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Review Paper

Flavone acetic acid—from laboratory to clinic and back

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Flavone acetic acid ester (NSC 293015, LM 985) emerged from a series of flavonoids from Lyonnaise Industrielle Pharmaceutique (Lipha) screened by the National Cancer Institute. LM 985 showed modest but sufficient activity in the P388 pre-screen to progress to secondary evaluation on the solid colon 38, where significant activity was seen. On the basis of this particular profile LM 985 was selected by the Cancer Research Campaign (CRC) UK for phase I clinical trial. It was not recommended for phase II trial because of drug associated hypotension and the fact that it appeared to act as a pro-drug for flavone acetic acid (NSC 347512, LM 975, FAA) which was shown to be responsible for the dramatic solid tumor activity in mice. This was manifested as dramatic hemorrhagic necrosis and involves a complex mechanism of action. FAA proceeded to clinical trial but unfortunately no anti-tumor activity was seen. A large amount of effort has been channelled into identifying the mechanisms of action of FAA in mice and it is clear that activity relies on a number of factors. Subcutaneous tumors respond dramatically whereas ascites tumors and tumor deposits in other sites are usually less responsive. Establishment of a tumor blood vasculature system appears necessary for response and adequate drug concentrations within a therapeutic window are necessary. From the large body of information available at present the most likely explanation for discrepancies in activity between mouse and man seems to relate to differences in the ability of the immune system to respond to FAA, although variation in the composition of the vasculature cannot yet be ruled out. Analogs of this type of compound are worth pursuing but it is necessary to examine them in appropriate model systems in order to predict for possible clinical activity.

Key words: Anti-tumor activity, flavone acetic acid, mechanisms.

Introduction

Screening methods utilized for anti-cancer drug discovery are currently under great debate. This is largely due to the fact that clinical responses of common solid tumors to drugs are disappointing. Since the pre-clinical National Cancer Institute (NCI) screens were based largely on leukemia with high growth fractions, it is not surprising that these models identified agents that are active in high growth fraction solid tumors and hematological malignancies rather than slow growing tumors. In an attempt to improve the situation the NCI has radically altered its screening programme to one that is largely based on *in vitro* cytotoxicity testing in human tumor cell lines. The new screen is based on the concept that histiospecific drug cytotoxicity exists and that differences can be detected in cell lines representative of different human cancers. Alongside this changing approach in drug development, basic scientists are attempting to identify the mechanisms which underlie both the growth and control of cancer cells with a view to identifying new targets. It is quite clear that for those individuals involved in new drug discovery to move into the area of rational drug design, it is essential that more detailed knowledge of the biology of the tumor systems employed is accrued. The importance of tumor biology and the relationship between the tumor and the host has been brought to the fore in the pre-clinical and clinical evaluation of an interesting flavonoid compound, flavone acetic acid (LM 975, NSC 347512, FAA). This compound has been studied extensively and we have no doubt that laboratories involved in its pre-clinical evaluation have learnt much about identifying compounds which have indirect anti-tumor properties, but also about possible pitfalls in pre-clinical evaluation. FAA (Figure 1) had the advantage of being a novel chemical structure compared with existing anti-cancer drugs and belonging to a class of chemical with a wide range of biological properties.[1]

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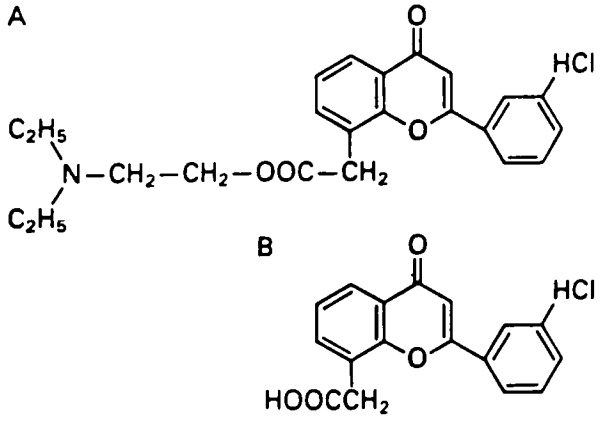

Figure 1. Structural formulae of (A) LM985 and (B) LM975.

Early in the NCI drug development program more than 200 flavonoids were screened against murine tumor systems, including adenocarcinoma 755, sarcoma 180 and leukemia L1210. None of these flavonoids was shown to be active in this screen but, on re-testing in 1971, quercetin showed modest activity against P388 leukemia implanted in the peritoneal cavity.[2]

NCI went on to screen a series of flavones from Lyonnaise Industrielle Pharmaceutique (Lipha) in the screen which had emerged in 1975. They used P388 as a pre-screen followed by a new panel of solid tumors for secondary evaluation.[3]

Flavone acetic acid ester, NSC 293015 (LM 985) (Figure 1) emerged as a lead compound from this screen as it showed good activity against P388 and also against the generally refractory subcutaneously implanted colon adenocarcinoma 38.[2] LM 985 was also shown to produce cures against subcutaneous colon 38 tumors at doses greater than 200 mg/kg intraperitoneally whereas when the drug was given by the intravenous route a dose of 50 mg/kg proved to be toxic (Table 1). On the other hand, FAA was shown to be active against colon 38 by both routes (Table 2).

