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Vascular Collapse After Flavone Acetic Acid: A Possible Mechanism of its Anti-tumour Action

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Abstract—*Flavone acetic acid (FAA, LM 975) causes regression and growth retardation in several solid murine tumours. The mechanism of action is unknown, although various lines of evidence suggest an indirect cytotoxic effect. We have carried out preliminary studies on the effect of FAA on relative blood flow in six experimental murine tumours using ^{86}RbCl extraction. We have also measured growth delay after treatment with the same dose of FAA (200 mg/kg). The data show that the drug induces a drop in tumour perfusion within 6 h of treatment in all of the tumours, and that this can be correlated with the growth delay measured. We conclude that vascular collapse may be an important component of the action of this drug, and that further investigation of this phenomenon is warranted.*

INTRODUCTION

MOST experimental chemotherapy is designed to be more effective on malignant cells than on normal cells. However, substances are sometimes found which are effective *in vivo* as antitumour agents, without an obvious biological basis for their antitumour activity. Flavone acetic acid (FAA, LM-975) has been shown to cause significant regression and growth retardation in a variety of experimental murine tumours [1–3]. It is only slightly cytotoxic to cells *in vitro* and there is no obvious rationale for any normal/malignant cell differential [1, 2, 4–6]. Furthermore, the sensitivity of different tumour lines *in vitro* and *in vivo* does not correlate well [2, 3]. The rapid necrosis that characterizes the solid tumour response has been likened to the effects of tumour necrosis factor [7], and this has led to studies of natural killer cells, of macrophages and of other biological response modifiers [3, 8–10]. Thus far the mechanism of its action is totally unknown.

FAA differs from most cytotoxic agents in a number of aspects:

1. it induces *rapid* necrosis, with tumour cell death apparent within 4–6 h [3, 6];
2. it is more effective on solid tumours than on ascites, lymphomas or leukaemias [1, 2, 5];
3. it is more effective on tumours grown subcutaneously than as ascites or as small lung nodules [11];
4. it is more effective on established (5–10 mm) tumours than on newly implanted tumour cells [3, 12];
5. it is more effective *in vivo* than a similar concentration *in vitro* [3, 13];
6. it is ineffective on tumour cells in perfusion chambers, and is not simply related to pharmacokinetics, implying host interactions are needed [3, 11].

Because of the pattern of patchy necrosis reported by several groups [3, 6, 14, 15] we decided to investigate whether all or part of the antitumour action of this drug was mediated through an indirect mechanism. This histological picture should not occur with agents causing random cell death but might be expected to result from loss of tumour blood vessels. We have repeatedly focused attention on the rapidly proliferating immature vascular networks in tumours as a potential target in tumour therapy [16, 17]. Because each tumour capillary is already displaced from adjacent capillaries beyond the optimum diffusion radius for nutrients, the loss of any individual blood vessel in tumours can have catastrophic results for the dependent tumour cells. This contrasts with normal vascular networks where a redundant reserve of collateral vesssels is usually available for increasing blood flow under stress.

In the present study six types of experimental tumour, varying widely in histology and growth rate, were studied. Treatment efficacy was measured using growth delay and ^{86}RbCl extraction

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was used as a measure of vascular perfusion. The tumours chosen are all syngeneic tumours in CBA or WHT mice which arose 7–31 years ago and have been maintained both in a frozen bank and by serial subcutaneous transplantation in the strains of origin. They are believed to be free of transplantation artefacts [18].

MATERIALS AND METHODS

Each tumour was implanted subcutaneously on the rear dorsum of groups of syngeneic inbred mice, either using a trochar to implant fragments of approximately 1 mm^3 or a needle to inject 0.05 ml of a coarse tumour cell brei. The mice were then observed at regular intervals and were selected for treatment when the tumours reached the predefined size range (6–7 mm mean diameter). For the fast growing tumours treatment commenced in the first 2–3 weeks after implant, whereas for the slow growing CaHAL and SaS the treatment began approximately 20 weeks after implant, when they had reached the required size.

Flavone acetic acid (LM 975) was obtained as the sodium salt, freeze dried, from Lipha Pharmaceuticals. After dissolving in distilled water the 100 mg/ml solution was kept at 4°C prior to administration. A final concentration of 20 mg/ml was injected intraperitoneally at a dose of 200 mg/kg to tumour-bearing animals. The mice used in this study were both male and female and weighed between 25 and 40 g. Tumours were measured regularly after treatment, either three or two times per week for the fast and slow growing tumours respectively.

