PERGAMON

European Journal of Cancer 36 (2000) 2411–2419

European Journal of Cancer

www.ejconline.com

Therapeutic effects of the combination of fenretinide and all-*trans*-retinoic acid and of the two retinoids with cisplatin in a human ovarian carcinoma xenograft and in a cisplatin-resistant sub-line

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Received 13 April 2000; received in revised form 22 June 2000; accepted 23 June 2000

Abstract

We previously showed that fenretinide (4-HPR), a synthetic derivative of all-*trans* retinoic acid (RA), is effective in mice bearing the human ovarian carcinoma IGROV-1 and it significantly enhances the antitumour activity of cisplatin on the same tumour. The present study examined the therapeutic effects of the combination of 4-HPR and RA and of the two retinoids with cisplatin as intracavitary treatments of mice bearing IGROV-1 and IGROV-1/cisplatin tumours, the latter derived from a sub-line with an *in vivo* reduced sensitivity to cisplatin. 4-HPR, as a single agent, was effective against both tumours, whereas RA had no effect. In IGROV-1 tumour-bearing mice, the combination of RA and 4-HPR significantly improved the efficacy of 4-HPR, resulting in an antitumour activity similar to that obtained with cisplatin alone. N-(4-methoxyphenylretinamide), the main metabolite of 4-HPR, had no antitumour effect and it did not increase 4-HPR activity in IGROV-1 tumour-bearing mice. In the same tumour model, 4-HPR and RA separately increased cisplatin activity, even though for RA the increase was not statistically significant. In contrast, the association of the two retinoids together with cisplatin did not produce any benefit and resulted in increased toxicity. In IGROV-1/cisplatin tumour-bearing mice, the association of 4-HPR (but not of RA) to cisplatin significantly increased cisplatin activity, resulting in the reversal of cisplatin resistance. These findings demonstrate that 4-HPR may be effective and enhance cisplatin sensitivity in cisplatin-sensitive and -resistant ovarian tumours and that the association of RA and 4-HPR may result in increased 4-HPR antitumour activity.

Keywords: Fenretinide; Cisplatin resistance; Ovarian carcinoma; Retinoids; Combination therapy

1. Introduction

Vitamin A (retinol) and its derivatives are essential for the normal processes of development, growth, vision, reproduction, cell differentiation and immune system integrity. Retinoids are the natural and synthetic derivatives of vitamin A. They are compounds of clinical interest for the treatment and prevention of a variety of neoplasms.

Fenretinide or N-(4-hydroxyphenyl)retinamide (4-HPR) is a synthetic amide of all-*trans* retinoic acid (RA) which has shown reduced toxicity relative to RA, while maintaining a significant biological activity. In animal models, 4-HPR has shown chemopreventive efficacy against mammary gland, urinary bladder, seminal vesicle and prostate carcinogenesis and therapeutic efficacy against mammary gland, ovary and prostate tumours [1]. *In vitro* studies have demonstrated that 4-HPR has significant antiproliferative activity associated with the induction of apoptosis in several tumour cell types including breast, prostate, leukaemia, head and neck, neuroblastoma and ovary [1]. The mechanism of action of 4-HPR has not yet been clarified. Some studies suggest that the effects of the retinoid are mediated through RA receptors (RAR) and retinoid X receptors (RXR) signalling [2–5]. Other results suggest that 4-HPR can act as a pro-oxidant and induce apoptosis by eliciting

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0959-8049/00/$ - see front matter
PII: S0959-8049(00)00335-X

oxidative stress [6,7]. 4-HPR is currently under clinical investigation in several cancer types as a therapeutic and preventive agent. In humans it has already shown efficacy in oral leucoplakia [8], lichen planus [9] and actinic keratoses [10]. Recently, reductions in the incidence of contralateral breast cancer and ipsilateral breast cancer reappearance have been reported in premenopausal women treated with 4-HPR after having undergone surgery for early breast cancer [11]. Results of the same trial suggested a chemopreventive effect of 4-HPR in ovarian cancer [11,12]: a significant reduction in the incidence of ovarian cancer was observed in 4-HPR-treated patients compared with controls during the treatment period.

Ovarian cancer is a major cause of death in women due to the disease being in an advanced stage at diagnosis in two-thirds of cases. Despite recent advances in the treatment, survival of patients with advanced stages remains poor. Cisplatin is one of the effective cytotoxic drugs used in the therapy of ovarian cancer, but its efficacy is clinically limited because some tumours are initially unresponsive to cisplatin or become so during treatment. For this reason, agents to be used as alternative therapeutic approaches or that can interfere with the still controversial mechanisms of cisplatin resistance are needed.

