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In vivo effects of tumor necrosis factor-α or flavone acetic acid in combination with doxorubicin on multidrug-resistant B16 melanoma

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Having observed that tumor necrosis factor (TNF)-α and doxorubicin (DXR) produce a synergistic inhibition of melanoma B16 and also of its multidrug resistant (MDR) variant *in vitro*, we tested whether this interaction would occur *in vivo* as well. C57BL/6 mice with s.c. tumors were treated with TNF or flavone acetic acid (FAA), a biological response modifier, in simultaneous or sequential combination with DXR. The agents were administered systemically. Overall, the results were negative, apart from a trend towards slight synergy, found in the chemosensitive melanoma, when TNF was given 1 or 2 days before DXR. The effects of FAA and DXR were found to be subadditive or antagonistic. However, an encouraging new finding was that FAA has significant inhibitory effects on the MDR B16 melanoma.

Key words: B16 melanoma, doxorubicin, flavone acetic acid, multidrug resistance, tumor necrosis factor-α.

Introduction

Multidrug resistance (MDR) associated with various mechanisms, including the overexpression of a multidrug efflux pump known as P-glycoprotein (P-gp), is capable of reducing the effectiveness of anticancer treatment.[1,2] Cytokines applied alone or in combination with antineoplastic agents have been considered to be capable of bypassing MDR.[3,4] Indeed, interferon (IFN)-α[5] or tumor necrosis factor (TNF)-α[6–9] in conjunction with established antitumor agents have produced synergistic inhibition of various MDR tumor cell lines *in vitro*. However, information on the efficacy of such combinations on *in vivo* MDR tumors is scarce. Since we have observed that TNF is synergistic with doxorubicin (DXR) in inhibiting the *in vitro* growth of mouse B16 melanoma and of its MDR, P-gp overexpressing, form,[8] we tested here whether this synergy could occur *in vivo*. We report also on the effects of the combination of DXR with flavone acetic acid (FAA). In fact, the latter drug induces *in vivo* the activity of cytokines like TNF and IFNs, and this is probably related to its antitumor effects.[10–13]

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Materials and Methods

Drugs

DXR hydrochloride was supplied by Farmitalia Carlo Erba (Milan, Italy). FAA was generously provided by Lipha (Lyon, France) and was dissolved in 4% sodium bicarbonate. Recombinant mouse TNF-α (specific activity 2.7×10^8 U/mg protein, lot no. B4767) was supplied by Genzyme (Cambridge, MA) and further diluted with cold PBS containing 2% pooled normal C57BL/6 mouse serum.

Tumors and assay of *in vitro* cell proliferation

Mouse B16 melanoma cells, sensitive (B16) or resistant (B16-DXR) to DXR, were obtained as described (8). The two cell lines were grown in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% fetal calf serum (Gibco), 1% penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were incubated in humidified 5% CO_2/air at 37°C and were subcultured twice weekly. For the assays of cell proliferation, inocula of 1×10^5 cells/ml were seeded into 24-well culture plates (Nunc, Roskilde, Denmark) and 24 h later TNF, FAA, DXR or combinations of these were added. After a further 72 h the viable cells were counted by Trypan blue exclusion. The evaluation of the effectiveness of the combined treatments was performed by isobologram analysis.[14]

In vivo experiments

Female C57BL/6 mice were purchased from Harlan Nossan (Correzzana, Italy) and were 8–10 weeks of

age when used in the experiments. At day 0, 1×10^6 exponentially growing cells from the *in vitro* culture of B16 or B16-DXR melanomas were inoculated s.c. in the left axillary region of the animals. The survival time of the mice was subsequently observed. An unpaired Student's *t*-test was used to analyze the effect of the treatments on the survival of the mice. The significance of the changes in body weight was analyzed using the Student's *t*-test for paired data.

Results

In vitro sensitivity of the cell lines to the agents used alone or incombination

The MDR characteristics of B16-DXR, which include cross-resistance to vincristine and overexpression of P-gp, have already been described.[8] Table 1 reports the inhibitory activity of DXR, FAA and TNF on B16 and B16-DXR. B16-DXR melanoma was 15-fold less sensitive to DXR than the parent line; it was equally sensitive to FAA and more sensitive to TNF. Figure 1 shows the effects of TNF in combination with DXR: in both B16 and B16-DXR melanoma a synergy occurred at moderate doses of the agents and was confirmed by isobologram analysis (Figure 1). On the other hand, the effects of the combination of FAA with DXR were found to be merely additive (Figure 2).

