all-trans Retinoic Acid Enhances Cisplatin-induced Apoptosis in Human Ovarian Adenocarcinoma and in Squamous Head and Neck Cancer Cells

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ABSTRACT

Cisplatin exerts its cytotoxicity by inducing apoptosis. Similarly, all-trans retinoic acid (ATRA) causes apoptosis in certain cells. We studied the interaction of cisplatin and ATRA in human ovarian adenocarcinoma cells 2008, in human head and neck squamous carcinoma cells UMNSCC10b, and in their respective cisplatin-resistant sublines. ATRA enhanced the cytotoxicity of cisplatin. The interaction of the drugs was synergistic in combination index-isobologram analyses (combination index <0.5 at 50% cell survival) in all of the cell lines tested. ATRA inhibited the cellular accumulation of the cisplatin analogue [3H]cis-dichloroethylenediamineplatinum(II) by 22-33% in three of four cell lines tested but did not alter the cellular content of reduced glutathione. The expression of Bcl-2 relative to Bax decreased more after combined treatment with cisplatin and ATRA than after either drug alone. The apoptotic mechanism of cell death was confirmed by demonstrating cleavage of poly(ADP-ribose)polymerase and by morphological analysis. The combined treatment with ATRA and cisplatin induced apoptosis in significantly more cells than either drug alone. We conclude that ATRA enhances the cytotoxicity of cisplatin by facilitating apoptosis in ovarian and head and neck carcinoma cells.

INTRODUCTION

DDP is one of the most effective drugs in the treatment of solid tumors including epithelial ovarian carcinoma and head and neck squamous carcinoma. It exerts its cytotoxic action by forming intra- and interstrand adducts in DNA, which induce apoptosis (1) both in ex vivo tumor cells (2) and in tissue culture models (3). Therapy with DDP is limited by cumulative renal and neural toxicity; therefore, it would be advantageous to increase the therapeutic index of DDP by increasing its efficacy or by decreasing the toxic side effects.

Resistance to DDP develops frequently in tumors. It results from a number of different mechanisms, and it is a major cause for failure of treatment (reviewed in Ref. 4). Bcl-2 counters apoptosis by various mechanisms, one of which is by forming a heterodimer with Bax, a homologous apoptosis-promoting protein. Rather than the absolute cellular content of either Bcl-2 or Bax, the relative amounts of Bcl-2, Bax, and other proteins determine the apoptotic response (reviewed in Ref. 5). Some DDP-resistant ovarian carcinoma cell lines overexpress Bcl-2, and exogenous overexpression of Bcl-2 inhibits apoptosis (3), indicating that Bcl-2 may play a role in the cellular resistance to DDP in this type of tumor.

ATRA binds to the nuclear retinoic acid receptors and activates their functions as transcription factors. It is metabolized to 9-cis and 13-cis retinoic acid in human cells (reviewed in Ref. 6). 9-cis retinoic acid is a high-affinity ligand for retinoic acid receptors but in addition also activates retinoic acid X receptors. Thus, ATRA can directly and indirectly activate a wide range of retinoid receptors (reviewed in Ref. 7). ATRA has numerous cellular effects including differentiation and growth arrest in ovarian carcinoma (8, 9) and in squamous head and neck carcinoma cells (10). ATRA induces apoptosis in many tissue culture models including ovarian carcinoma (11). It enhances the cytotoxicity of DDP in head and neck and ovarian carcinoma cells in vitro (12, 13). 9-cis retinoic acid has been reported to increase the efficacy of DDP in an in vivo model of human squamous head and neck carcinoma (14).

The different mechanisms of action of DDP and ATRA as well as their lack of overlapping toxicity make them potential candidates for combined therapy. In this study, we have used the...
mathematically rigorous technique of CI analysis to examine the nature of the interaction between ATRA and DDP in human ovarian carcinoma and squamous head and neck carcinoma cell lines and investigated potential mechanisms of the observed synergy.

