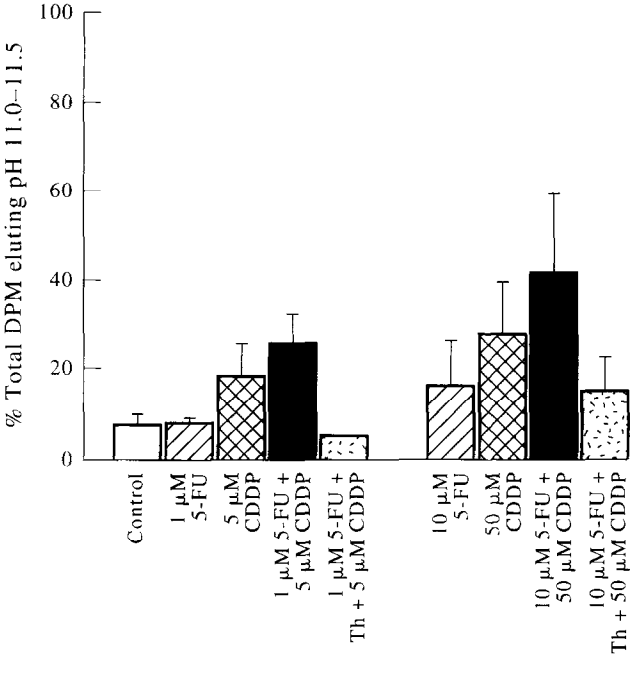

Figure 4. pH step alkaline elution of [^{3}H]-labelled DNA fragments expressed as the per cent of total DNA that eluted with pH step 11.0–11.5. The cells were pulsed with 10 μCi [^{3}H]thymidine for the final 2 h of drug exposure. Successive alkaline elutions were carried out at pH 11.3, 11.5, and 12.1. The results are the mean ± the SE of at least three separate experiments.

Alkaline elution analysis revealed that the degree of parental and nascent single strand DNA fragmentation was greater when cells were treated with the combination of 5-FU followed by CDDP compared with the reverse sequence or either drug alone. The concurrent administration of thymidine protected cells and decreased the amount and size of the DNA fragments, suggesting that a DNA directed 5-FU mechanism of action was important in increasing CDDP induced DNA damage.

The optimal schedule of combination therapies of 5-FU and CDDP appears to be tumour and cell line dependent. Several studies have demonstrated synergistic interactions in human cancer cell lines and rodent bearing transplantable tumours only when CDDP is given prior to 5-FU. In CAL 27 and CAL 33 human head and neck carcinoma cell lines, Etienne and colleagues demonstrated synergistic activity only with pre-exposure of cells to CDDP prior to 5-FU [13]. In A2780 human ovarian cancer cells, Scanlon and coworkers detected a synergistic interaction when cells were treated with CDDP for 30 min followed by 5-FU for 30 min [12]. These investigators observed less than 14% cytotoxicity for either agent alone versus 76% for the sequence of CDDP followed by 5-FU and 59% using the reverse sequence. The sequence of CDDP followed by 5-FU resulted in a 2.5-fold increase in 5, 10 methylene tetrahydrofolate and tetrahydrofolate pools accompanied by a 2.5-fold enhancement of ternary complex formation. Shirasaka and colleagues demonstrated that intraperitoneal CDDP followed by a 6 day continuous infusion 5-FU had synergistic cytotoxicity in Yoshida sarcoma and P388 lymphoma cells transplanted into rodents [14]. These investigators also noted that CDDP inhibited incorporation of exogenous L-methionine into ascitic tumour cells, and increased the levels of reduced folates 2–3-fold which resulted in enhanced TS inhibition. In our study, CDDP had no effect on intracellular reduced folate levels. Van der Wilt and coworkers [34] tested various combinations of CDDP and 5-FU in conjunction with WR 2721 in two colon tumour models, Colon 26 and Colon 38, in Balb/C mice. The optimal cytotoxic effect was found with 5-FU/CDDP delivered together 30 min after WR2721. The increased efficacy of this treatment schedule could not be explained by enhanced inhibition of TS. Other investigators have also found that the sequence of 5-FU followed by CDDP results in enhanced cytotoxicity. Palmeri and colleagues demonstrated that CDDP given 12–24 h after the initial dose of 5-FU was the optimal sequence in mice bearing L1210 cells. They noted that CDDP had no effect on TS activity, but recovery of TS inhibition was significantly delayed. More recently, Esaki and colleagues have also demonstrated that only the sequence of 5-FU followed by CDDP after a 24 h drug-free delay is synergistic in a human squamous carcinoma cell line HST-1 [19]. In this study, TS inhibition and intracellular folate pools were not evaluated. While thymidine did not appear to protect cells, their findings suggested that the efficiency of DNA repair was decreased; thereby, potentiating the CDDP-induced cytotoxicity in these cells. Their data are consistent with the increase in single strand DNA fragments that we have observed when cells are treated with the combination of 5-FU and CDDP, and suggests that pretreatment with 5-FU enhances CDDP-induced DNA damage.

Previous studies have demonstrated that thymidine starvation can provoke physical alteration of DNA in mammalian cells, such as hypermethylation and increased uracil incorporation into DNA [35]. This leads to an increase in the number of uracil-DNA glycosylase susceptible sites and results in increased excision of uracil-containing DNA with resultant DNA strand breaks. Moreover, cells that contain a mutant or defective DNA glycosylase have been shown to be resistant to thymineless induced cell death [35]. While 5-FU itself can directly induce DNA damage due to direct DNA incorporation, we have found no evidence for any increase in 5-FU incorporation into either DNA or RNA in the H548 cell line [36]. Thus, the increase in DNA fragments is most likely the result of 5-FU-induced thymidine depletion resulting in defective repair of CDDP associated DNA damage.

In conclusion, the schedule of 5-FU followed by CDDP appears to be maximally growth inhibitory in human H548 colon carcinoma cells. Our data suggest that 5-FU modulates the cytotoxic effect of CDDP in H548 cells and results in increased DNA fragmentation which may be partially protected by the administration of thymidine.

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