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Activity of aphidicolin glycinate alone or in combination with cisplatin in a murine ovarian tumor resistant to cisplatin*

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Summary. Aphidicolin, a reversible inhibitor of DNA polymerase α and δ, has recently been reported to reverse the resistance to cisplatin (DDP) of an ovarian cancer cell line. We investigated the pharmacokinetics of aphidicolin in mice and examined its activity either alone or in combination with DDP in the DDP-sensitive M5076 (M5) murine reticular cell sarcoma as well as in a DDP-resistant subline (M5/DDP). The drug was cleared from plasma very rapidly (clearance, 41.6 ml min^{-1} kg^{-1}), showing a half-life of 15 min. Aphidicolin concentrations in the tumor were approximately 50% of those found in plasma at steady state. Using several dose schedules and continuous infusions we failed to detect significant antitumor activity for aphidicolin glycinate. Potentiation of the activity of DDP by aphidicolin glycinate was moderate in mice bearing M5 tumor as well as in those bearing M5/DDP tumor. These data do not support the possible clinical use of aphidicolin in combination with DDP. However, further studies should be carried out in different tumor models before this possibility is conclusively ruled out.

Introduction

Aphidicolin (Aphi) is a reversible inhibitor of DNA polymerase α and δ [4, 6–8, 10] that has shown antiviral and antiproliferative effects in vitro [5] and moderate antitumor activity in vivo against several murine models (P388 leukemia, B16 melanoma, colon 26 adenocarcinoma, CD8F1 mammary adenocarcinoma, and LX-1 subrenal capsular lung-cancer xenograft) (Winograd et al., submitted for publication). In addition, Aphi has been reported to counteract resistance to cisplatin (DDP) in a human ovarian-cancer cell line, possibly by inhibiting the repair of drug-induced DNA damage. The low solubility of Aphi in physiological solvents has hindered the clinical development of this compound. Recently, ICI Pharmaceuticals synthesized aphidicolin-17 glycinate hydrochloride (AphiG), a water-solube analog of Aphi that retains the antitumor activity of the parent drug.

Concomitantly with a phase I study of AlphiG [15], we undertook a series of animal studies to elucidate some aspects of the pharmacokinetics and activity of AphiG so as to pursue its clinical investigation in the most rational manner possible. The main questions addressed were:

1. What is the most appropriate dose schedule for the administration of AphiG alone and in combination with DDP?
2. Can AphiG reverse the resistance of tumors to DDP?

Materials and methods

Animals. Female C57BL/6 mice weighing 20±2 g (Charles River, Calco, Italy) were used for these experiments.

Pharmacokinetics studies. Female C57BL/6 mice were given an i.v. dose of 100 mg/kg AphiG. Animals were killed at 5, 10, 30, 45, 60, and 90 min after treatment, and blood was collected separately from four animals at each of these times. All plasma samples were immediately frozen at –20° C until analysis. For the pharmacokinetics studies in which AphiG was given as a 24-h infusion, plasma was collected at 4–6 h after the beginning of the infusion and at the end of the infusion together with tumor samples.

Plasma samples were extracted as previously described by Rotondo et al. [11]. The same extraction procedure was used for tumor samples after homogenization. Briefly, samples (200 µl) were extracted with 5 ml of a hexam-isopropanol (10:1, v/v) mixture, dried under vacuum, and supplemented with aphidicolane as the external standard. Following their derivatization, the samples were assayed for Aphi using gas chromatography-mass spectrometry as described elsewhere [11]. The only modification applied was the replacement of the on-column injector with a split-splitless injector to minimize column degradation due to the deposition by the on-column injector of the entire sample and solvent onto the liquid phase of the column.