In vivo effects of the combination of TNF or FAA and DXR

We initially tested a virtually simultaneous combination of TNF or FAA with DXR. In the experiment of Table 2 the administration of the agents was started the day after the implantation of B16-DXR tumor cells. DXR, TNF and their combination slightly, although non-significantly, reduced the survival of the mice. FAA alone or, to a lesser extent, in combination with DXR, exhibited activity. The experiments of Table 3 were carried out on established tumors and the administration of the agents was initiated on day 9 for the B16 bearing animals and on day 7 for the B16-DXR ones. At these days the tumor areas were 123 ± 38 and 253 ± 69 mm^3, respectively. DXR prolonged significantly the survival only in the B16 mice; TNF was marginally active and FAA had significant effects on both forms. The combinations had subadditive or antagonistic effects. The results on survival were well correlated to the growth rates of the tumors in the various groups (data not shown). There were no early deaths or cures in the treated groups. The animals treated with the combinations underwent some loss of weight, and Tables 2 and 3 report the peak variations of their body weight with respect to the pretreatment weight.

Next, the effects of administering non-simultaneous schedules of combination on established B16 or B16-DXR tumors were investigated. Table 4 presents the combined results of two separate experiments: TNF or FAA were given on day 7 and DXR either on day 5, 8 or 9. TNF or DXR as single agents had no significant activity while FAA had superimposable efficacy on both tumors. For the combinations, there was a trend towards a slight synergy when DXR was administered after TNF, either on day 8 or 9, in the B16 mice. This difference in survival was significant ($p < 0.05$) when compared to the control group, but not to the TNF or DXR groups, only when the data for the 2 days were pooled. The same schemes modestly prolonged the survival also in the B16-DXR mice. However, in this

Table 1. Concentrations of TNF, FAA or DXR required for partial growth inhibition of B16 or B16-DXR melanoma

Tumor	IC_{30} TNF (ng/ml)	IC_{50} FAA (μg/ml)	IC_{50} DXR (ng/ml)
B16	25.2 ± 2.0	197.2 ± 13.2	10.7 ± 0.5
B16-DXR	10.4 ± 1.2	189.5 ± 16.67	157.1 ± 17.4

Values are the mean ± SD of at least three independent experiments carried out on triplicate or duplicate plates. IC_{30} and IC_{50}: 30% and 50% inhibitory concentration. For further details, see Materials and methods.

Table 2. Effects of TNF or FAA in simultaneous combination with DXR on the survival of mice bearing early B16-DXR melanoma

Treatment	*n*	Mean survival (days ± SD)	T/C	Variation of body weight* (%)
Saline	8	21.5 ± 2.5		102.4[c]
TNF	7	20.4 ± 2.8	94.8	101.5
FAA	7	27.7 ± 5.4[a]	128.8	101.4
DXR	7	21.2 ± 3.5	98.6	100.6
TNF + DXR	7	20.0 ± 2.6	93.0	96.2[d]
FAA + DXR	7	24.5 ± 1.2[b]	113.9	93.0[d]

B16-DXR melanoma cells (1×10^6) were inoculated on day 0 s.s in C57BL/6 mice. On day 1 and 3 the animals received TNF 0.33 μg/mouse i.v., or FAA 150 mg/kg i.v., or DXR 5 mg/kg i.p., or the same doses of TNF or FAA 1 h after DXR 5 mg/kg i.p. *n*, number of animals. T/C: survival of the treated animals/survival of the control animals × 100. *Mean % variation in body weight on day 5 versus day 1. [a]$p < 0.05$ versus control group. [b]$p < 0.05$ versus control group and DXR, not significant versus FAA. [c]$p < 0.05$ versus pretreatment weight. [d]$p < 0.01$ versus pretreatment weight.

