

Modulation of Drug Resistance Mediated by Loss of Mismatch Repair by the DNA Polymerase Inhibitor Aphidicolin¹

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ABSTRACT

Loss of expression of mismatch repair (MMR) proteins leads to resistance of tumor cells to a variety of DNA-damaging agents, including bifunctional alkylating and monofunctional methylating agents such as *cis*-diaminedichloroplatinum II (CDDP) and *N*'-methyl-*N*-nitrosourea (MNU). It has been suggested that coupling to cell death does not occur in the absence of MMR, but instead, DNA lesions are bypassed during replication, giving a drug-tolerant phenotype. In the present study, we have used aphidicolin (Ap), an inhibitor of DNA polymerases, to study the role of replicative bypass in drug resistance mediated by loss of MMR. We have examined the survival of matched ovarian carcinoma cell lines with known MMR status after sequential treatment with CDDP or MNU and Ap. We show that Ap increases the sensitivity of MMR-deficient cell lines to CDDP and MNU to a greater extent than their MMR-proficient counterparts. Furthermore, loss of MMR correlates with loss of CDDP-induced G₂ arrest, but this is partially restored after Ap treatment. These data support Ap sensitizing drug-resistant cancer cells that have lost MMR to CDDP and MNU and suggest that the potential use of Ap as a modulator of drug resistance should be targeted to MMR-defective tumors.

INTRODUCTION

CDDP³ is a clinically important antitumor drug that is particularly effective against ovarian and testicular tumors (1). It is generally accepted that DNA is a crucial target for CDDP cytotoxicity (2). The main lesions formed by CDDP are intrastrand cross-links between purine bases, with 1,2 cross-links accounting for 90% of lesions, and 1,3 cross-links accounting for an additional 5% (3). As with most chemotherapeutic agents, the clinical effectiveness of CDDP is often reduced by the acquisition of resistance (4). Although drug uptake and metabolism can be important for drug responsiveness (5), an inability to couple DNA damage to an apoptotic signal pathway can also lead to CDDP resistance (6, 7).

Studies suggest that MMR proteins are able to link CDDP-induced DNA damage to cell death in a variety of carcinoma cells (6, 8). It has been suggested that in the absence of MMR, coupling to apoptosis does not occur, but instead, CDDP-induced intrastrand cross-links are bypassed during replication, giving a CDDP-resistant phenotype (9, 10). Such a MMR-dependent mechanism of resistance may have widespread implications in cancer chemotherapy because loss of expression of MMR proteins has been correlated not only with resistance to CDDP (and carboplatin) but also with resistance to 6-thioguanine, doxorubicin, etoposide, ionizing radiation, and monofunctional methylating agents such as temozolomide and MNU (6, 7, 11).

Recent *in vitro* studies have shown that DNA polymerase δ and DNA polymerase ϵ are able to bypass 1,2 cross-links induced by CDDP in structures that resemble replication forks (12, 13). Ap is an antibiotic that inhibits DNA polymerases α , δ , and ϵ by binding to polymerase nucleotide binding sites (14). In the present study, we have used Ap as a tool to study the role of DNA polymerases and replicative bypass in CDDP resistance mediated by a loss of MMR.

Studies investigating the modulatory effect of Ap on CDDP resistance have been performed in the past and have produced conflicting results. Some studies have suggested that Ap can increase both CDDP-induced DNA damage and cytotoxicity (15–17), whereas others have concluded that Ap has no effect on CDDP-induced DNA damage or cytotoxicity (18, 19). All of these studies have been performed using cells with unknown MMR status. Consequently, conclusions were drawn without considering the role of MMR in CDDP resistance or how Ap may alter MMR function. In contrast, our investigations involved quantifying the survival of paired ovarian carcinoma cell lines with known MMR status after sequential treatment with CDDP or MNU and Ap. We show that Ap increases the sensitivity of MMR-deficient lines to CDDP and MNU by a greater extent than MMR-proficient lines. This supports Ap sensitizing drug-resistant cancer cells that have lost MMR to CDDP or MNU and is consistent with Ap inhibiting replicative bypass preferentially in MMR-deficient cells.