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Table 1. Plasma and tumor levels of Aphi in mice treated with continuous infusions of AphiG

AphiG	Time from the start of the infusion	Levels of Aphi	
		Plasma (μg/ml)	Tumor (μg/g)
30 mg kg^{-1} h^{-1} × 24 h	4 h	1.7 ±0.4	–
(total dose; 720 mg/kg)	24 h	2.43 ±0.2	0.915 ±0.1
60 mg kg^{-1} h^{-1} × 24 h	6 h	3.4 ±0.8	
(total dose; 1440 mg/kg)	24 h	5.5 ±1.2	2.2 ±0.6

Data represent mean values ± SD

Table 2. Effect of daily and acute administration of AphiG either alone or in combination with DDP to mice bearing M5 and M5/DDP tumors

Treatment (days)						
DDP		AphiG	M5		M5/DDP	
			T/C%	TD	T/C%	TD
–		40 mg/kg q3h × 4/day i.p. (3–10)	107	0/10	92	1/10
–		50 mg/kg q3h × 3/day i.p. (3–10)	107	0/10	108	0/10
–		40 mg/kg q3h × 4/day i.p. (3–5, 7–9)	104	0/10	91	0/10
–		50 mg/kg q3h × 3/day i.p. (3–5, 7–9)	104	0/10	91	0/10
–		100 mg/kg q3h × 3/day i.p. (3, 7, 11)	113	1/10	89	3/10
6 mg/kg i.p. (3, 7)		–	160	0/10	107	1/10
6 mg/kg i.p. (3, 7)	+	40 mg/kg q3h × 4/day i.p. (3–10)	50	6/10	115	1/10
6 mg/kg i.p. (3, 7)	+	50 mg/kg q3h × 3/day i.p. (3–10)	178	1/10	123	1/10
6 mg/kg i.p. (3, 7)	+	40 mg/kg q3h × 4/day i.p. (3–5, 7–9)	167	0/10	117	0/10
6 mg/kg i.p. (3, 7)	+	50 mg/kg q3h × 3/day i.p. (3–5, 7–9)	183	0/10	107	4/10
4 mg/kg i.p. (3, 7, 11)		–	150	1/10	112	0/10
4 mg/kg i.p. (3, 7, 11)	+	100 mg/kg q3h × 3/day i.p. (3, 7, 11)	57	7/10	129	0/10

TD, Toxic deaths; T/C%, median survival of treated mice/controls × 100

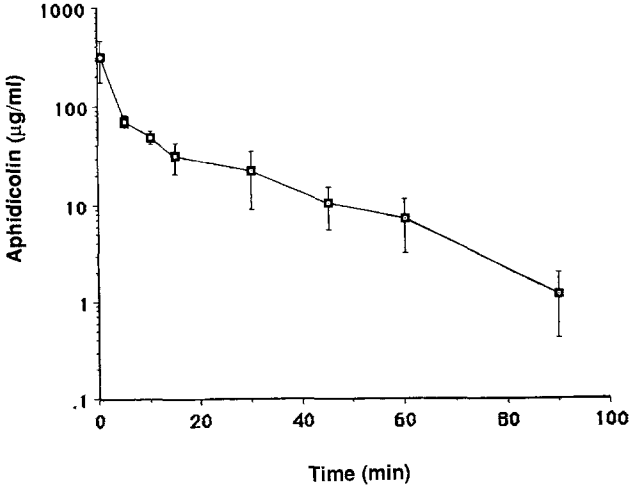

Fig. 1. Disappearance of Aphi from the plasma of mice treated i.v. with 100 mg/kg AphiG

The plasma concentrations of Aphi versus time were fitted to the standard equation for a two-compartment model [2] using a nonlinear-fitting computer program [12]. The area under the plasma concentration-time curve (AUC) was determined by the trapezoidal rule. The terminal half-life ($t_{1/2}\beta$ and plasma clearance (CR_p) were calculated using the equations $t_{1/2}\beta = 0.693/\beta$ and $CR_p = \text{dose}/AUC_{0-\infty}$, respectively.