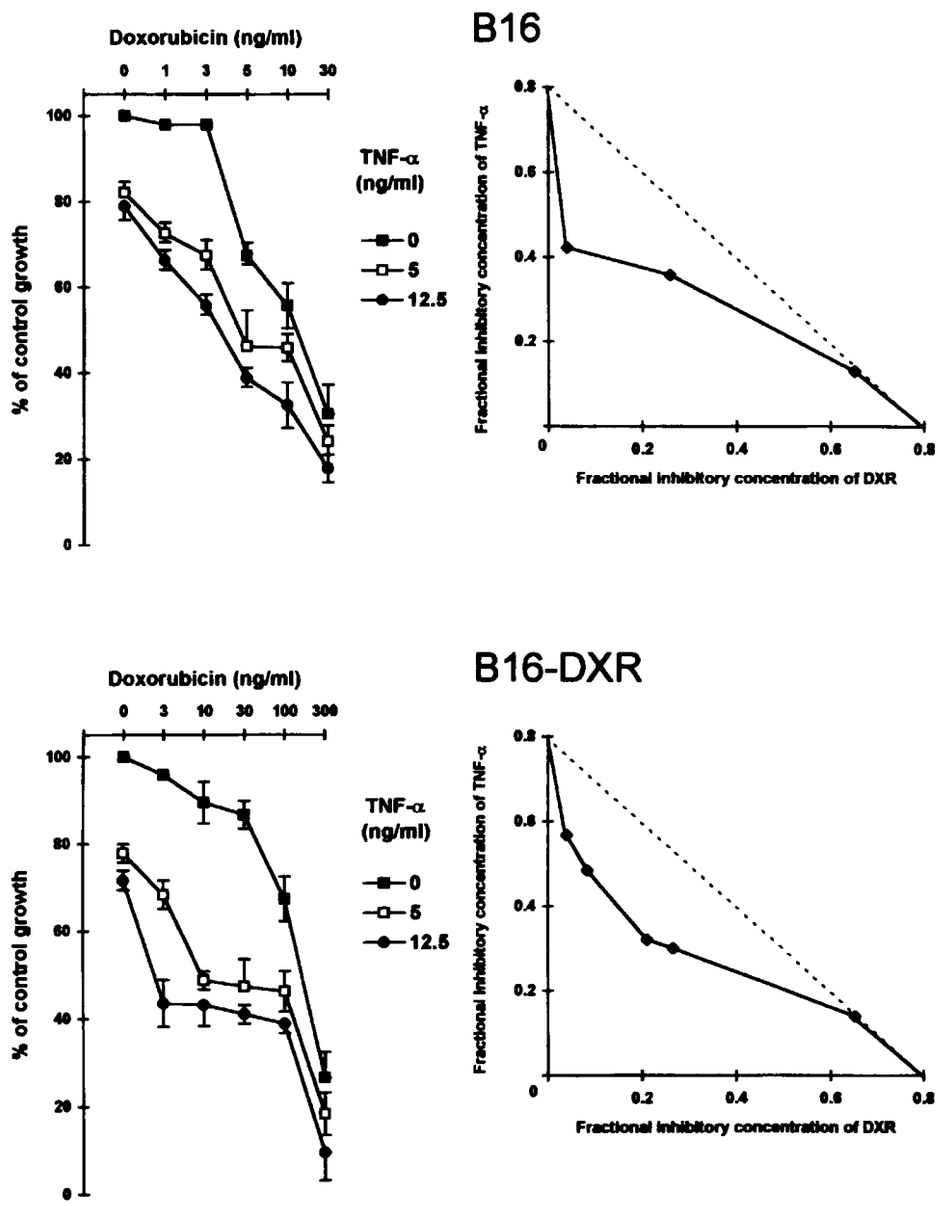

Figure 1. Inhibitory effects of the combination of TNF and DXR on B16 or B16-DXR melanoma. Data are expressed as percentage of control growth and are the mean ± SD of three independent experiments carried out on triplicate plates. The isobologram analyses shown on the right were generated according to Berenbaum.[14]

case the results were roughly equal to the sum of the effects of TNF and DXR given separately. Finally, the combinations of FAA and DXR produced sub-additive or antagonistic effects in the two tumor forms.