MATERIALS AND METHODS

Cell Lines and Drug Sensitivity Assays. The following human cell lines were used: (a) A2780, a MMR-proficient ovarian carcinoma line; (b) A2780/cp70, a CDDP-resistant A2780 derivative known to be MMR defective due to loss of expression of the MMR protein MLH1 (8, 20); and (c) the A2780/cp70 derivatives cp70-ch2 and cp70-ch3 that have been transfected with human chromosomes 2 and 3, respectively (9). The transfer of chromosome 3 containing a wild-type copy of the *hMLH1* gene into A2780/cp70 restores MLH1 expression and MMR activity, whereas the transfer of chromosome 2 has no effect on MLH1 expression or MMR activity (data not shown). All cell lines were maintained as monolayers in RPMI 1640 with 10% FCS and grown at 37°C in 95% air:5% CO₂. All cell lines were free of *Mycoplasma* infection.

Drug sensitivity assays were performed by seeding cells (5×10^5) into flasks on day 1 and exposing cells to CDDP (Sigma) for 1 h or MNU (Sigma) for 2 h on day 4. This was followed immediately by 24-h exposures to Ap (Sigma), after which cells were re-seeded at 10^3 cells/dish into at least five dishes per treatment, per experiment. Surviving colonies were counted after an additional 10 days of growth.

Cell Cycle Analysis by Flow Cytometry. Proportions of cells in different phases of the cell cycle were assessed by the incorporation of BrdUrd in a 1–4-h pulse, propidium iodide staining, and flow cytometric analysis, as described previously (21).

Statistics. The mean colony counts over the replicates for each set of experimental conditions are the basic data used for the statistical analysis. These data are then transformed to give the odds of colony formation as follows: (mean colony count)/(1000 – mean colony count). The analysis of covariance technique was used to analyze the data. The odds of colony formation for each cell line, as observed on the first replicate with no cisplatin and no modifier present, is taken as the covariate reflecting the difference between the experiments (this replicate is omitted from the mean count calculation outlined above). To make the data conform to the assumptions of

Received 9/4/98; accepted 3/5/99.

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¹ Supported by grants from the Association for International Cancer Research and the Cancer Research Campaign UK (to R. B.).

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³ The abbreviations used are: CDDP, *cis*-diaminedichloroplatinum II; MMR, mismatch repair; Ap, aphidicolin; MNU, *N*'-methyl-*N*-nitrosourea; BrdUrd, bromodeoxyuridine; SF, surviving fraction; O⁶-meGua, O⁶-methylguanine; O⁶-beGua, O⁶-benzylguanine.

the analysis of covariance, a square root transformation is then applied to the odds of colony formation and the covariate.

The *P* quoted for each pair of cell lines is for the three-way interaction (Cell line) × (Ap) × (CDDP or MNU dose). This interaction relates to the null hypothesis that the change in sensitivity to CDDP or MNU brought about by Ap is the same for both cell lines.

RESULTS

The Effect of Ap on CDDP Cytotoxicity. We have tested the hypothesis that Ap may specifically sensitize MMR-deficient, CDDP-resistant tumor cells by inhibiting replicative bypass in paired ovarian carcinoma cell lines of differing MMR status (as described in “Materials and Methods”). In the first instance, the effect of Ap on cell survival and cell cycle progression was characterized in the four cell lines. IC₅₀ estimations obtained from clonogenic survival assays performed after a 24-h exposure to Ap are shown in Table 1. These IC₅₀ estimates did not correlate with MMR status and CDDP sensitivity but did correlate with the proportion of S-phase cells (as measured by flow cytometry) present in each cell line at the time Ap exposure was begun (*r*² = 0.79). This suggests that a cell line with a higher basal level of S-phase cells has increased polymerase activity and requires a higher concentration of Ap to inhibit this activity. Flow cytometric analysis demonstrated that a 24-h exposure to Ap at the IC₅₀ concentration inhibits the progression of the cells through the cell cycle by inducing a reversible G₁-S-phase block (data not shown) and inhibiting replicative DNA synthesis (Table 1). Comparable cell cycle data with Ap have been published previously (22).