MATERIALS AND METHODS

Cell Lines. The experiments were performed with the human ovarian serous adenocarcinoma cell line 2008 (15) and its DDP-resistant subline 2008/C13*5.25 (16). In addition, the interaction of ATRA with DDP was studied in the human squamous head and neck cancer cell line UMSCC10b (17) and its DDP-resistant subline UMSCC10b-Pt/15S (18). The cells were maintained in a 5% CO2 atmosphere at 37°C in RPMI 1640 (Irvine Scientific, Irvine, CA) supplemented with 2 mM glutamine and 10% heat-inactivated FCS without antibiotics. The plating efficiencies of 2008, 2008/C13*5.25, UMSCC10b, and UMSCC10b-Pt/15S were 63, 68, 61, and 54%. All experiments were performed with exponentially growing cells. All cell lines tested negative for contamination with *Mycoplasma* spp.

Chemicals. DDP was a gift from Bristol-Myers Squibb (Princeton, NJ). ATRA and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO).

Drug Treatment. DDP was dissolved in 0.9% (w/v) saline. ATRA was dissolved in DMSO; stocks of ATRA were kept frozen under nitrogen until use. ATRA was added to the cells in various dilutions such that the concentration of DMSO was constant and did not exceed 0.1% (v/v); control cells were treated with equal concentrations of DMSO. The concentration of 0.1% DMSO was previously shown not to interfere with the growth of 2008 and UMSCC10b cells. The exposure to ATRA was continuous starting either at the same time as the treatment with DDP or 72 h prior to DDP. The medium was replaced with new ATRA-containing medium after 3 days. In contrast, the cells were exposed to DDP for 1 h, after which the medium was replaced.

Clonogenic Assays and Drug Interactions. For clonogenic assays, the cells were trypsinized, cell clusters were disrupted by repetitive pipetting, and the absence of cell clusters was confirmed by microscopy. Three hundred cells were seeded on 60-mm plastic dishes in 5 ml of media. Clones of more than 50 cells were scored visually after 9–10 days. Drug interactions were analyzed by the CI method (19). The drug concentrations ranged from 0 to 15 µM for ATRA in all cell lines, 0 to 12 µM DDP in 2008, 0 to 60 µM DDP in 2008/C13*5.25, 0 to 15 µM in UMSCC10b, and 0 to 40 µM DDP in UMSCC10b-Pt/15S cells. The median-effect principle was applied to determine the dose-effect parameters for DDP, ATRA, and a mixture of both drugs corresponding to the ratio of the IC50 for each individual drug in each cell line. The median effect equation is expressed as $f_e(D) = f(D)_{ATRA} + f(D)_{DDP}$ (Eq. A), and can be linearized as $\log(f_e/D_{ATRA}) = -m\log(D_{ATRA})$ (Eq. B), where $D$ is the concentration of the drug or the sum of concentrations of both drugs, respectively, $D_{ATRA}$ is the median effective dose, $f_e$ is the fractional inhibition of colony formation, $f_{ATRA}$ is the fraction unaffected $(1 - f_e)$, and $m$ is the exponent defining the sigmoidal shape of the dose-effect curve. The parameters of the median effect equation $(D_{ATRA}, m)$ for both drugs alone and in combination were determined from Eq. B by linear regression analysis. The isoeffective dose $D_i$ for any effect level for each drug and their combination were calculated by rearranging the median effect Eq. A as follows: $D_i = D_{ATRA}(1 - f_i)^{1/m}$ (Eq. C). Synergism or antagonism for ATRA plus DDP was determined on the basis of the multiple drug equation of Chou and Talalay (19) and was quantitated by the CI. The CI for $x\%$ inhibition of colony formation was calculated for mutually exclusive effects as $CI = (D_{ATRA}/D_{ATRA} + D_{DDP}/D_{DDP})^{1/x}$ (Eq. D), where $(D_{ATRA})$ and $(D_{DDP})$ are the doses of single drug ATRA or DDP required to produce $x\%$ inhibition; $D_{ATRA}$ and $D_{DDP}$ are the doses in combination that can also inhibit colony formation by $x\%$. If $CI = 1$, Eq. D describes a classical isobole. A CI of $<1$, $=1$, or $>1$ signifies synergism, additivity, or antagonism, respectively. The CI formula (Eq. D) allows the construction of $f_e$-CI plots such as shown in Fig. 1. Each experiment was performed with triplicate cultures for each data point and was repeated independently at least twice.