Discussion

Many reports have suggested that TNF may interact favorably with conventional antitumor drugs, including DXR, in inhibiting neoplastic cell growth.[6–9,15–18,21] *In vitro*, such synergy may be implemented at the site of various direct mechanisms of the agents[6–9,15–18,21], and, in general, is achieved when the drug application precedes or is simultaneous with that of TNF.[6,8,15–18,21] However, *in vivo* this may also depend on immunological or pharmacokinetic interactions[19–23] and often be favored when TNF is administered before.[19,20,22,23] Currently, the combined use of TNF and cytotoxic

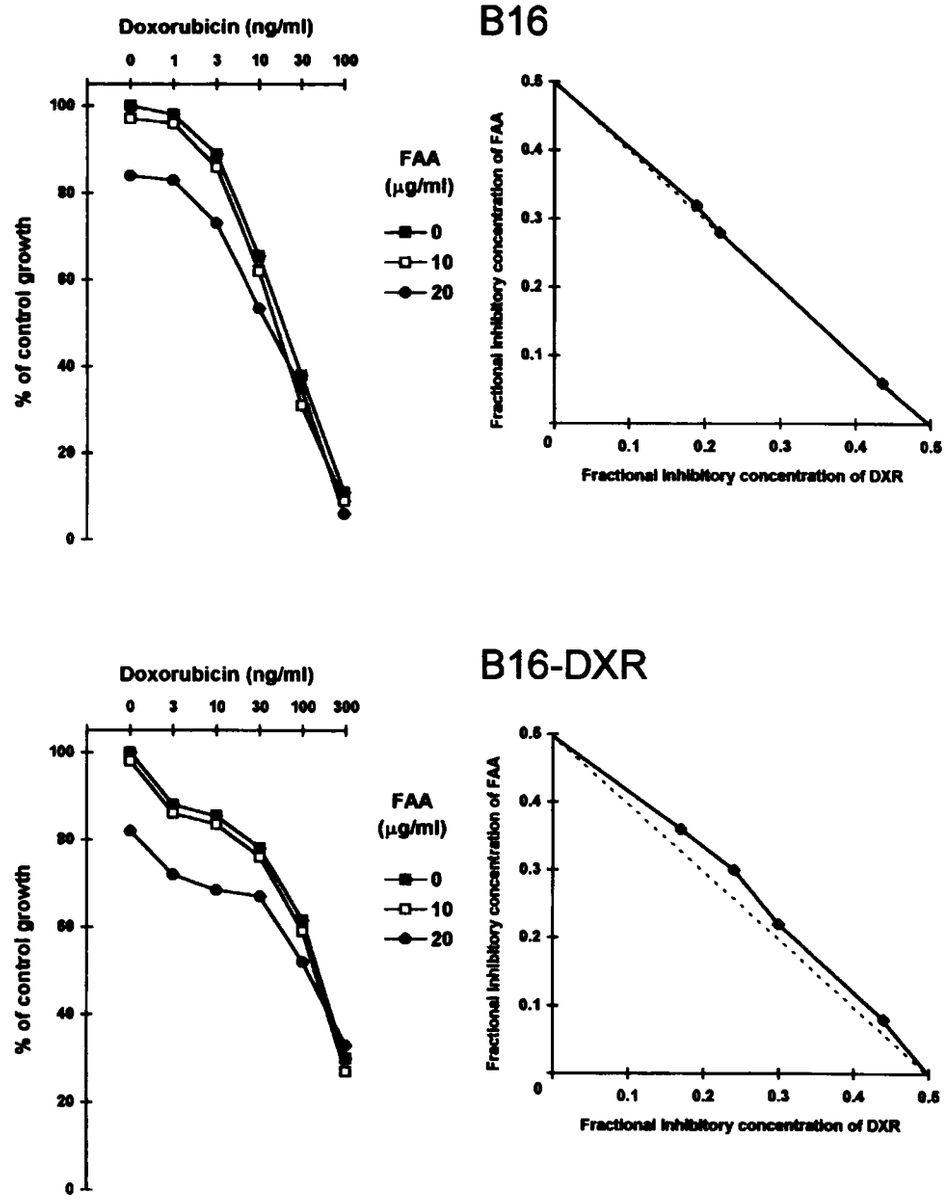

Figure 2. Inhibitory effects of the combination of FAA and DXR on B16 or B16-DXR melanoma. Data are expressed as percentage of control growth and are the mean of three independent experiments carried out on triplicate plates. The SD are omitted, but they were regularly less than 10% of the mean. The isobologram analyses shown on the right were generated according to Berenbaum.[14]

agents is the subject of extensive clinical research.[24–26]

MDR represents one of the main reasons for the failure of anticancer therapy. Clearly, new strategies are required to overcome this process. On the other hand, there is a lack of information on the activity of combinations of TNF and drugs on *in vivo* MDR tumors. Thus, in this paper we were interested to study *in vivo* the possible effects of the combination of TNF and DXR on B16 melanoma and its DXR-resistant, MDR, variant. The *in vitro* data had indicated that the two agents exert a modest but reproducible antiproliferative synergy on these tumors.