Antitumor activity studies. For these studies, female C57BL/6 mice underwent i.m. transplantation of 5×10^5 viable cells of M5076 (M5) murine ovarian reticular cell sarcoma (Mason Research Institute, DTC Animal and Human Tumour Bank) [13] and of M5/DDP (an M5 tumor subline isolated in our laboratory that is partially resistant to DDP [1]). The tumors were maintained by i.m. passage in the same mouse strain every 3 weeks.

DDP was kindly supplied by Bristol Meyers (Wallingford, Conn., USA); AphiG was generously donated by ICI Pharmaceuticals (Alderly Park, Cheshire, UK) in vials containing 250 mg of hydrosoluble powder. DDP was dissolved in 0.9% NaCl solution and given i.p. AphiG was dissolved in sterile water and given either i.p. or as a continuous infusion using Alzet mini-osmotic pumps (Charles River, Calco, Italy) for delivery of the drug at a controlled rate. The minipumps were implanted in the s.c. space in the backs of the mice slightly posterior to the scapulae.

The antitumor activity of AphiG following its administration alone or in combination with DDP was investigated using several dosing schedules: i.p. injections given daily and continuous s.c. infusion for 24, 48, and 72 h. The tumor volume in treated and untreated mice was calculated by the formula $V = (LW^2/2)$, where L represents the average length and W, the width of the tumor, both being expressed in millimeters; the volume was converted to weight in grams, assuming unit density and spheroidal shape, whereby the short axes are of the same length (width and depth). Each group consisted of ten animals, and data were expressed as mean values ± SE. The median survival of treated mice was expressed as a percentage of the median survival of untreated mice × 100

Table 3. Effect of continuous s.c. infusions of AphiG given alone or in combination with DDP to mice bearing M5 and M5/DDP tumors

Treatment (days)						
DDP		AphiG	M5		M5/DDP	
			T/C%	TD	T/C%	TD
–		30 mg kg^{-1} h^{-1} × 24 h	125	0/10	103	0/10
–		60 mg kg^{-1} h^{-1} × 24 h	–	–	103	0/10
–		30 mg kg^{-1} h^{-1} × 48 h	–	–	107	0/10
–		7.5 mg kg^{-1} h^{-1} × 72 h	129	1/10	103	0/10
–		15 mg kg^{-1} h^{-1} × 48 h	–	–	103	0/10
–		15 mg kg^{-1} h^{-1} × 72 h	–	–	107	3/10
8 mg/kg i.p. (3)		–	137	0/10	112	0/10
8 mg/kg i.p. (3)	+	7.5 mg kg^{-1} h^{-1} × 72 h	152	0/10	113	0/10
8 mg/kg i.p. (3)	+	15 mg kg^{-1} h^{-1} × 48 h	–	–	113	2/10
8 mg/kg i.p. (3)	+	15 mg kg^{-1} h^{-1} × 72 h	–	–	33	6/10