Cellular Pharmacokinetics. The cellular pharmacokinetic studies were performed with [3H]DEP, a radiolabeled analogue of DDP. [3H]DEP produces the same type of DNA adducts as DDP (20). Its cellular pharmacokinetics have been well characterized and shown to parallel those of DDP (21). Five hundred thousand cells were seeded into a 60-mm dish and exposed to 5 µM ATRA or an equal volume of DMSO (controls) for 72 h, at which point they were treated with 5 µM [3H]DEP with a specific activity of 500 µCi/µmol for 60 min. Thereafter, the cells were washed with ice-cold PBS and lysed overnight in 2 ml of NaOH (1 M). [3H]DEP was measured by liquid scintillation counting, and cellular protein was assayed by the Brad...
ford Coomassie Blue method. Each experiment was performed with triplicate cultures.

Intracellular Glutathione. Cytosolic GSH was assayed as described previously (22). Briefly, after labeling with monobromobimane, the cellular thiols were separated by reversed phase HPLC and were detected and quantitated with on-line fluorometry. Calibration curves with known amounts of GSH were constructed daily.

Immunoblots. Cells were lysed on ice in 150 mM NaCl containing 5 mM EDTA, 1% Triton X-100, 10 mM Tris/HCl (pH 7.4), 5 mM DTT, 0.1 mM phenylmethylsulfonylfluoride, and 5 mM e-aminoacaproic acid. After centrifugation, 50 μg of protein were denatured by boiling in an equal volume of 130 mM Tris/HCl (pH 6.8) containing 20% glycerol, 4.6% SDS, and 0.02% bromophenol blue. For the assay of PARP, the cells were lysed in a buffer containing 63.5 mM Tris (pH 6.8), 6 mM urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, and 5% β-mercaptoethanol. Nuclear PARP was released by sonication for 20 s, and the proteins of 100,000 cells were denatured by heating to 65°C for 15 min. The proteins were separated by SDS-PAGE on an 8% gel for PARP or a 12% gel for Bcl-2 and Bax, respectively. They were electrotransferred onto a polyvinylidene fluoride membrane (Immobilon P; Millipore, Bedford, MA). Bcl-2 and Bax were detected using the monoclonal antibodies Bcl-2(100) and Bax(P19), respectively (Santa Cruz Biotechnology, Santa Cruz, CA). PARP was detected with the monoclonal antibody C-2-10 (23) purchased from Dr. Guy G. Poirier (Université Laval, Quebec, Canada). The binding of antibody was detected by peroxidase-conjugated sheep anti-mouse antibodies (Amersham Corp., Arlington Heights, IL) and generation of chemoluminescence by ECL (Amersham). X-ray films were used to create a permanent record, and the bands of interest were quantitated with a LKB Ultrascan XL densitometer.

Morphological Assay for Apoptosis. Cells (5 × 10^5) were seeded into 10-cm dishes and were treated with drugs as described above. The medium containing floating cells and the adherent cells after trypsin treatment were centrifuged and resuspended in 100 μl of PBS. The cells were immediately stained with acridine orange and ethidium bromide (X25 solution: 0.01% w/v of both stains in PBS) and examined independently by two investigators (B. C. and S. A.) using fluorescence microscopy (24). Apoptotic cells showed condensed and often fragmented nuclei and could be easily distinguished from normal and from necrotic cells. In addition, this method permits the distinction between early apoptotic cells with intact cytoplasmic membranes (green fluorescent condensed nuclei) and late apoptotic cells (red fluorescent condensed nuclei).

Table 1  Cytotoxicity of DDP and ATRA in clonogenic assays in human ovarian adenocarcinoma (2008) and squamous head and neck carcinoma (UMSCC10b) cells and in their respective DDP-resistant sublines

<table>
<thead>
<tr>
<th></th>
<th>IC_{50} (μM)</th>
<th>DDP (μM)</th>
<th>ATRA (μM)</th>
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<tbody>
<tr>
<td>2008</td>
<td>7.3 ± 2.9</td>
<td>3.8 ± 1.0</td>
<td></td>
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<tr>
<td>2008/C13*5.25</td>
<td>50 ± 5.9</td>
<td>1.3 ± 0.3</td>
<td></td>
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<tr>
<td>UMSCC10b</td>
<td>7.7 ± 1.5</td>
<td>4.9 ± 1.1</td>
<td></td>
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<tr>
<td>UMSCC10b-Pr/15S</td>
<td>15.5 ± 1.2</td>
<td>1.4 ± 1.0</td>
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* 1-h exposure.