Clonogenic assays in which CDDP incubations (1 h) were followed by Ap incubations (24 h) were performed using the determined IC₅₀ concentrations of Ap appropriate for each cell line. For both sets of paired cell lines, data from triplicate experiments were transformed to odds of colony formation (as detailed in “Materials and Methods”). In the A2780/A2780/cp70 cell line pair, the differing slopes of the cells treated with CDDP alone show that the MMR-defective A2780/cp70 cells (Fig. 1A, *iii*) are more resistant than the MMR-proficient A2780 cells (Fig. 1A, *i*), as demonstrated previously (6). A2780 had a SF of 0.24 at 10 μM CDDP for 1 h, whereas A2780/cp70 had a SF at this concentration of 0.85. However, when Ap is added, the slopes for the two cell lines are very similar (Fig. 1A, *ii* and *iv*), indicating that Ap reduces the relative CDDP resistance in the MMR-defective A2780/cp70 cells compared to the parental line. The null hypothesis that the change in sensitivity to CDDP mediated by Ap is the same for both cell lines was rejected (*P* = 0.02).

The chromosome transfer cell lines show the same MMR dependence as the parental lines; thus, the differing slopes of the cells treated with CDDP alone demonstrate that the MMR-defective cp70-ch2 cells (Fig. 1B, *iii*) are more resistant than the MMR-proficient