TD, Toxic deaths; T/C%, median survival of treated mice/controls × 100

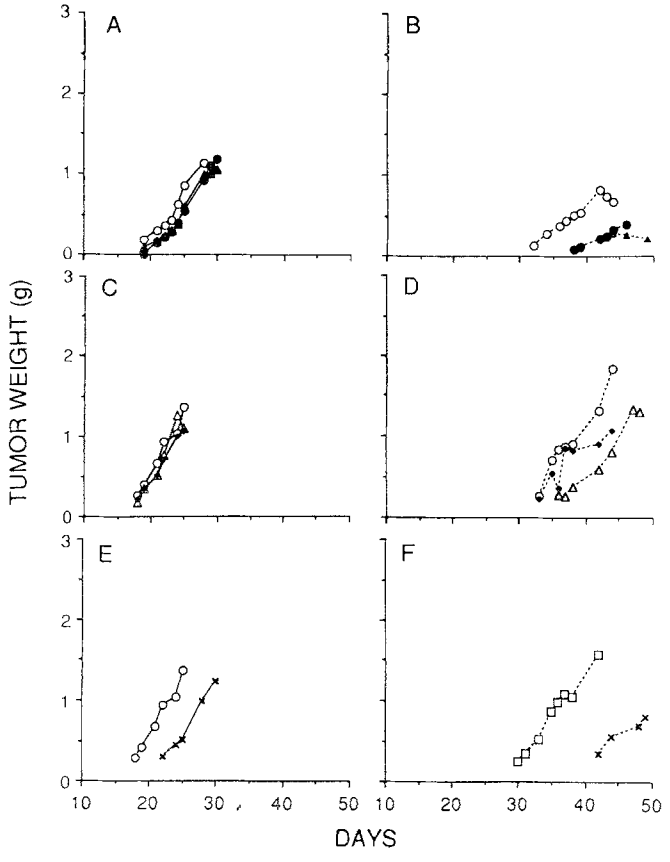

Fig. 2 A–F. Growth-inhibitory effect of AphiG given i.p. alone or in combination with i.p. DDP to mice bearing M5 tumors. *A, C, E* Treatment with AphiG alone (*solid lines*). *B, D* Treatment with AphiG in combination (*dotted lines*) with 6 mg/kg DDP (days 3 and 7) or **F** 4 mg/kg DDP (days 3, 7, and 11). ○——○, Controls (0.9% NaCl; ○- - - -○, 6 mg/kg DDP (days 3 and 7); □- - - -□, 4 mg/kg DDP (days 3, 7, and 11); ●, AphiG, 40 mg/kg q3h × 4/day (days 3–10); ▲, AphiG, 50 mg/kg q3h × 3/day (days 3–10); ◆, AphiG, 40 mg/kg q3h × 4/day (days 3–5 and 7–9); △, AphiG, 50 mg/kg q3h × 3/day (days 3–5 and 7–9); X, AphiG, 100 mg/kg q3h × 3/day (days 3, 7, and 11)

(T/C%). A drug was considered to be active if it produced a T/C value of over 125% according to the National Cancer Institute (NCI) criteria for evaluation of drugs against solid tumors [3].

Results

Pharmacokinetics studies

Figure 1 shows the disappearance of Aphi from the plasma of mice treated i.v. with AphiG at a dose of 100 mg/kg. The drug disappeared rapidly from plasma (clearance, 41.6 ml min^{-1} kg^{-1}), showing a half-life of 15 min. Further studies were done to evaluate the plasma and tumor levels of Aphi following AphiG doses of 30 or 60 mg kg^{-1} h^{-1} given as 24-h continuous infusions to mice bearing M5/DDP tumors (Table 1). The plasma steady-state levels of Aphi were correlated with the dose. The levels of Aphi detected in the tumor were approximately 2.5 times lower than those found in plasma at both of the drug doses tested.

Antitumor activity studies

Figures 2 and 3 show the growth-inhibitory activity of several schedules of AphiG given i.p. alone or in combination with DDP to mice bearing M5 and M5/DDP tumors, respectively. In both tumors, AphiG given alone showed marginal growth-inhibitory activity (Figs. 2E, 3E) but failed to improve the median survival of mice (Table 2). Its combination with DDP produced a moderate effect in delaying the appearance of both tumors, which then grew at the same rate as the control lesions (Figs. 2 and 3, panels B, D, F), but AphiG again failed to enhance the antitumor activity of DDP (Table 2). The toxicity of AphiG alone was very low except when the drug was given at 100 mg/kg × 3/day (days 3, 7, and 11), which caused 4 toxic deaths among the 20 animals treated. When AphiG was given in combination with DDP, an increase in toxicity was observed (20 vs 5 toxic deaths).