Continuous exposure.

RESULTS

Interaction of DDP with ATRA. DDP and ATRA were cytotoxic as single agents and inhibited colony formation in human ovarian adenocarcinoma (2008) and head and neck squamous carcinoma (UMSCC10b) cells and in their respective DDP-selected sublines 2008/C13*5.25 and UMSCC10b-Pr/15S (Table 1). The nature of the interaction of DDP and ATRA was analyzed by the CI method (19). This procedure is based on the median effect principle and on isobologram analysis and allows the characterization of drug interactions with a single number, the CI. A CI <1, =1, and >1 implies synergy, additivity, and antagonism, respectively. Fig. 1 shows that a significant synergistic interaction was consistently observed in the ovarian and the head and neck carcinoma cell lines at most levels of inhibition of colony formation as demonstrated by a CI <1. Synergy was present when the exposure to DDP (1 h) and to ATRA (continuous) was started simultaneously and also when the cells were preincubated with ATRA for 72 h prior to the exposure to DDP and continuously thereafter (CIs for the simultaneous start of treatment at a fractional inhibition of 0.5: 2008, 0.39; 2008/C13*5.25, 0.17; UMSCC10b, 0.41; UMSCC10b-Pr/15S, 0.16). However, in all cell lines tested, the interaction of the drugs was not synergistic when ATRA was present only prior to but not after DDP (data not shown). It is evident from Fig. 1 that a higher degree of synergism resulted from pretreating the cells with ATRA for 72 h prior to the exposure to DDP (plots marked “ATRA-3d”; CIs at a fractional inhibition of 0.5: 2008, 0.08; 2008/C13*5.25, 0.17; UMSCC10b, 0.31). This schedule, consisting of a continuous exposure to ATRA starting 72 h prior to a 1-h exposure to DDP and continuing thereafter, was used for the subsequent studies. As an example, Fig. 2 illustrates that continuous treatment with 5 μM ATRA starting 72 h before DDP increased the slope of the dose-response curve of DDP in both ovarian (2008) and head and neck (UMSCC10b) carcinoma cells.

Cellular Pharmacokinetics. The accumulation of DDP is a major determinant of its cytotoxicity (4). [3H]DEP is an analogue

Statistical Analysis. One-way factorial ANOVA and repeated measures ANOVA were calculated with Statview 4.5 for Windows (Abacus Concepts, Berkeley, CA). Post-hoc comparisons were performed using the Schef"f procedure.
Synergy between all-trans Retinoic Acid and Cisplatin

Fig. 3  Fraction of apoptotic cells after treatment with DDP (2008, 5 μM; UMSCC10b, 10 μM; 1 h exposure), ATRA (2008, 5 μM; UMSCC10b, 1 μM; continuous exposure), or the combination of ATRA and DDP; DDP was added after a 72 h preincubation with ATRA. Selected significance levels are indicated below the bars: **, P < 0.001; *, P < 0.03 (ANOVA, Scheffé test). The number of apoptotic cells was not significantly different between controls and cells treated with ATRA alone. Means of three independent experiments are shown; bars, SD. ■, early apoptosis; □, late apoptosis.

of DDP that forms the same DNA adducts as DDP and has very similar cellular pharmacokinetic properties (21). Preincubation with 5 μM ATRA for 72 h reduced the accumulation of [3H]DDP by 22–33% in three of four cell lines tested (2008: 59.6 ± 5.2 in controls versus 46.6 ± 2.2 pmol/mg protein in cells treated with ATRA, P < 0.01; 2008/C13*5.25: 38.4 ± 8.6 versus 42.8 ± 2.1 pmol/mg protein, P = 0.37; UMSCC10b: 74.0 ± 6.5 versus 49.4 ± 3.1 pmol/mg protein, P < 0.01; UMSCC10b-Pt/155: 50.5 ± 1.6 versus 41.3 ± 3.8 pmol/mg protein, P = 0.02; n = 3, mean ± SD, two-sided unpaired t test). These results indicate that the synergy between ATRA and DDP is not mediated by increased cellular accumulation of DDP.