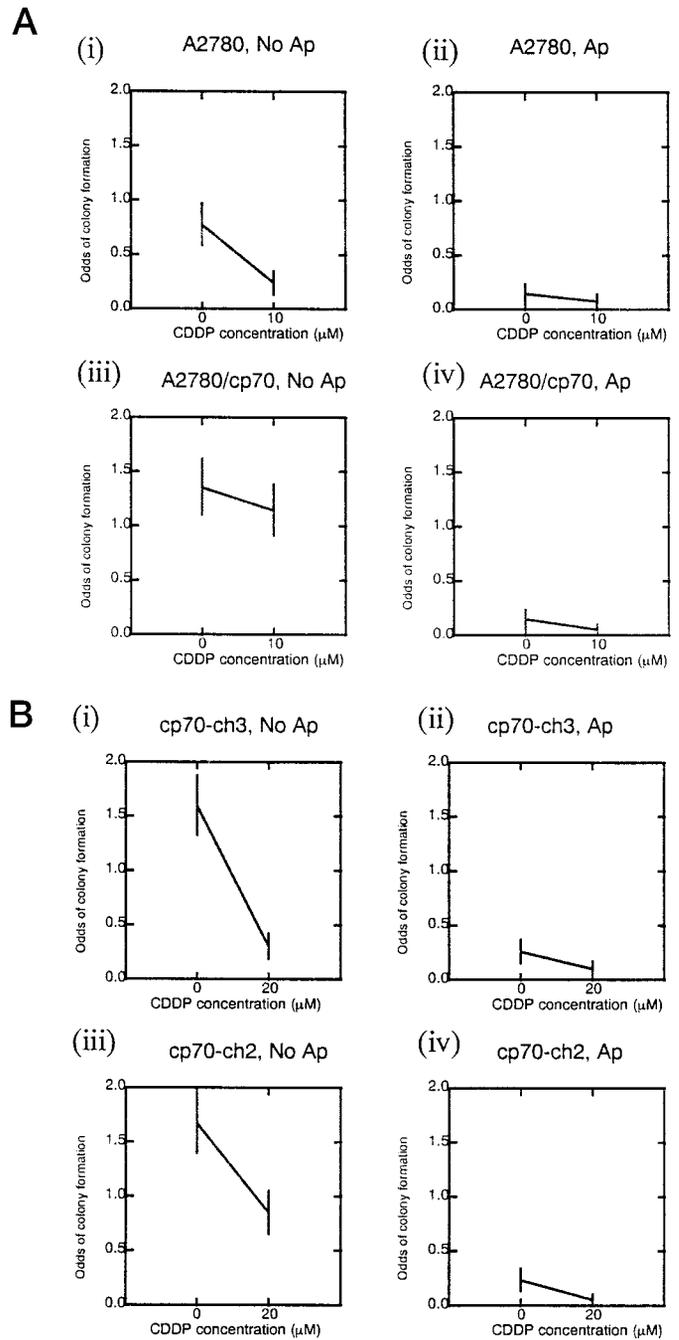


Fig. 1. The effect of Ap on CDDP cytotoxicity. Odds of colony formation in A2780/A2780/cp70 (A) and cp70-ch3/cp70-ch2 (B) paired cell lines. All cultures were treated with 10 (A) or 20 μM (B) CDDP for 1 h; half of the cultures (A, *ii* and *iv*; B, *ii* and *iv*) were also treated with Ap for 24 h (using the IC₅₀ concentration of AP appropriate for each cell line) beginning immediately after CDDP removal. Clonogenic survival was quantified as detailed in “Materials and Methods.” Graphs show the mean levels and associated 95% confidence intervals.

cp70-ch3 cells (Fig. 1B, *i*). The cp70-ch2 cells had a SF of 0.84 at 20 μM CDDP for 1 h, whereas cp70-ch3 cells had a SF of 0.36 at this concentration. Thus, as demonstrated previously for colon cell lines (7), restoration of MMR by chromosome transfer sensitizes these ovarian cell lines to CDDP. However, when Ap is added, the slopes in the two cell lines are very similar (Fig. 1B, *ii* and *iv*), indicating that Ap reduces CDDP resistance in the MMR-defective cp70-ch2 cells. Again, the significance was demonstrated by the rejection of the null hypothesis (*P* = 0.02).

The Effect of Ap on MNU Cytotoxicity. The development of CDDP resistance by a loss of MMR has a precedent in the develop-

Table 1 Ap sensitivity and cell cycle arrest

Cell line	Ap IC ₅₀ (μM) ^a	Percentage of cells in S phase ^b	
		pre-Ap ^c	post-Ap (IC ₅₀) ^d
MMR proficient cells			
A2780	1.9	30.3 ± 2.3	2.9 ± 0.6
cp70-ch3	3.1	38.5 ± 2.1	3.2 ± 1.1
MMR-deficient cells			
A2780-cp70	2.4	37.0 ± 3.1	3.0 ± 1.3
cp70-ch2	2.1	34.2 ± 2.2	2.5 ± 0.6

^a The concentration at which a 50% survival of clonogenic cells is seen after a 24-h exposure to Ap.

^b The percentage of cells in the S-phase fraction is calculated from the number of BrdUrd-positive cells expressed as a percentage of total cells. Errors are SEs of the mean.

^c The percentage of cells in S phase at time Ap exposure was begun.

^d The percentage of cells in S phase after a 24-h exposure to Ap at the IC₅₀ concentration.