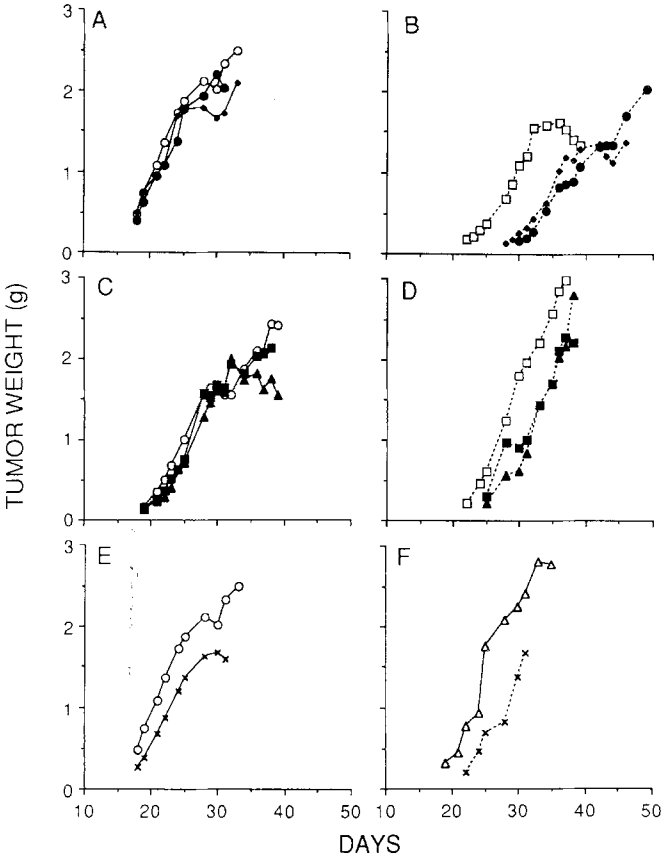

Fig. 3 A–F. Growth-inhibitory effect of AphiG given i.p. alone or in combination with i.p. DDP to mice bearing M5/DDP (DDP-resistant subline) tumor. **A, C, E** Treatment with AphiG alone (*solid lines*). **B, D** Treatment with AphiG in combination (*dotted lines*) with 6 mg/kg DDP (days 3 and 7) or **F** with 4 mg/kg DDP (days 3, 7, and 11). ○——○, Controls (0.9% NaCl); □- - - -□, 6 mg/kg DDP (days 3 and 7); △——△, 4 mg/kg DDP (days 3, 7, and 11); ◆, AphiG, 40 mg/kg q3h × 4/day (days 3–10); ●, AphiG, 50 mg/kg q3h × 3/day (days 3–10); ▲, AphiG, 40 mg/kg q3h × 4/day (days 3–5 and 7–9); ■, AphiG, 50 mg/kg q3h × 3/day (days 3–5 and 7–9); X, AphiG, 100 mg/kg q3h × 3/day (days 3, 7, and 11)

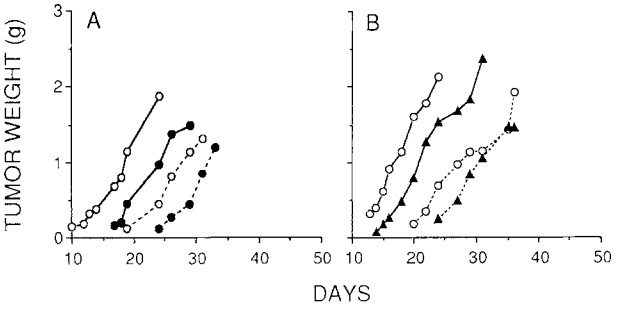

Fig. 4 A, B. Growth-inhibitory effect of continuous s.c. infusion of AphiG given alone (*solid lines*) or in combination (*dotted lines*) with DDP (8 mg/kg i.p., day 3) at 4 h after the implantation of mini-osmotic pumps in mice bearing M5 tumor. ○——○, Controls (0.9% NaCl); ○- - - -○, 8 mg/kg i.p. DDP (day 3); ▲, AphiG, 30 mg kg^{-1} h^{-1} × 24 h; ●, AphiG, 7.5 mg kg^{-1} h^{-1} × 72 h