Intracellular GSH. Treatment of 2008 cells with ATRA (5 μM, 72 h) had no significant effect on the intracellular content of GSH (20 ± 2.3 μmol/ml in control cells versus 22 ± 9.3 μmol/mg protein in ATRA-treated cells; mean ± SD, n = 4, P = 0.75).

Mechanism of Cell Death. ATRA is known to induce apoptosis in certain ovarian carcinoma cells (11). Similarly, DDP exerts its cytotoxic action by activating the apoptotic pathway of cell death (1). Therefore, we studied the induction of apoptosis in the ovarian (2008) and in head and neck carcinoma (UMSCC10b) cells. Both cell lines were treated with DDP (5 μM in 2008, 10 μM in UMSCC10b), ATRA (5 μM in 2008, 1 μM in UMSCC10b) and the combination of both drugs; the typical morphology of apoptosis was evident 48 h after treatment with DDP when stained with acridine orange and ethidium bromide and was examined by fluorescent microscopy (24). Fig. 3 shows that in both cell lines, the combined treatment with ATRA and DDP resulted in about 1.5 (UMSCC10b) to 2 (2008) times the number of apoptotic cells when compared to either drug alone. In addition, the fraction of cells in late apoptosis was significantly higher after combined treatment, suggesting an earlier onset or a more rapid completion of apoptosis after pretreatment with ATRA. Necrotic cells constituted less than 1% of the cells following any treatment and in controls.

To further confirm that apoptosis was the mechanism of cell death induced by DDP and ATRA in 2008 human ovarian adenocarcinoma cells, we studied the cleavage of PARP. PARP is cleaved specifically by apopain/CPP32, a protease that is necessary to initiate apoptosis (25). Fig. 4 demonstrates that cleavage of PARP was not detected in untreated cells. However, the typical M, 85,000 cleavage product (23) was present in the nuclear extracts 48 h after treatment with DDP and with the combination of ATRA and DDP. In agreement with the morphological finding of only modest induction of apoptosis, it was almost undetectable after treatment with ATRA alone.

Bcl-2 and Bax are homologous proteins involved in the regulation of apoptosis with Bax acting as a promoter and Bcl-2 as an inhibitor (26). We measured their expression by immunoblot in ovarian cancer cells (2008) after treatment with DDP (5 μM, 1 h), ATRA (5 μM, continuous exposure), and the combination of ATRA (72 h pretreatment) and DDP. The expression of Bax remained essentially unchanged after treatment with either DDP or ATRA alone or in combination. The expression of Bcl-2 and, therefore, the ratio of Bcl-2:Bax declined rapidly after treatment with DDP. Fig. 5A illustrates that the combined treatment with ATRA and DDP resulted in further suppression of Bcl-2 relative to Bax (P = 0.03 for differences between treatments, repeated measures ANOVA). Fig. 5A shows a representative immunoblot demonstrating the decline in Bcl-2 expression after combined treatment with ATRA and DDP. At the chosen dose, ATRA by itself produced a decline in the ratio of Bcl-2:Bax only after prolonged exposure.

DISCUSSION

This study of the interaction of DDP and ATRA produced two important findings: (a) ATRA synergistically enhanced the cytotoxicity of DDP in human ovarian adenocarcinoma cells (2008), in human squamous head and neck carcinoma cells (UMSCC10b), and in their respective DDP-resistant sublines (2008/C13*5.25 and UMSCC10b-Pt/155); and (b) ATRA seems to enhance the potency of DDP by increasing the susceptibility of the cells to undergo apoptosis.
Cells is presently unknown. The schedule dependence of the interaction occurs in
When the continuous treatment with ATRA was started 72 h prior to exposure to ATRA, drugs. This finding is of interest because the lower drug concentration range for both types of cells (2008 and UMSCC1Ob) and also in the cisplatin-resistant sublines (2008/Cl3*5.25 and UMSCC1Ob-PtJl15S). The greatest degree of synergy occurred in the low concentration range for both drugs. This finding is of interest because the lower drug concentrations are readily attainable in the plasma of patients (28, 29).