The perfusion of the vasculature was assessed using $^{86}RbCl$, a radioactive tracer which distributes freely into tissue and exchanges with tissue K^+. If tumours are excised 60–90 s after injection into the tail vein, the radioactivity in each tissue, or in a tumour, can be used to calculate the relative blood flow to that tissue [19–21]. The tumours and samples of normal tissues were removed immediately after sacrificing the mice by neck fracture, and the samples were counted in a Wallac 1282 compugamma counter.

RESULTS

The results are summarized in Table 1. The six types of tumour showed a range of growth rates, with volume doubling times ranging from 2 days in SaF to 10 days in SaS and CaHAL. Figure 1 shows the growth curves for tumours growing in control animals (no treatment) and in mice given 200 mg/kg FAA when the tumours were 6–7 mm diameter. In all of the tumours FAA produced some inhibition of growth, but the magnitude varied considerably with tumour type. Growth delays were calculated as the time required for treated tumours to grow to 3 mm larger than the original size at treatment minus the time for control tumours to do the same. These values ranged from 2 to 61 days. By translating growth delay into specific growth delay, it is possible to compare directly the effectiveness of treatment in the different tumours. If a doubling time is needed to counteract a halving in cell number, the specific growth delay can be used to estimate how many halvings of cell number has occurred and can be converted to percentage cell survival. These values are also indicated in Table 1. They show a 40-fold range of cell survival after a fixed dose.

The measurements of vascular perfusion were made at a single time point, 6 h after administering 200 mg/kg FAA. As shown in Table 1, a marked reduction in ^{86}Rb activity was apparent after FAA treament. Again, the magnitude of the effect varied with tumour type. In contrast to this reduction in tumour blood flow, an increased perfusion was measured in the kidney and intestine of treated animals. Of the other normal tissues investigated, liver showed no change, but a decrease was measured in muscle. These values are presented in Table 2.

Table 1. Effect of FAA on six murine tumours*

Tumour	Mouse strain	Volume doubling time (days)	Growth delay (days) 6.5–9.5 mm	Specific growth delay†	Percentage cell survival	Relative perfusion at 6 h (per cent control)
SaF	CBA	2.2 ± 0.1 (5)‡	11.5 ± 2.1 (5)	5.2 ± 1.0	2.7	7.9 ± 0.8 (6)
CaNT	CBA	3.9 ± 0.6 (5)	6.2 ± 0.4 (5)	1.6 ± 0.3	33.0	20.5 ± 2.9 (5)
SaNeO	WHT	5.0 ± 0.8 (4)	9.2 ± 3.8 (4)	1.8 ± 0.8	28.7	13.8 ± 2.6 (6)
SaHM	CBA	6.6 ± 0.9 (4)	2.4 ± 1.1 (4)	0.4 ± 0.2	75.8	33.1 ± 5.0 (4)
SaS	CBA	9.7 ± 0.8 (5)	2.8 ± 2.1 (6)	0.3 ± 0.2	81.2	46.0 ± 4.3 (5)
CaHAL	WHT	9.8 ± 0.6 (7)	61.1 ± 13 (5)	6.2 ± 1.4	1.3	6.75 ± 1.3 (5)

*200 mg/kg single dose i.p.
†Growth delay ÷ volume double time.
‡Number of animals.