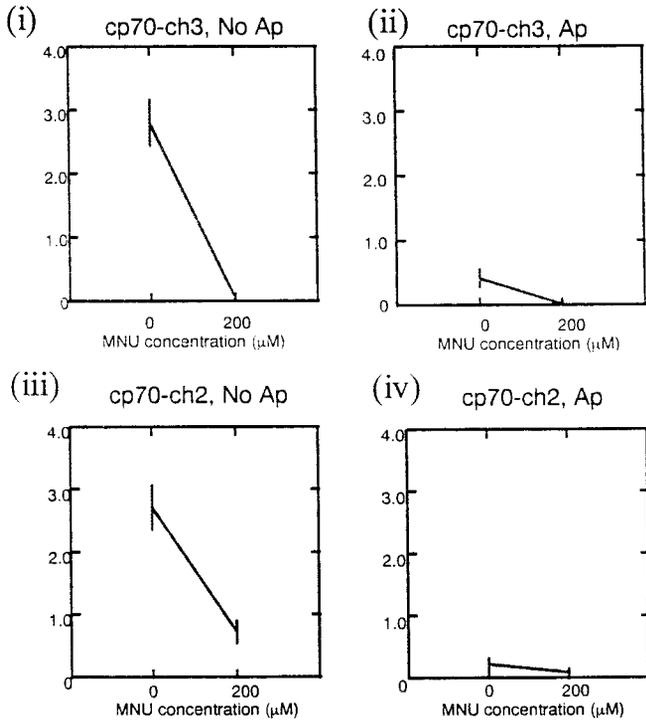


Fig. 2. The effect of Ap on MNU cytotoxicity and odds of colony formation in cp70-ch3/cp70-ch2 paired cell lines. All cultures were treated with 200 μM MNU for 2 h; half of the cultures (ii and iv) were also treated with Ap for 24 h (the IC_{50} concentration of AP appropriate for each cell line) beginning immediately after MNU removal. Clonogenic survival was quantified as detailed in "Materials and Methods." Graphs show the mean levels and associated 95% confidence intervals.

ment of resistance to DNA methylation damage, in particular, the potentially lethal O^6 -meGua lesion (11). Thus, it is possible that MMR-deficient cells may have the ability to perform replicative bypass not only of 1,2 intrastrand cross-links produced by CDDP but also of O^6 -meGua lesions produced by methylating agents such as MNU. To determine whether Ap specifically sensitizes MMR-deficient tumor cells to MNU, identical studies to those performed with CDDP have been performed with MNU, using clonogenic assays to measure cell sensitivities.

Initial clonogenic assays performed with MNU and Ap in this study incorporated a 4-h O^6 -beGua pretreatment. The basis for this was that O^6 -beGua treatment has been shown to deplete O^6 -meGua DNA methyltransferase activity *in vitro* (23). O^6 -meGua DNA methyltransferase is a DNA repair enzyme that can repair O^6 -meGua lesions induced by MNU, resulting in a MNU-resistant phenotype. However, studies have shown that the inclusion of O^6 -beGua when treating the A2780/ A2780/cp70 paired cell lines with MNU did not significantly alter the MMR-dependent MNU resistance of the cell lines (20). Similarly, in the present studies, O^6 -beGua pretreatment had no significant effect on the modulatory activity of Ap (data not shown); thus, it was eliminated from the subsequent protocols.

In the cp70-ch3/cp70-ch2 cell line pair, the differing slopes of the cells treated with MNU alone demonstrate that the MMR-defective cp70-ch2 cells (Fig. 2iii) are more resistant than the MMR-proficient cp70-ch3 cells (Fig. 2i). This demonstrates that MNU sensitivity correlates with the presence of MMR in these cell lines. The cp70-ch2 cell line had a SF of 0.6 after 200 μM MNU for 2 h, whereas cp70-ch3 had a SF of 0.04 after the same treatment. When Ap is added, the slopes in the two cell lines become similar (Fig. 2, ii and iv), indicating that Ap reduces MNU resistance in the MMR-defective cp70-ch2 cells. The null hypothesis that the change in sensitivity to MNU by Ap is the same for both cell lines was rejected ($P = 0.023$).

Ap Modulation of CDDP-induced G_2 -M-phase Arrest. CDDP treatment of tumor cells can inhibit their progression through the cell cycle by inducing a G_2 -M-phase arrest, with this arrest having been implicated in CDDP-induced apoptosis (24). Furthermore, CDDP-induced G_2 -M-phase arrest appears to be correlated with MMR status in ovarian carcinoma cell lines (20). Thus, cell lines deficient in MMR and resistant to CDDP, including A2780/cp70, demonstrate reduced G_2 -M-phase arrest after CDDP damage compared to MMR-proficient lines such as A2780. To determine whether the chromosome transfer cell lines cp70-ch3 and cp70-ch2 differ in their ability to arrest in G_2 -M phase compared with the parental A2780/cp70 cell line, cell cycle analysis has been performed. As shown in Fig. 3A, the MMR-proficient cp70-ch3 cells show a marked increase in cells with a 4 N DNA content 24 h after a 1-h CDDP exposure (20 μM), which is indicative of a G_2 -M-phase arrest, whereas the MMR-deficient A2780/cp70 and cp70-ch2 cell lines show no such increase.