The first evidence of an *in vivo* therapeutic effect of a retinoid against ovarian tumours came from our study on the efficacy of 4-HPR against the human ovarian carcinoma IGROV-1 [13]. We showed that intracavitary 4-HPR treatment of mice bearing intraperitoneal (i.p.) IGROV-1 tumours caused a significant increase in survival time and also enhanced the antitumour activity of cisplatin against the same tumour. On the basis of such experimental findings and of the promising preliminary clinical findings of 4-HPR protection from a second ovarian tumour [11,12], we further investigated the possible role of 4-HPR in ovarian cancer treatment. Since in clinical practice polychemotherapy with cytotoxic agents has proven superior to monotherapy, the modality might also be used for other classes of antiproliferating drugs like retinoids. Moreover, the combination of antitumour agents with different mechanisms of action may result in improved efficacy. We investigated the potential of the combination of 4-HPR with another retinoid, and we analysed the modulation of cisplatin sensitivity by the two retinoids in one cisplatin-sensitive and one cisplatin-resistant ovarian tumour. RA was chosen as the retinoid to be tested in association with 4-HPR because in human acute promyelocytic cells this association has been shown to result in enhanced differentiation [14,15]. Moreover, since mice metabolise 4-HPR to N-(4-methoxyphenyl)retinamide (4-MPR) to a great extent [16], we tested whether the antitumour activity of 4-HPR was due to its activation to 4-MPR or if it was increased by its association with this retinoid.

2. Materials and methods

2.1. Animals and tumours

Female CD-1 nu/nu mice (7–9 weeks old) were supplied by Charles River (Calco, Italy) and kept under standard laboratory conditions according to the guidelines of our Institute. They were kept in laminar air-flow rooms in sterilised cages, with bedding, food and acidified water. The IGROV-1 tumour, a human epithelial ovarian adenocarcinoma line (kindly supplied by Dr Benard, Villejuif, France), was grown as ascites and maintained *in vivo* as i.p. transplants. The cisplatin-resistant IGROV-1 ovarian tumour was established *in vivo* starting from the *in vitro* cell line IGROV-1/Pt 1, a generous gift from Dr Perego [17]. Cultured cells were injected i.p. in mice, and an *in vivo* cell line, named IGROV-1/cisplatin, was established by subsequent mouse-to-mouse transplant of the ascitic-growing tumours. IGROV-1 and IGROV-1/cisplatin were maintained *in vivo* for no more than 10 consecutive passages. For experiments, mice were injected i.p. with 2.5×10^6 IGROV-1 or 18×10^6 IGROV-1/cisplatin tumour cells in 0.2 or 0.5 ml 0.9% (w/v) NaCl solution, respectively. From preliminary experiments, these numbers of cells were the lowest ones to cause 100% takes (i.e. they correspond to the respective oncogenic doses of 100%); injected mice died with similar median survival times (18 and 19.5 days after IGROV-1 and IGROV-1/cisplatin cell injection, respectively). Six animals per group were used in each experiment, which was performed at least twice. Animal studies were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori (Milan, Italy) and were carried out in accordance with the guidelines of the UK. Coordinating Committee on Cancer Research [18].

2.2. Drugs and treatments

4-HPR, 4-MPR, kindly provided by the R.W. Johnson Pharmaceutical Research Institute (Spring House, PA, USA), and RA (Sigma, Milan, Italy) were dissolved in absolute ethanol and then diluted in a sterile 0.9% (w/v) NaCl solution containing 1.65 mg/ml bovine serum albumin (Sigma) at a final ethanol concentration of 5% (v/v). Retinoids were freshly prepared once a week, protected from light and kept at 4°C. Cisplatin (Platamine, Pharmacia Upjohn, Milan, Italy) was dissolved in sterile distilled water, diluted in a 0.9% NaCl solution, protected from light and administered immediately after preparation. Drug treatment started one day after tumour cell injection. Retinoids were administered i.p., five days per week, for three weeks. When 4-HPR was combined with RA or 4-MPR, RA or 4-MPR were given first and 4-HPR immediately after. Cisplatin was administered i.p. once a week for three weeks.

When cisplatin was associated with the retinoids, it was administered 3 h before them according to the schedule previously found to be effective for the association with 4-HPR [13]. All drugs were delivered at a volume of 10 ml/kg of body weight. Controls were treated with vehicles in the same volumes and sequences of the combination experiments. The survival times of control and treated mice were registered and the respective median survival times (MST) calculated. For each drug treatment, the percentage increase in life span (%ILS) was calculated from the formula $\%ILS = (T-C)\times 100/C$, where T = MST of treated mice and C = MST of control mice. Tumour-free mice surviving longer than 100 days after tumour injection were considered long-term survivors (LTS). Toxicity was assessed as lethal toxicity and as body weight loss (BWL). Lethal toxicity was defined as any death which occurred earlier than that of the controls and without tumour. BWL was evaluated as the percentage difference in mice mean body weight after the last treatment with respect to the beginning of the treatment. Per cent BWL = 100−(BW at the end of treatment/BW on day 1)×100.