We employed the species-specific cytokine and DXR at doses which, in combination, had acceptable toxicity. TNF alone had only marginal antitumor

Table 3. Effect of TNF or FAA in simultaneous combination with DXR on the survival of mice bearing established B16 or B16-DXR melanoma

Tumor	Treatment	*n*	Survival in days (mean ± SD)	T/C	Variation of body weight* (%)
B16	saline	8	25.6 ± 4.5		103.3[d]
B16	TNF	7	27.7 ± 4.1	108.2	105.4[d]
B16	FAA	7	33.8 ± 5.9[a]	132.0	101.8
B16	DXR	7	31.8 ± 5.4	124.2	97.9[d]
B16	TNF + DXR	7	27.0 ± 5.3	105.4	92.8[e]
B16	FAA + DXR	7	33.0 ± 2.4[b]	128.9	95.2[d]
B16-DXR	saline	9	21.0 ± 3.0		100.9
B16-DXR	TNF	7	23.5 ± 3.6	111.9	102.1
B16-DXR	FAA	7	28.1 ± 3.8[a]	133.8	100.1
B16-DXR	DXR	7	22.8 ± 2.7	108.5	100.6
B16-DXR	TNF + DXR	7	23.8 ± 3.2	113.3	95.7[e]
B16-DXR	FAA + DXR	7	26.2 ± 3.9[c]	124.7	94.0[e]

B16 or B16-DXR melanoma cells (1×10^6) were inoculated on day 0 s.c. in C57BL/6 mice. On days 9 and 11 (B16 melanoma) or 7 and 9 (B16-DXR melanoma) the animals received TNF 0.33 μg/mouse i.v., or FAA 150 mg/kg i.v., or DXR 5 mg/kg i.p., or the same doses of TNF or FAA 1 h after DXR 5 mg/kg i.p. *Mean % variation in body weight on day 15 versus day 9 (B16) or on day 11 versus day 7 (B16-DXR). [a]$p < 0.01$ versus control group. [b]$p < 0.01$ versus control group, not significant versus FAA or DXR. [c]$p < 0.05$ versus control group, not significant versus FAA or DXR. [d]$p < 0.05$ versus pretreatment weight. [e]$p < 0.01$ versus pretreatment weight.

Table 4. Effects of TNF or FAA in sequential combinations with DXR on the survival of mice with established B16 or B16-DXR melanoma

Tumor	Treatment	*n*	Survival in days (mean ± SD)	T/C
B16	saline	17	26.6 ± 4.9	–
B16	TNF	14	27.6 ± 3.7	103.7
B16	FAA	14	33.1 ± 4.7[a]	124.4
B16	DXR day 5	7	29.0 ± 3.7	109.0
B16	DXR day 8	7	28.1 ± 4.7	105.6
B16	DXR day 9	7	26.5 ± 3.1	99.6
B16	TNF + DXR day 5	7	27.2 ± 2.4	102.2
B16	TNF + DXR day 8	7	30.2 ± 4.3	113.5
B16	TNF + DXR day 9	7	30.1 ± 3.1	113.1
B16	FAA + DXR day 5	7	34.8 ± 7.4[b]	130.8
B16	FAA + DXR day 8	7	31.7 ± 4.4[c]	119.1
B16	FAA + DXR day 9	7	31.2 ± 2.8[d]	117.2
B16-DXR	saline	16	20.4 ± 3.3	–
B16-DXR	TNF	14	22.0 ± 3.4	107.8
B16-DXR	FAA	14	24.8 ± 3.5[a]	121.5
B16-DXR	DXR day 5	7	20.0 ± 2.1	98.0
B16-DXR	DXR day 8	7	22.4 ± 2.9	109.8
B16-DXR	DXR day 9	7	23.0 ± 2.5	112.7
B16-DXR	TNF + DXR day 5	7	21.2 ± 3.6	103.9
B16-DXR	TNF + DXR day 8	7	23.5 ± 2.9[e]	115.1
B16-DXR	TNF + DXR day 9	7	24.1 ± 4.5[e]	118.1
B16-DXR	FAA + DXR day 5	7	23.4 ± 2.5[d]	114.7
B16-DXR	FAA + DXR day 8	7	25.4 ± 4.9[b]	124.5
B16-DXR	FAA + DXR day 9	7	24.7 ± 2.4[b]	121.0