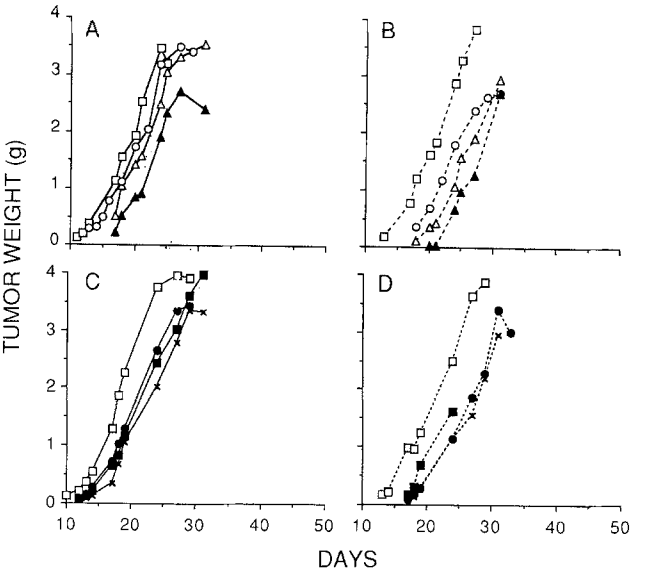

Fig. 5 A–D. Growth-inhibitory effect of continuous s.c. infusion of AphiG given **A, C** alone (*solid lines*) or **B, D** in combination (*dotted lines*) with 8 mg/kg i.p. DDP (day 3) at 4 h after the implantation of mini-osmotic pumps in mice bearing M5/DDP (DDP-resistant subline) tumor. □——□, Controls (0.9% NaCl); □- - - -□, 8 mg/kg DDP (day 3); ○, AphiG, 30 mg kg^{-1} h^{-1} × 24 h; △, AphiG, 60 mg kg^{-1} h^{-1} × 24 h; ▲, AphiG, 30 mg kg^{-1} h^{-1} × 48 h; ●, AphiG, 7.5 mg kg^{-1} h^{-1} × 72 h; ■, AphiG, 15 mg kg^{-1} h^{-1} × 48 h; X, AphiG, 15 mg kg^{-1} h^{-1} × 72 h

Figures 4 and 5 show the growth-inhibitory activity of several schedules of AphiG given s.c., by continuous infusion either as a single agent or in combination with DDP to mice bearing M5 and M5/DDP tumors, respectively. The inhibitory effects of AphiG on tumor growth following prolonged infusions were also modest. In addition, the activity of DDP against M5 or M5/DDP tumors was manifested only in terms of a delay in tumor appearance, and the shift toward the right of the tumor-growth curve was moderate. The lack of antitumor activity observed for the combination of DDP and AphiG is summarized in Table 3 in terms of survival duration (no increase in T/C values was observed). The toxicity of AphiG was again markedly increased by the DDP treatment (4/80 vs 8/40 toxic deaths; Table 3).

Discussion

The present study showed that AphiG had some inhibitory effect on the growth of both M5 and M5/DDP murine tumors. However, this effect was moderate and did not result in a significant increase in the survival of the animals.

The pharmacokinetics studies demonstrated that Aphi was cleared very rapidly from mouse plasma. Considering that Aphi is a reversible inhibitor of DNA-polymerase α and δ, it seems logical to assume that its continuous infusion would enable the attainment of stronger pharmacologic effects. However, in both M5 and M5/DDP tumors, even 72-h infusions resulted in only marginal antitumor activity. In the DDP-resistant A2780cp cell line, Aphi per se caused

no cytotoxicity but significantly enhanced the cells' sensitivity to DDP [9]. By analogy, we hypothesized that the drug might also potentiate the effect of DDP in the M5/DDP cell line, which showed low sensitivity to Aphi. None of the experiments testing several dose schedules of AphiG demonstrated that Aphi was capable of counteracting the mechanisms of resistance to DDP in the M5/DDP tumor, although an additive effect for Aphi and DDP was detected in both M5 and M5/DDP tumors.