Next we examined whether the increased sensitization to CDDP by Ap in the MMR-deficient cp70-ch2 cells is accompanied by a restored G_2 -M-phase arrest. Fig. 3B reveals that the addition of Ap (IC_{50}) for

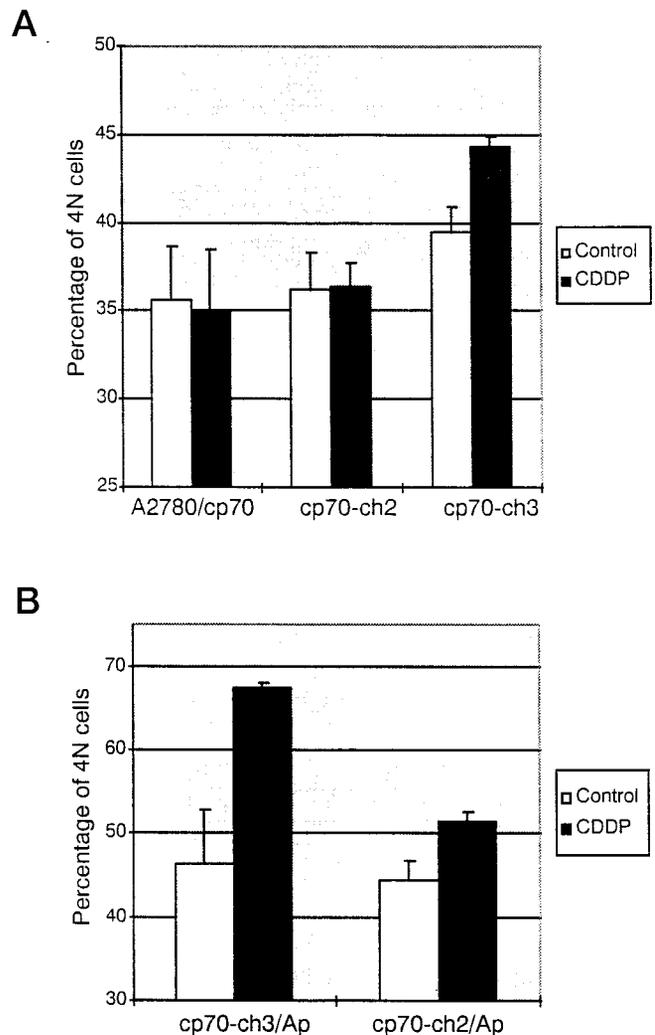


Fig. 3. CDDP-induced cell cycle arrest. A, CDDP induced changes in the proportion of cells with 4 N DNA content in the A2780/cp70, cp70-ch3, and cp70-ch2 cell lines. Cells were exposed to CDDP (20 μM) for 1 h. A 4-h BrdUrd (10 mM) pulse was performed 24 h after CDDP treatment. B, CDDP and Ap induced changes in the proportion of cells with 4 N DNA content in the cp70-ch3/cp70-ch2 paired cell lines. Cells were seeded on day 1 and exposed to CDDP (20 μM) and Ap (the IC_{50} concentration of AP appropriate for each cell line) for 24 h over days 4 and 5. A 1-h BrdUrd (10 mM) pulse was performed 24 h after completion of the Ap incubation. The values shown were calculated from the means of at least two repeat measurements, analyzing more than 15,000 events. Bars, SE.