2.3. In vitro *assays for evaluation of IGROV-1/cisplatin sensitivity to cisplatin*

For the experiments on IGROV-1/cisplatin tumour-bearing mice, parallel *in vitro* assays were performed on the same tumours in order to check the stability of cisplatin resistance. Ascitic-growing IGROV-1/cisplatin tumour cells were collected from the peritoneum and after washing they were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum, in 5% CO_2 at 37°C. The sensitivity of the *in vitro* grown cells to cisplatin was tested at the second transplant generation and compared with that of IGROV-1, the cisplatin-sensitive cell line and IGROV-1/Pt1, the cisplatin-resistant cell line from which IGROV-1/cisplatin tumours were derived. Twenty-four hours after seeding, tumour cells were treated with various concentrations of cisplatin for 1 h. Drug was then removed and three days later, cell number was determined by trypsinised cell count using a ZBI electronic particle Counter (Coulter Luton, UK). Data are means of three replicates.

2.4. Statistical analysis

The homogenicity of the results obtained in each experiment was checked by two-way analysis of variance. Afterwards, data were compared according to the two-tailed Student's *t*-test and the logrank test for the means of survival times and survival curves, respectively.

3. Results

3.1. Effect of the combination of 4-HPR and RA on IGROV-1 ovarian tumour

The antitumour activity of 4-HPR, RA and their association was tested against the ascitic-growing IGROV-1 ovarian tumour by administering the drugs i.p. 4-HPR and RA were delivered (five times/week for three weeks) at daily doses of 120 mg/kg and 10 mg/kg, respectively. The dose of 4-HPR was chosen on the basis of its previously demonstrated efficacy in the tumour [13]. This dose is equivalent, on a mg/m^2 basis, to a 600 mg/day dose in the clinic, i.e. it is three times higher than the dose tested in the breast tumour prevention trial [11]. The RA dose was the maximum tolerated dose as evaluated in pilot experiments in healthy animals. The same doses were also used when the two drugs were administered in combination. The results of three experiments with homogeneous data are shown in Fig. 1 and Table 1. 4-HPR caused a slight (43%), but statistically significant increase in survival time compared with controls ($P < 0.001$). RA was ineffective. In spite of the lack of activity, when RA was associated with 4-HPR, it produced an increase in survival time (112%) that was statistically significant compared with that of 4-HPR alone ($P < 0.05$). The increase in lifespan (ILS) obtained with the combination of 4-HPR with RA was similar to that obtained with the effective drug cisplatin (111%, see Table 2 below).

The combination of the two retinoids did not result in severe toxicity. Treated mice did not show any sign of apparent toxicity. No deaths occurred, and body weight loss, which was lower than 10%, was similar to that caused by RA alone.

3.2. Effect of the combination of 4-HPR with 4-MPR on IGROV-1 ovarian tumour

We also tested whether the antitumour activity of 4-HPR was due to its metabolic activation to 4-MPR or was increased by its association with the retinoid. 4-HPR is most likely the active form of the drug, since its major metabolite 4-MPR, at doses similar (120 mg/kg) or higher (180 mg/kg) than those used for 4-HPR, was inactive in the tumour model (data not shown). Moreover, 4-MPR did not enhance 4-HPR activity (data not shown).

3.3. Effect of 4-HPR and RA administered with cisplatin on IGROV-1 ovarian tumour

We previously showed [13] that 4-HPR significantly increases the activity of cisplatin in the IGROV-1 tumour model. In the present study, we investigated the effect of 4-HPR and RA when associated with cisplatin