The discrepancy between the results obtained by Masuda et al. [9] in the A2780cp cell line and those obtained by us in M5/DDP tumor may be attributable to many factors. One possibility would be that the mechanisms of resistance in the A2780cp cell line differ from those in the M5/DDP tumor. A2780cp cells have been reported to be resistant to DDP because they can repair DDP-induced DNA damage more efficiently than the parent DDP-sensitive cell line [9], and Aphi counteracted the resistance of these cells to DDP by inhibiting DNA-repair mechanisms. Our in vivo system is less characterized by this phenomenon. We can exclude the possibility that the resistance of M5/DDP tumor to DDP might be attributable to pharmacokinetic causes (unpublished data obtained in our laboratory), as has recently been found in other resistant murine tumors [14]. The possibility that the resistance of M5/DDP might be due to intracellular levels of reduced glutathione that are higher than those in the sensitive line can also be excluded (data not shown). We know that the G2 block caused by DDP is more rapidly reversed in the M5/DDP than in M5 tumors, suggesting a more rapid repair of the DNA damage induced by DDP, although we do not have direct evidence for this. We therefore believe that the resistance of M5/DDP to DDP is attributable to more efficient repair of drug-induced DNA lesions [1].

There may be other reasons for the contrast between the results obtained by Masuda et al. [9] in the A2780cp cell line and those obtained by us in M5/DDP tumor. The Aphi tumor concentrations achieved in the present study may not have been sufficiently high or long-lasting to be effective. Very limited information is available on the Aphi concentrations required to inhibit the repair of DDP-induced DNA damage. Masuda et al. [9] found a dose-dependent capacity of Aphi to inhibit DNA repair of DDP-induced DNA damage in A2780cp cells (but not in the DDP-sensitive parental cell line), which became evident at concentrations as low as 1 μg/ml, with maximal inhibition being observed following incubation of cells with Aphi at 4 μg/ml for 6 h after DDP treatment. In addition, 24 h treatment of cells with Aphi at 2 μg/ml resulted in a 3.5-fold increase in the cytotoxicity of DDP in the A2780cp cell line. We achieved Aphi concentrations of approximately 2 μg/g tumor tissue after 24 h treatment of mice with 60 mg kg^{-1} h^{-1} AphiG but observed no apparently remarkable effect.

Although the concentrations of Aphi detected in the tumors and plasma of mice in the present study were on the same order of magnitude of those found to be active in vitro, it may be that these extrapolations were incorrect due to differences in the availability of drug to cells growing in monolayers as compared with cells organized in a tissue. In addition, the concentrations of Aphi that are effective against A2780cp cells and those that are effective against M5/DDP cells may not necessarily be the same. On the other hand, the doses used by us could not be further increased due to the occurrence of toxic deaths. In fact, although we did not investigate the mechanisms of toxicity in detail, it was clear that the combination of DDP and Aphi was much more toxic than was either drug used alone.

Another possible reason for the disparate results could be that A2780cp cells were treated while they were confluent and after their exposure to inhibitors of DNA synthesis. In fact, the assay carried out by Masuda et al. [9] to evaluate the DNA repair had to be performed in cells that were incapable of replicative DNA synthesis. Moreover, the cytotoxicity of DDP in the presence or absence of Aphi was investigated in quiescent cells. Only after treatment were the A2780cp cells seeded at low concentrations for evaluation of their clonogenic capacity. Of course, the effects of DDP on quiescent cells cannot be compared with those occurring in growing tumors. If the data obtained in quiescent A2780cp cells cannot be reproduced in growing cells undergoing DNA synthesis, they could be irrelevant to the attempt to counteract the resistance of human tumors to DDP by their treatment with a combination of Aphi and DDP.

In conclusion, although our negative data cannot necessarily be generalized to apply to human tumors, the positive in vitro data thus far obtained in a questionable experimental system do not provide a rationale for the clinical development of Aphi. More in vitro and in vivo data on the activity of Aphi alone or in combination with DDP are needed before any further clinical investigation is pursued.

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