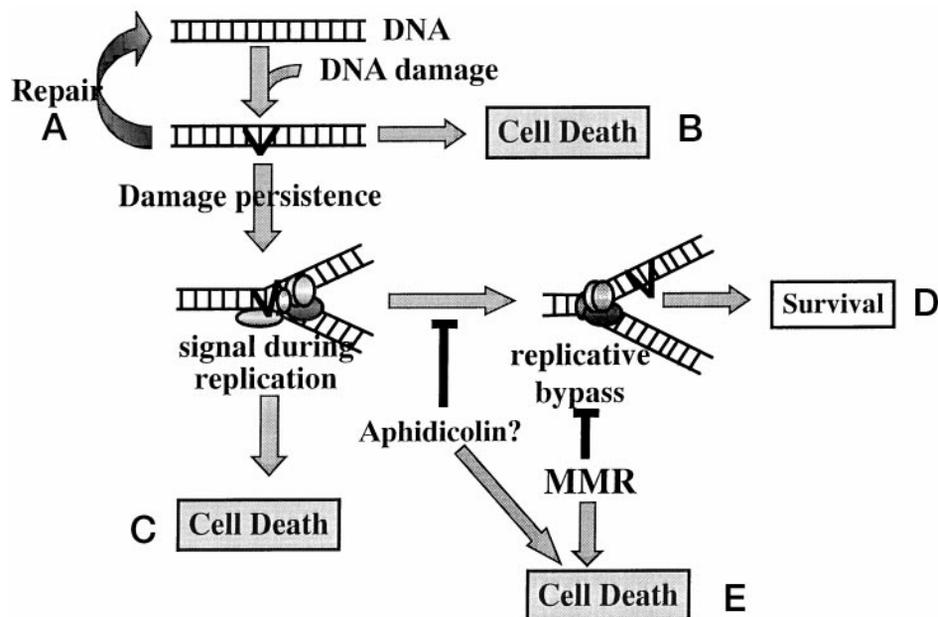


Fig. 4. Model of the effect of Ap on replicative bypass. We suggest that DNA damage may be repaired (A) or may be cytotoxic and induce immediate cell death (B). Alternatively, the lesion may persist and produce a signal during replication that triggers a cell death pathway (C). In certain circumstances, these lesions may be bypassed during replication, allowing survival (D). We propose that in a MMR-proficient environment, this bypass is inhibited, allowing additional cell death (E). Ap may inhibit bypass of CDDP and MNU lesions in the MMR-defective cell lines by virtue of its ability to block polymerase δ and ϵ function, resulting in further cell death (E).

24 h after a 1-h CDDP (20 μ M) exposure increases the proportion of cells with 4 N DNA in both the cp70-ch3 and cp70-ch2 cell lines as compared to those treated with Ap alone. In contrasting studies with CDDP as a single agent (Fig. 3A), there is a marked increase in the proportion of cells with 4 N DNA in the MMR-deficient cp70-ch2 cells treated with both CDDP and Ap compared to those treated with Ap alone (Fig. 3B). The increase of 4 N cells in the presence of Ap correlates with the reduction in CDDP resistance seen in the presence of Ap in these cells (Fig. 1B). Taken together, these observations suggest that there is a correlation between MMR status, CDDP sensitivity, and G₂-M-phase arrest and that Ap is able to restore CDDP-induced G₂-M-phase arrest in a MMR-deficient background.

DISCUSSION

We have shown that Ap has a greater effect on sensitization to CDDP and MNU in MMR-defective cells than in their MMR-proficient counterparts. These observations lead to the question: how does Ap alter drug cytotoxicity in a MMR-dependent manner? We favor a model whereby Ap inhibits the bypass of DNA lesions that may occur in the absence of MMR. The model has stemmed from several bodies of evidence. The first is the ability of Ap to inhibit DNA polymerase δ and ϵ , both of which are able to bypass DNA adducts such as CDDP-induced 1,2 cross-links *in vitro* (14, 19). The second is that increased replicative bypass of CDDP lesions has been demonstrated in CDDP-resistant ovarian cell lines (10). Thirdly, increased recombination-dependent replicative bypass has been proposed to lead to CDDP resistance in yeast and mammalian cells (9). A diagram of this replicative bypass model is shown in Fig. 4. We suggest that DNA damage, such as that induced by CDDP and MNU, may be processed in a number of ways within a cell. The initial lesion may be repaired (Fig. 4A) or may be cytotoxic and induce immediate cell death (Fig. 4B). Alternatively, the lesion may persist and produce a signal during replication that triggers a cell death pathway (Fig. 4C). In the case of CDDP and MNU, the persistent lesions are likely to be the 1,2 intrastrand cross-link and the O⁶-meGua lesions, respectively, because these are the most potent and prevalent lesions induced by the compounds (2, 11). In certain circumstances, these lesions may be bypassed during replication, allowing survival (Fig. 4D). We propose that in a MMR-proficient environment, such as that seen in the A2780