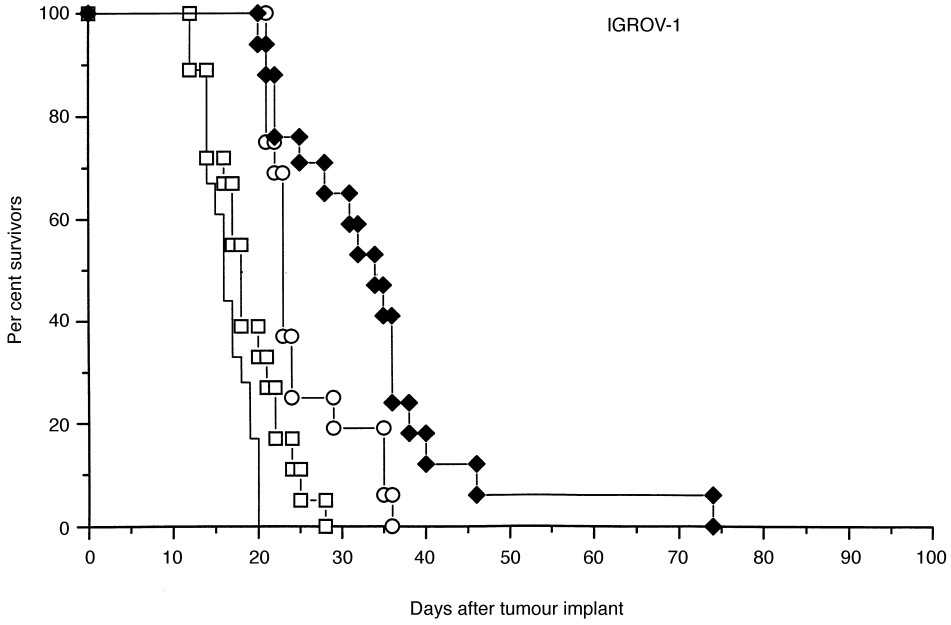

Fig. 1. Activity of fenretinide (4-HPR) alone and combined with all-*trans* retinoic acid (RA) on intraperitoneal (i.p.) IGROV-1 ovarian tumours. Nude mice were injected i.p. with 2.5×10^6 IGROV-1 tumour cells. 4-HPR (○) and RA (□) were administered, starting on day 1, five days/week for three weeks at doses of 120 and 10 mg/kg of body weight, respectively. In the combination of 4-HPR with RA (◆), the retinoids were given subsequently at the above doses, starting with RA. Controls (—) were treated with vehicles.

as single agents or in combination. Cisplatin was administered at a dose and with a schedule previously shown to be effective in the experimental system, i.e. i.p. at 6 mg/kg, once a week for three weeks. 4-HPR and RA were administered as described above. Results are reported in Fig. 2 and Table 2. All the treatments were effective and caused a statistically significant increase in the survival time of treated mice compared with controls ($P<0.001$, logrank test). Cisplatin alone determined a 111% ILS and 1/17 long-term survivors. When 4-HPR was associated with cisplatin, the result was a statistically significant increase in the activity of cisplatin alone ($P<0.05$, logrank test), with a 178% ILS and 3/17 long-term survivors. As observed when associated with 4-HPR, when RA, which was not active as a single agent (Fig. 1 and Table 1), was administered with cisplatin, this resulted in a considerable, even though not statistically significant ($P=0.118$, logrank test), increase in cisplatin activity (172% ILS for cisplatin associated with RA versus 111% ILS for cisplatin alone). The association of cisplatin with the two retinoids together did not produce any benefit compared with cisplatin alone ($P=0.64$, logrank test). The lack of a beneficial effect was probably due to the toxicity of the combination of the three drugs. The treatment regimen resulted in a body weight loss greater than that caused by the other treatments, and 1/18 mice died without tumour before the controls.

Table 1
Activity of 4-HPR and RA alone and in combination on intraperitoneal (i.p.) IGROV-1 ovarian tumour

					Toxicity	
Treatment	Dose (mg/kg)	*n*	MST	% ILS	% BWL	No. toxic deaths/total *n* of mice
Vehicles	–	18	16 (12–20)	–	–	–
4-HPR	120	16	23 (21–36)[a]	43	0	0/16
RA	10	18	17.5 (12–28)	9	−8	0/18
4-HPR + RA	120 + 10	17	34 (20–74)[a,b]	112	−8	0/17

4-PR, fenretinide; RA, all-*trans* retinoic acid; MST, median survival time in days, range in parenthesis; %ILS, percentage increase in life span = (T−C)×100/C, where T = MST of treated mice and C = MST of controls; % BWL, percentage mean body weight loss = 100−(BW at the end of treatment/BW on day 1)×100.

[a] $P<0.001$ Student's *t*-test versus controls.

[b] $P<0.05$ Student's *t*-test versus 4-HPR.

Table 2
Activity of cisplatin alone and combined with fenretinide (4-HPR) and retinoic acid (RA) on intraperitoneal (i.p.) IGROV-1 ovarian tumours

Treatment	Dose (mg/kg)	MST	% ILS	LTS	Toxicity	
					% BWL	No. toxic deaths/total *n* of mice
Vehicles	–	18 (14–25)	–	0/18	–	–
Cisplatin	6	38 (21–84)	111	1/17[a]	−4	0/17
Cisplatin + 4-HPR	6 + 120	50 (38–99)	178	3/17[a,b]	−6	0/17
Cisplatin + RA	6 + 10	49 (30–95)	172	1/17[a]	−9	0/17
Cisplatin + RA + 4-HPR	6 + 10 + 120	40 (20–90)	122	2/17[a]	−16	1/18

MST, median survival time in days, range in parenthesis; % ILS, percentage increase in life span = (T−C)×100/C, where T = MST of treated mice and C = MST of controls; LTS, long-term survivors at day 100, tumour free; % BWL, percentage mean body weight loss = 100−(BW at the end of treatment/BW on day 1)×100.