Enhancement of the cytotoxicity of DDP by ATRA has been described previously in human teratocarcinoma cells (27), squamous head and neck carcinoma cells (12), and very recently in ovarian cancer cells (13). In the present study, the synergistic nature of the cytotoxic effects of ATRA and DDP in the ovarian carcinoma cells (2008 and 2008/C13*5.25) and in the squamous head and neck cancer cells (UMSCC1Ob and UMSCC1Ob-PtJ15S) was demonstrated by the mathematically rigorous CI method (19). Simultaneous treatment with ATRA and DDP followed by continuous exposure to ATRA produced CIs well below 1 in the wild-type cells (2008 and UMSCC1Obb) and also in the cisplatin-resistant sublines (2008/C13*5.25 and UMSCC1Ob-PtJ15S). The greatest degree of synergy occurred in the low concentration range for both drugs. This finding is of interest because the lower drug concentrations are readily attainable in the plasma of patients (28, 29).

When the continuous treatment with ATRA was started 72 h prior to the exposure to DDP, we observed an additional increase in synergy. This observation is consistent with the multistep mechanism of action of ATRA that involves transport to the nucleus, activation of the retinoic acid receptor, and binding to retinoic acid response elements with consecutive transcriptional regulation of target genes. Furthermore, isomerization of ATRA to 9-cis retinoic acid with activation of retinoic acid X receptors is possible, but the extent to which this isomerization occurs is unknown. The schedule dependence of the interaction of ATRA and DDP will have to be considered in future clinical trials.

Cellular sensitivity to DDP can be modified by a variety of mechanisms. Diminished drug accumulation is the single most important determinant of the cytotoxicity of DDP and is present in about 80% of cisplatin-selected cell lines (4). In this study, ATRA promoted the toxicity of DDP but did not increase the accumulation of the DDP analogue [3H]DEP in cells but rather caused diminished accumulation. Furthermore, depletion of GSH has been shown to sensitize cells to DDP (16). However, we did not observe an alteration in the cellular content of GSH after a 72-h exposure to 5 μM ATRA in 2008 cells. Thus, enhanced accumulation of DDP and changes in the cellular concentration of GSH did not explain the synergy.

Apoptosis is regulated by numerous proteins (reviewed in Refs. 5 and 30), among which Bcl-2 inhibits and Bax promotes apoptosis. DDP induces cell death by apoptosis (1–3). In this study, DDP caused a rapid decline in the ratio of Bcl-2 and Bax that was further enhanced by concurrent treatment with ATRA. In 2008 cells, ATRA by itself reduced the expression of Bcl-2 relative to Bax only after prolonged exposure. Whether the observed decline of the Bcl-2:Bax ratio was the cause or the effect of apoptotic cell death cannot be decided based on the present data. In recent reports, ATRA enhanced the toxicity of cytarabine by inducing apoptosis and down-regulating Bcl-2 in leukemic stem cells and acute myelogenous leukemia blasts (31). This effect may be mediated by retinoic acid X receptors after isomerization of ATRA to 9-cis retinoic acid (32). DDP-induced apoptosis as assessed by cellular morphology was enhanced by ATRA at a time when ATRA alone induced only a moderate amount of apoptosis in 2008 cells. These findings are compatible with the observed temporal changes of the Bcl-2:Bax ratio, with the PARP cleavage pattern (23), and with a recent report by Jozan et al. (33). The temporal and quantitative relationships between drug exposure, biochemical processes, and morphological markers of apoptosis will need to be clarified more in detail to design optimal treatment schedules.

In addition to cisplatin and cytarabine, ATRA also interacts with IFN-α by modulating the amounts and state of activation of some components of the IFN signaling pathways in acute promyelocytic leukemia and breast cancer cells (34–36). To date, the clinical application of ATRA is limited largely to the treatment of acute promyelocytic leukemia. Similar to 13-cis retinoic acid (reviewed in Ref. 37), ATRA as a single drug had only modest activity in phase II studies in solid tumors (38–40).

Taken together, the present data support the conclusion that ATRA facilitates apoptosis induced by DDP in 2008 ovarian carcinoma and in UMSCC1Ob head and neck carcinoma cells. Considering the different clinical toxicities of ATRA and DDP and the synergistic interaction of the two drugs in vitro, the combined treatment of malignant tumors with ATRA and DDP merits further clinical investigation.

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