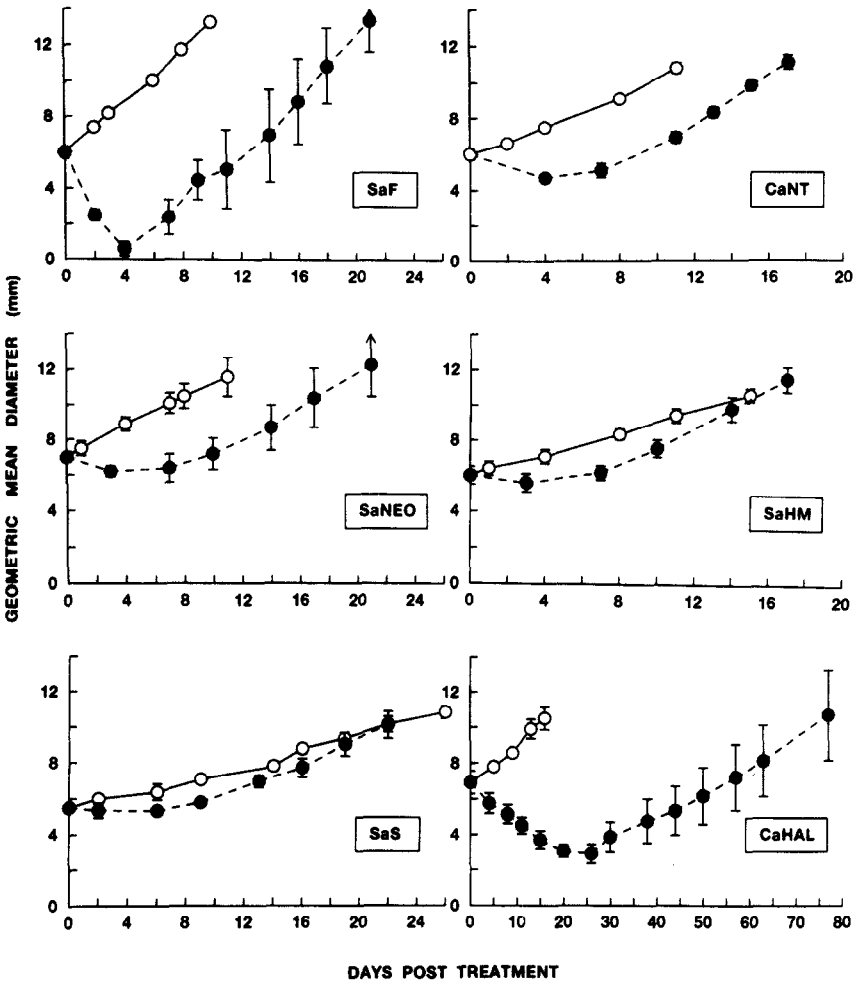

Fig. 1. Growth curves for untreated tumours and tumours in mice given 200mg/kg *flavone acetic acid when the tumours were* 6–7 mm *mean diameter. Each point represents the mean ± 1 S.E.M. of 4–6 tumours.*

Table 2. Normal tissue perfusion

Tissue	Percentage injected ^{86}Rb activity/g		Treated/Control %
	Untreated	6 h post FAA	
Muscle (gastrocnemius)	2.7 ± 0.2	2.3 ± 0.2	85.2 ± 9.7
Liver	3.1 ± 0.3	3.1 ± 0.1	100.0 ± 10.2
Gut (jejunum)	8.2 ± 0.6	9.5 ± 0.6	115.9 ± 11.2
Kidney	42.3 ± 2.1	57.1 ± 3.2	135.0 ± 10.1

The dose of 200 mg/kg FAA used in the present study was chosen on the basis of published LD_{50} data [1, 5]. We did, however, administer the drug to both CBA and WHT mice to confirm the safety margin in our own strains of animals. The LD_{50} was 400–450 mg/kg for male and female albino WHT mice, but was greater than 450 and 500 mg/kg in CBA females and males (at which doses no deaths were seen). However, we have found the drug to be much more toxic in tumour-bearing animals, with an apparent dependence on both tumour type and tumour size. The cause of death is at present unknown, but it occurs within 24 h and is preceded by a severe drop in body temperature.

DISCUSSION

The data reported indicate that a single dose of 200 mg/kg FAA is effective in inducing some growth delay in the six tumours studied. The degree of responsiveness varies considerably, the derived cell

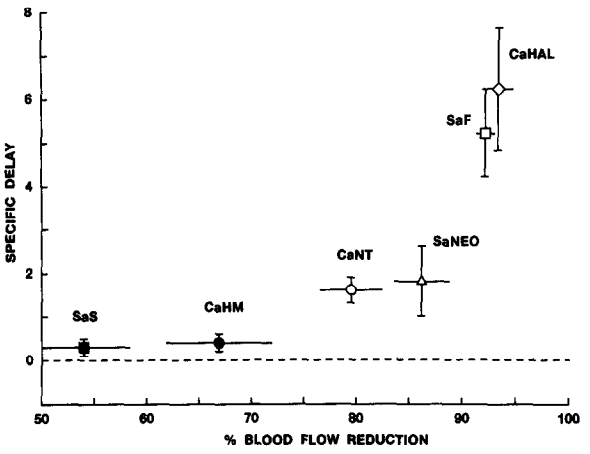

Fig. 2. Specific growth delay as a function of the percentage reduction in tumour blood flow measured by ^{86}RbCl extraction 6 h after treatment. Vertical and horizontal bars represent ± 1 S.E.M. of 4–6 tumours.