[a] $P < 0.01$ Logrank test versus controls.

[b] $P < 0.05$ Logrank test versus cisplatin.

3.4. Effect of 4-HPR, cisplatin and their combination on IGROV-1/cisplatin ovarian tumour

To further investigate the effect of the retinoids combined with the cytotoxic drug cisplatin, we tested whether the combined treatment could circumvent cisplatin resistance. Starting from an *in vitro* cell line made resistant to cisplatin (IGROV-1/Pt1) [17], we established an *in vivo* tumour line (IGROV-1/cisplatin) by subsequent mouse-to-mouse transplant of the ascitic-growing tumour.

IGROV-1/cisplatin tumour cells, when re-established in tissue culture and tested in an *in vitro* cytotoxicity assay, showed the same resistance index (RI) (ratio between the IC_{50}s of the resistant and sensitive cells, RI = 12) (Fig. 3) as the *in vitro* grown line IGROV-1/Pt1 [17].

In spite of an *in vitro* RI of 12, cisplatin treatment caused, *in vivo*, a significant increase in survival time (Fig. 4, Table 3). However, the antitumour activity of cisplatin in the resistant line IGROV-1/cisplatin was significantly lower ($P \leqslant 0.01$ logrank test) than that in the sensitive line IGROV-1. This was evaluated by comparison of the survival curves of cisplatin-treated mice bearing the sensitive (Fig. 2) and the resistant (Fig. 4) line, after assessment of no difference in the survival curves of the respective untreated groups.

When 4-HPR was tested against the IGROV-1/cisplatin tumours (Fig. 4, Table 3), it was as effective as cisplatin (74% ILS). The combination of 4-HPR with

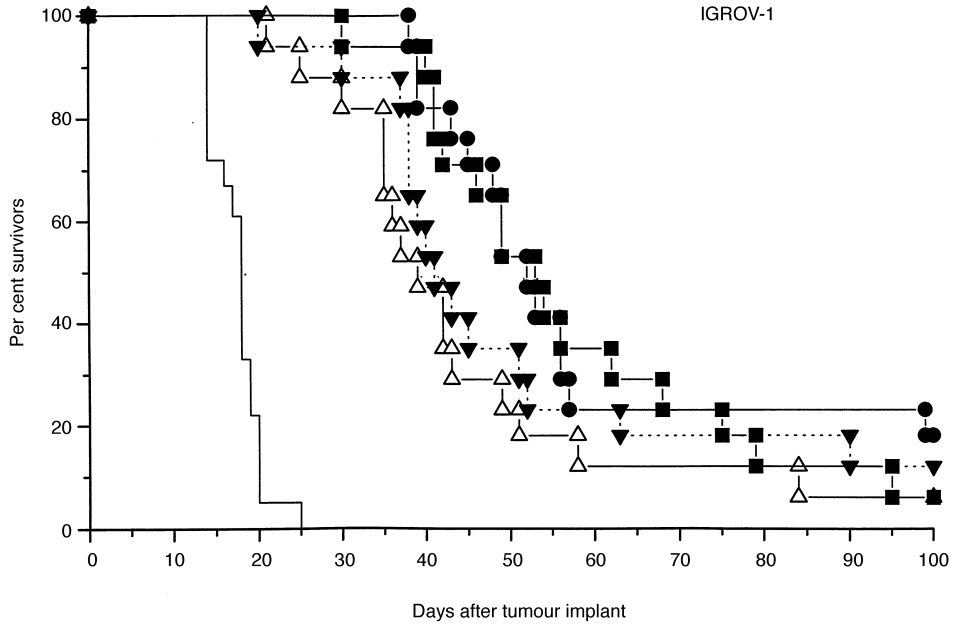

Fig. 2. Activity of cisplatin alone and combined with fenretinide (4-HPR) and retinoic acid (RA) on intraperitoneal (i.p.) IGROV-1 ovarian tumour. Nude mice were treated i.p. starting one day after i.p. injection of 2.5×10^6 IGROV-1 tumour cells. 4-HPR and RA were given five days/week for three weeks at doses of 120 and 10 mg/kg, respectively. Cisplatin was administered on a weekly schedule for a total of three injections at the dose of 6 mg/kg of body weight, alone (Δ) or 3 h before 4-HPR (●) or RA (■), or RA + 4-HPR (▼). Controls (—) were treated with vehicles.