B16 or B16-DXR melanoma cells (1×10^6) were inoculated on day 0 s.c. in C57BL/6 mice. On day 7 some animals received TNF 0.33 μg/mouse i.v. or FAA 180 mg/kg i.v. Other animals received DXR 8 mg/kg i.p. either on day 5,8 or 9. In the combination groups the animals were treated on day 7 with the same doses of TNF or FAA and with DXR 8 mg/kg i.p. either on day 5, 8 or 9. [a]$p < 0.01$ versus control group. [b]$p < 0.01$ versus control group, not significant versus FAA or DXR on the same day. [c]$p < 0.05$ versus control group, not significant versus FAA or DXR on the same day. [d]$p < 0.05$ versus control group and DXR on the same day, not significant versus FAA. [e]$p < 0.05$ versus control group, not significant versus TNF or DXR on the same day.

effects which, however, were consistently present in all the experiments carried out on mice bearing established tumors.Working with higher doses of the cytokine we have invariably observed a low inhibitory activity on established, but not on early B16 melanomas (unpublished observations). This probably suggests that this activity does not depend on the direct cytotoxicity or immunological mechanisms of the cytokine. Neither DXR effects were great, even in the parent melanoma. On the other hand, B16 melanoma of C57BL/6 mice is a very aggressive tumor and it has already been described that TNF and DXR have, respectively, low and moderate efficacy on its growth *in vivo*.[21,27,28] Overall the results with the various schemes of combination were also disappointing, apart from a trend towards a slight synergy encountered in B16, but not in B16-DXR, when DXR was given after the cytokine. Since chemosensitivity seemed to be a requisite for this result, one might adopt the view that TNF may enhance the effects of DXR by inducing vascular damage and timed permeability changes which could increase the drug concentrations near the tumor cells.[22]

Nevertheless, the present results did not corroborate the *in vitro* data where the synergy was present in both tumors, and best achieved when the administration of DXR preceded or was simultaneous with that of TNF.[8] On the other hand, the work of others[29] has recently pointed out that TNF antitumor effects *in vivo* do not correlate with TNF cytotoxicity *in vitro* and that they may rather depend on other factors, including the secretion of vascular permeability factor (VPF) by tumor cells. Apart from this, we have already commented on the fact that the *in vitro* synergy of TNF and DXR was modest, i.e. in the range of a 10–30% gain in cytotoxicity (Figure 1). In addition, we have previously shown[8] that this synergy requires at least a 2-day exposure to the agents to take place. These conditions cannot easily be obtained *in vivo* after systemic administration. For example, the serum half-life of TNF after i.v. injection is reportedly short, i.e. about 20 min.[30] Perhaps it would be worthy to see whether the use of liposome-encapsulated TNF could improve the effects of the combination with DXR by increasing the availability of the cytokine at the tumor site.[31]

We also studied FAA and the drug showed significant effects on the survival of the animals bearing B16 or B16-DXR melanomas. This is not in contrast with the results observed with TNF, since many reports have indicated that FAA is a biological response modifier whose antitumor activity depends on a pleiotropic induction of cytokines, not only TNF but also IFNs, and cell effectors in the host.[11–13] We also observed activity of FAA *in vitro*, but with high concentrations and prolonged exposure times that most likely, owing to pharmacokinetic reasons,[18,32] cannot be achieved *in vivo*. Thus, it is quite questionable whether this direct cytotoxicity of FAA contributed to its *in vivo* effects. The combination of FAA and DXR was even less advantageous than that of TNF and DXR. It exhibited additive effects *in vitro* and, even more importantly, subadditive or antagonistic effects *in vivo*. Thus, it is possible that an immunosuppressive effect of DXR may have disturbed the induction of biological activities by FAA.

The excellent activity exhibited by FAA on various experimental tumors has not been confirmed by the clinical data accumulated so far.[13] It has been proposed, however, that analogs like 5, 6-dimethylxanthenone-4-acetic acid may retain activity in man.[13] With this in view, the present observation that FAA has efficacy on a MDR tumor like the B16-DXR melanoma is, to our knowledge, rather new and might be of interest.

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