survival values ranging from 1 to 81% (Table 1). Although these data consist of only small numbers of animals per treatment group, repeat experiments have shown the patterns of sensitivity to be reproducible. A marked reduction in tumour blood flow was measured at 6 h after treatment. Again a range of responses was seen, with the greatest blood flow reductions being measured in the tumours showing the greatest drug sensitivity. This is illustrated in Fig. 2. Whether tumour response is expressed as delay or specific delay, the degree of early vascular damage shows some correlation with the eventual outcome of treatment. Although FAA also produced a decrease in blood flow to muscle, the increased perfusion measured in two other normal tissues might indicate some generalized vasodilation, which could in itself account for the reduced flow to the tumours.

More detailed studies are in progress to follow the time course of changes in perfusion. In at least three of these tumours, a significant reduction in blood flow is detectable within 30 min of treatment and persists for at least 24 h.

In our view, the pattern of response seen after FAA carries all the hallmarks of an ischaemic necrosis resulting from vascular collapse. The histological picture of FAA treated tumours shows focal necrosis which extends to form a sea of dead cells, in which occasional intact vessels with a surrounding cuff of viable cells can be seen. This characterizes vascular-mediated damage, by contrast with individual cell death where live and dead cells would be more randomly scattered [22]. The greater effect reported for solid than ascites tumours and for large rather than small tumours would support this hypothesis.

Identifying the vascular component of the effects of FAA does not, however, identify its target population. It may be more effective in killing immature, rapidly proliferating endothelial cells, which are characteristic of the new blood vessels in solid tumours. This seems unlikely because of its speed of action. However, studies are in progress to investigate the response of both quiescent and proliferating endothelial cells in culture. Alternatively, coagulation pathways may be altered which are regulated by the production of several co-factors and modulators at the endothelial cell surface [23]. Some of these modulators, e.g. Factor VIII R-Ag and thrombomodulin, may be less abundant on the immature tumour endothelium. Furthermore, tumours are known to secrete substances which are pro-coagulant in nature [24]. In this respect the biochemical inhibition of ATPase pumps and prostaglandin cyclo-oxygenase that characterizes naturally occurring flavonoids may be important [25]. The increased toxicity in tumour-bearing mice is currently being investigated in relation to the coagulability of the blood (Murray *et al.*, in preparation).

Alternatively, the vascular effect may be mediated by a more general physiological response. The increased perfusion of some normal tissues (Table 2) may lead to a passive 'steal phenomenon', which has been demonstrated for a number of other vasoactive agents, including anaesthetics, antihypertensive agents and the radiosensitizer misonidazole [26–28]. It is then intriguing, however, to speculate as to why a similar quantitative shut down of the vasculature (e.g. with hydralazine) is so much less effective at causing growth delay, even in the same tumours (Hill *et al.*, in preparation). It seems likely that the vascular effects of FAA are more complex than simple hypotension, and probably involve several simultaneous actions of the drug.

In the clinic FAA has been administered as a slow infusion. A very large dose of 8.6 g/m^2 (up to 12–14 g per patient) can be given over a 6 h period [29]. This slow infusion was intended to minimize the recognized hypotensive side-effects, and to maximize the exposure time to the drug since this had been shown to be of major importance *in vitro*. The clinical results have so far been extremely disappointing, but this may be a function of this prolonged method of administration. By deliberately avoiding the vascular (hypotensive) action of the drug in patients, the marked vascular collapse in tumours may also be prevented. Preliminary studies in mice have shown that the drug is much less effective when administered chronically, both for growth delay and for vascular shut down (Hill, unpublished). It is obvious that any change of clinical practice towards more rapid administration would have to be approached with caution, since hypotension and a decreased platelet aggregation have both been noted as significant side-effects [29, 30].

We would emphasize that a vascular component of injury appears to be a common part of tumour response to many physical and chemical agents, and it may be wise to include monitoring of the vascular perfusion in assessing any new anti-cancer agent [31]. The mechanism by which such vascular collapse is induced may differ for different agents, and it is clearly important to investigate this with basic studies of vascular biology as well as studying gross tumour response.

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