cisplatin produced an increase in survival time (120%), which was statistically significant compared with that obtained with the two drugs as single agents. Moreover, the antitumour activity was similar to that obtained for cisplatin in the sensitive line: no statistically significant difference was found between the survival curve of IGROV-1 tumour-bearing mice treated with cisplatin and that of IGROV-1/cisplatin tumour-bearing mice

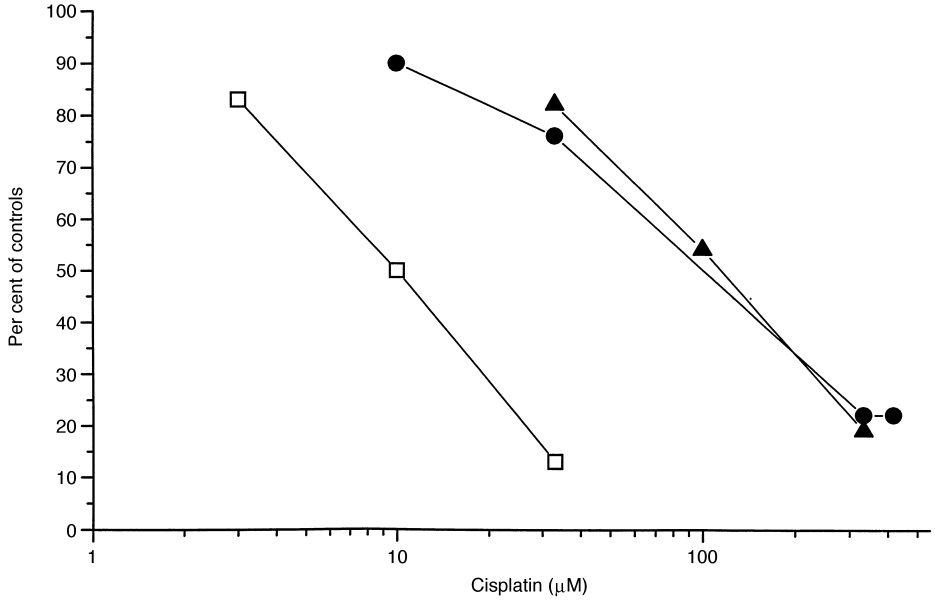

Fig. 3. *In vitro* sensitivity to cisplatin of IGROV-1/cisplatin ascitic grown cells, after re-establishment in tissue culture; IGROV-1 and IGROV-1/Pt1 cells were also tested for comparison. Twenty-four hours after seeding, cells were treated for 1 h with cisplatin and the surviving cell number was evaluated three days later. Data are expressed as a percentage of control untreated cells. Results are the means of three replications of one representative experiment performed at the tenth *in vivo* transplant generation of IGROV-1/cisplatin. The standard deviation (S.D.) never exceeded 15%. IGROV-1 (□); IGROV-1/Pt1 (▲); IGROV-1/cisplatin (●).