and cp70-ch3 cell lines, this bypass is inhibited, allowing additional cell death (Fig. 4E). The results presented in this study suggest that Ap may inhibit bypass of CDDP and MNU lesions in the MMR-defective A2780/cp70 and cp70-ch2 cell lines by virtue of its ability to block polymerase δ and ϵ function, resulting in further cell death (Fig. 4E).

An alternative explanation stems from the ability of Ap to inhibit polymerase α (14). DNA polymerase α is an essential DNA synthesis enzyme, and as a consequence of this, incubating cells with Ap induces a reversible late G₁ block in the cell cycle (Table 1). This raises the possibility that the modulatory effect observed in this study is simply a consequence of cell cycle delay. *In vitro* studies combining CDDP and Ap in A2780/cp70 cells have demonstrated that Ap increased CDDP cytotoxicity in A2780/cp70 cells, and this correlated with the ability of Ap to delay the removal of platinum-DNA adducts (15). Thus, Ap may reduce CDDP and MNU resistance in MMR-deficient cell lines by delaying the removal of DNA-drug adducts. However, certain CDDP-resistant human ovarian cells appear to be able to bypass CDDP DNA intrastrand cross-links during DNA replication (25). If a resistant cell is able to bypass an adduct, then the amount of time that an adduct is present should not affect cell survival, arguing against the significance of the ability of Ap to delay lesion removal.

Ap not only reduces resistance to CDDP in MMR-defective cells but also increases the proportion of cells with a 4 N DNA content, which is indicative of G₂-M-phase arrest, after CDDP treatment (Fig. 3B). Several studies have suggested a link between MMR proteins and the G₂-M-phase cell cycle checkpoint in response to DNA damage induced by CDDP, 6-thioguanine, methylating agents, and radiation (20, 26, 27). Davis *et al.* (27) have hypothesized that in a MMR-proficient environment, the MMR system binds and recognizes certain lesions, resulting in the formation of DNA breaks. These breaks produce a signal that results in G₂-M-phase cell cycle arrest and a loss of survival. Thus, in the absence of MMR, lesions are not recognized, DNA strand breaks are not generated, and G₂-M-phase arrest is unable to be induced. The ability of Ap to restore G₂-M-phase arrest may be due to a restoration of the induction of DNA strand breaks. If Ap is indeed inhibiting replicative bypass of CDDP lesions, then this inhibition could increase the probability of DNA breaks at lesion sites. These breaks, formed by increased stalling of the replication complex,

may produce signals similar to those created when MMR recognizes lesions and may result in the G₂-M-phase arrest and loss of survival that we have observed.

Several *in vivo* and *ex vivo* studies with CDDP and Ap have been conducted previously. An *in vivo* model study of CDDP-refractory human ovarian tumors has shown that the combination of CDDP and Ap was markedly superior to CDDP alone (16). Similarly, *ex vivo* investigations using cells from primary ovarian tumors demonstrated that Ap had a greater CDDP sensitization effect in cells that were initially more resistant to CDDP (17). Both studies provided results that encouraged further development of the CDDP/Ap combination. However, our results suggest that the true potential of the compounds would be realized by performing *in vivo* or, indeed, clinical studies with tumors harboring MMR defects. Defective MMR has been suggested to occur in 15–20% of sporadic ovarian tumors, with this figure rising in CDDP-resistant tumors (20, 28, 29). It is within these tumors that Ap may inhibit the bypass of potentially lethal DNA lesions induced by CDDP or methylating agents and increase the susceptibility of tumor cells to their toxic effects. Phase I trials of Ap glycinate have already indicated that clinical use of this agent is feasible (30), and further clinical investigation is clearly justified.

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Cancer Res 1999;59:2102-2106.

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