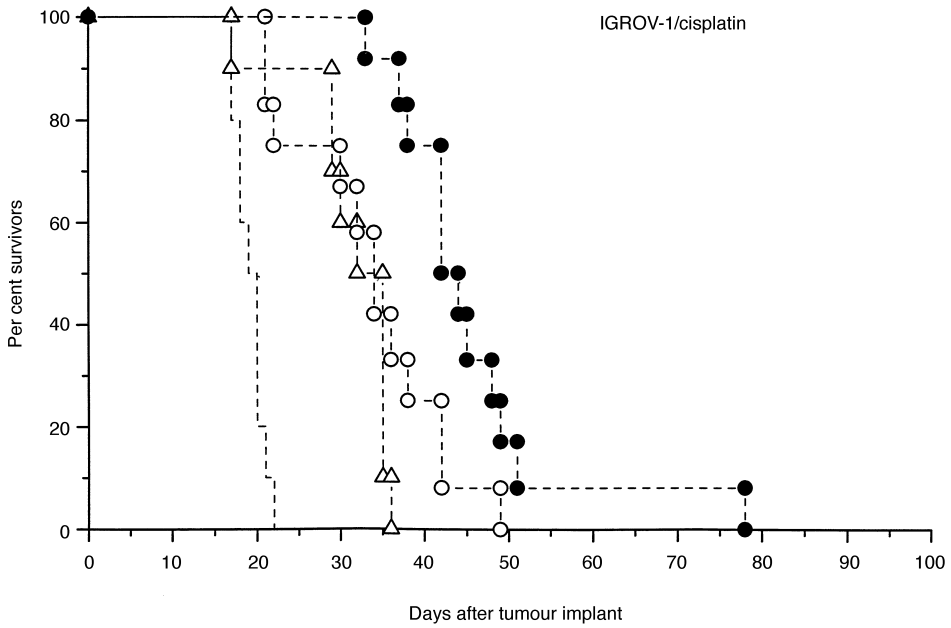

Fig. 4. Circumvention of drug resistance by the administration of fenretinide (4-HPR) with cisplatin in IGROV-1/cisplatin ovarian tumour. Nude mice were treated starting one day after intraperitoneal (i.p.) injection of 18×10^6 IGROV-1/cisplatin tumour cells. 4-HPR was administered by i.p. route five days/week for three weeks at a dose of 120 mg/kg of body weight (○). Cisplatin was given i.p. once a week for three weeks at the dose of 6 mg/kg of body weight alone (Δ) or in combination with 4-HPR (●). When the two drugs were associated, cisplatin was administered 3 h before 4-HPR. Control mice (— — –) were treated with vehicles.

treated with cisplatin and 4-HPR. No apparent increase in cisplatin toxicity was caused by its association with 4-HPR (Table 3).

The effect of RA alone, or added to cisplatin, on IGROV-1/cisplatin tumours was also tested. RA did not show any activity when administered alone, nor did it modify cisplatin activity (data not shown).

4. Discussion

We had previously shown that 4-HPR is effective *in vivo* against a human ovarian carcinoma and that it enhances the activity of cisplatin against the same tumour [13]. The present study demonstrated that: (a) the antitumour activity of 4-HPR and cisplatin against an ovarian tumour xenograft is enhanced by their association with RA; (b) 4-HPR is also active against a cisplatin-resistant ovarian tumour sub-line; and (c) 4-HPR, but not RA, maintained the ability to enhance cisplatin activity even in a cisplatin-resistant ovarian tumour sub-line with *in vivo* reduced cisplatin sensitivity.

The enhanced antitumour activity of 4-HPR when associated with RA was not due to the addition of 4-HPR growth-inhibitory effects to those of RA. In fact, RA administered alone was ineffective in increasing the survival time of IGROV-1 tumour-bearing mice. The antitumour activity of the combination of RA plus 4-HPR thus seems to be attributable only to 4-HPR and the observed potentiation, to the interaction of the two retinoids. The effects of the association of 4-HPR with RA have been investigated *in vitro* in other tumour cells. Discordant results, perhaps due to the different retinoid sensitivities of the treated cells, have been reported. In cervical carcinoma cells [7] and in malignant T-lymphoid cells [19], which are not sensitive to RA, RA did not affect the antiproliferative activity of 4-HPR. Conversely, in HL60 and in NB4 cells in which RA was found to be an effective inducer of differentiation, there was a joint effect between RA and 4-HPR [14,15]. In these tumour cells, 4-HPR was a poor inducer of differentiation, and in this case, 4-HPR therefore potentiated RA-induced differentiation. The authors showed that inhibition of RA catabolism by 4-HPR might be responsible for such potentiation. In IGROV-1 tumours growing *in vivo*, just the opposite results were apparently obtained. RA, which was ineffective, potentiated the antitumour activity of 4-HPR. However, we cannot exclude that even in IGROV-1 cells, 4-HPR increased the effectiveness of RA, perhaps by decreasing RA *in vivo* catabolism. Moreover, it has been shown that both RA and 4-HPR can induce in sensitive cells the metabolism of retinol to 4-oxo-retinol, which has growth-inhibitory effects in cells sensitive and resistant to RA and to 4-HPR [20]. The positive interaction between the two retinoids observed *in vivo* might be related to the ability of 4-HPR and/or RA to induce the production of 4-oxoretinol or other biologically active compounds.

Mice metabolise 4-HPR to 4-MPR, and they show a large amount of 4-MPR in their organs [16]. 4-MPR, which, in humans, reaches plasma concentrations comparable with those of 4-HPR [21], had no activity in IGROV-1 tumour-bearing mice, thus indicating that 4-HPR antitumour activity is not due to its metabolism to 4-MPR. Moreover, 4-MPR, at variance with RA, did not increase 4-HPR activity.

RA, in spite of its lack of activity as a single agent, increased not only the antitumour activity of 4-HPR, but also that of cisplatin. Several papers have reported the *in vitro* effects of the association of RA with cisplatin. In more than one study [22–24], potentiation of cisplatin cytotoxicity was found in RA-sensitive cells, but no effect [23,24] or even the opposite effect [22] was seen in RA-resistant cells. In one of the studies [24], the IGROV-1 cell line, the same one investigated here *in vivo*, was RA-resistant and RA did not potentiate cisplatin cytotoxicity in the cells. We have no explanation for the discrepancy between the *in vitro* and our *in vivo* results. We are currently investigating *in vitro* all the associations reported herein *in vivo* in order to understand whether the observed interactions take place at

Table 3
Circumvention of drug resistance by the administration of fenretinide (4-HPR) with cisplatin on intraperitoneal (i.p.) IGROV-1/cisplatin ovarian tumour

					Toxicity	
Treatment	Dose(mg/kg)	*n*	MST	% ILS	% BWL	No. toxic deaths/total *n* of mice
Vehicles	–	10	19.5 (17–21)	–	–	–
Cisplatin	6	10	33.5 (17–36)[a]	72	−6	0/10
4-HPR	120	12	34 (21–49)[a]	74	0	0/12
Cisplatin + 4-HPR	6 + 120	12	43 (33–78)[a,b]	120	−9	0/12

MST, median survival time in days, range in parenthesis; % ILS, percentage increase in life span = (T−C)×100/C, where T = MST of treated mice and C = MST of controls; % BWL, percentage mean body weight loss = 100−(BW at the end of treatment/BW on day 1)×100.

[a] $P < 0.001$ Student's *t*-test versus controls.

[b] $P < 0.01$ Student's *t*-test versus 4-HPR.

the tumour cell levels or whether they are indirectly mediated through the host.

Cisplatin is the major first-line drug used in the treatment of ovarian tumours, but one of the obstacles that limits its effectiveness is the acquisition of drug resistance. More effective treatment modalities or drugs able to modulate cisplatin resistance could improve the survival of affected women. The potential of 4-HPR as a single agent and associated with cisplatin was assessed in IGROV-1/cisplatin, a sub-line obtained from a cisplatin-resistant cell line with an *in vitro* RI of 12 [17]. In order to have growing tumours in all the injected mice, in the resistant cell line experiments, 7.2 times the number of cells had to be injected compared with the sensitive cell line experiments, but in spite of this, the survival time of the injected mice was similar. This suggests that, compared with IGROV-1 cells, IGROV-1/cisplatin cells are less oncogenic probably due to the *in vivo* growth of a lower percentage of tumour cells. The relationship between the *in vitro* and *in vivo* drug resistance of tumour cells is an important issue which has been seldom investigated. Our results provide evidence that, for the investigated tumour, an *in vitro* RI of 12 results in reduced *in vivo* sensitivity. Cisplatin treatment was less effective than in the sensitive line, but it still determined a significant increase in survival time. In contrast, 4-HPR was equally active against the two tumours, indicating no cross-resistance between 4-HPR and cisplatin. As previously found in IGROV-1 tumour-bearing mice [13], in mice bearing tumours derived from the less sensitive line IGROV-1/cisplatin, the combination of 4-HPR with cisplatin caused an increase in survival time that was significantly higher than that observed with either agent alone. The association of 4-HPR and cisplatin restored cisplatin sensitivity, since the antitumour activity was similar to that obtained by cisplatin in the sensitive line. We do not know whether the combination of 4-HPR with cisplatin might be effective in tumours which are completely unresponsive to cisplatin treatment *in vivo*.

At variance with 4-HPR, RA, which had improved cisplatin activity against IGROV-1 tumours, failed to enhance cisplatin activity against the cisplatin-resistant sub-line. Such findings suggest that 4-HPR and RA interfere with the mechanism of action of cisplatin at different levels. 4-HPR seems to interact with cisplatin in a pathway independent from cisplatin sensitivity and RA in one that was dependent on cisplatin sensitivity. *In vitro* results support the hypothesis for RA [25]. RA increased cisplatin cytotoxicity in a human germ-cell tumour cell line, but it reduced the cytotoxic effects of the drug in the corresponding cisplatin-resistant line [25]. As regards the combination of 4-HPR and cisplatin, in *in vitro* systems the combination was found to be more than additive in small-cell lung cancer cell lines [26], but antagonistic in breast cancer cells [27].

When 4-HPR and RA were used in combination or when each retinoid was added separately to cisplatin, there was no need to reduce the doses of the single agents. Enhanced efficacy occurred without apparently increasing the side-effects of either agent. In contrast, increased toxicity without an enhanced antitumour effect occurred when the two retinoids together were administered with cisplatin. Such findings suggest that the combination of retinoids with chemotherapy may result in an increase, but also in a decrease of the therapeutic index.

To conclude, the results of the study support and extend previous findings [13]. The encouraging results obtained with 4-HPR as a single agent or in combination with RA or with cisplatin, suggest that these combinations might be useful in cisplatin-sensitive and less sensitive ovarian tumours.

Acknowledgements

The study was supported by grants from the Associazione Italiana per la Ricerca sul Cancro, Milan, Italy. The authors wish to thank P. Perego for providing the IGROV-1/Pt1 cell line and L. Zanesi and B. Johnston for manuscript preparation.

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