Anti-tumor activity

These initial studies by the NCI prompted further study which identified for the first time an agent with very broad, perhaps nearly universal, solid tumor activity. Corbett *et al.*[4] found that FAA was active against a variety of transplantable solid tumors of mice (colon 51, 07, 10, 26, pancreatic ductal adenocarcinomas 02 and 03, mammary adenocarcinoma 16/C/Adr, M5076 reticulum cell sarcoma, and Glasgow's osteosarcoma). This group also demonstrated FAA to be selectively cytotoxic *in vitro* for solid tumors over leukemia's in L1210 and P388, and claimed a correlation between cellular selectivity *in vitro* with *in vivo* anti-tumor activity. *In vitro* studies, however, showed quite clearly that extremely high concentrations or long exposure times were necessary for direct cytotoxicity.[5–7] The latter study showed the FAA ester (LM 985) to be significantly more toxic than FAA itself, offering a possible explanation for the earlier observations that the ester had been active against P388[2] and that ascites tumors did not respond whereas solid subcutaneous tumors did.[8] Several studies were now suggestive that FAA was working

Table 1. Activity of FAA ester against subcutaneous colon adenocarcinoma 38: comparison of intraperitoneal and intravenous treatment

Intraperitoneal treatment, days 2 and 9				Intravenous treatment, days 2 and 9			
dose (mg/kg/ injection)[a]	per cent tumor inhibition on day 20[b]	no. of		dose (mg/kg/ injection)[a]	per cent tumor inhibition on day 20[b]	no. of	
		tumor-free mice on day 20	survivors/ total no. on day 20[c]			tumor-free mice on day 20	survivors/ total no. on day 20[c]
356	toxic[d]	6	6/10	50	toxic[d]	0	2/10
267	100	8	8/10	37.5	37	0	8/10
200	96	4	10/10	28.1	31	2	10/10
150	90	0	10/10	21.1	14	0	10/10
112.5	46	0	10/10	15.8	−9	0	9/10

[a]Bolus injections of drug in saline.
[b]Median weight of control tumours was 1100 mg.
[c]All 40 control animals were alive on day 20.
[d]Less than 65% of mice alive on day 20.
From Plowman *et al.*[2] with permission.

Table 2. Activity of FAA ester against subcutaneous colon adenocarcinoma 38: comparison of intraperitoneal and intravenous treatment

Intraperitoneal treatment, days 2 and 9				Intravenous treatment, days 2 and 9		
dose (mg/kg/ injection)[a]	per cent tumor inhibition on day 20[b]	no. of		per cent tumor inhibition on day 20[b]	no. of	
		tumor-free mice on day 20	survivors/ total no. on day 20[c]		tumor-free mice on day 20	survivors/ total no. on day 20[c]
356	100	7	7/10	toxic[d]	2	2/10
267	100	6	10/10	100	8	9/10
200	58	2	10/10	100	7	9/10
150	33	0	10/10	85	2	10/10
112	10	0	9/10	0	1	10/10

[a]Bolus injections of drug in 5% sodium bicarbonate.
[b]Median weight of control tumours was 1968 mg.
[c]Of 40 control mice, two died on day 20.
[d]Less than 65% of mice alive on day 20.
From Plowman *et al.*[2] with permission.

by a novel, possibly indirect, mechanism. Smith *et al.*[9] showed that as early as 4 h after treatment with FAA, treated colon tumors showed evidence of extensive hemorrhagic necrosis. After 24 h there were few tumor cells remaining which were recognizably viable. These authors claimed that the histological observations obtained in their study bore remarkable similarities to those reported for the action of tumor necrosis factor (TNF). Bissery *et al.*[10] treated Glasgow osteogenic sarcoma *in vivo* with 235 and 200 mg/kg FAA intravenously. They described extensive single-strand breaks in DNA beginning 5 h after treatment. Histopathology, however, reveals more rapid hemorrhagic necrosis so it is likely that this study is examining the consequence of cell death rather than its cause.[11] As mentioned earlier, *in vitro* studies with a variety of tumor cell lines had indicated that high concentrations of drug and long exposure times were necessary to achieve direct cytotoxicity. Further studies in this and other laboratories had demonstrated that the FAA concentrations *in vivo* appeared to be insufficient to be responsible for the anti-tumor activity seen *in vivo* based on a direct FAA cytotoxic effect. An Italian group[12] demonstrated that FAA had activity against chemically induced primary colon tumors in mice and studies in this laboratory[5] had the same outcome in MAC tumors. The appearance of treated MAC 26 tumors is shown in Figure 2. MAC 26 is a well differentiated, well vascularized colon adenocarcinoma which is highly responsive to FAA. A later study indicated massive tissue necrosis following FAA treatment against a highly refractory tumor

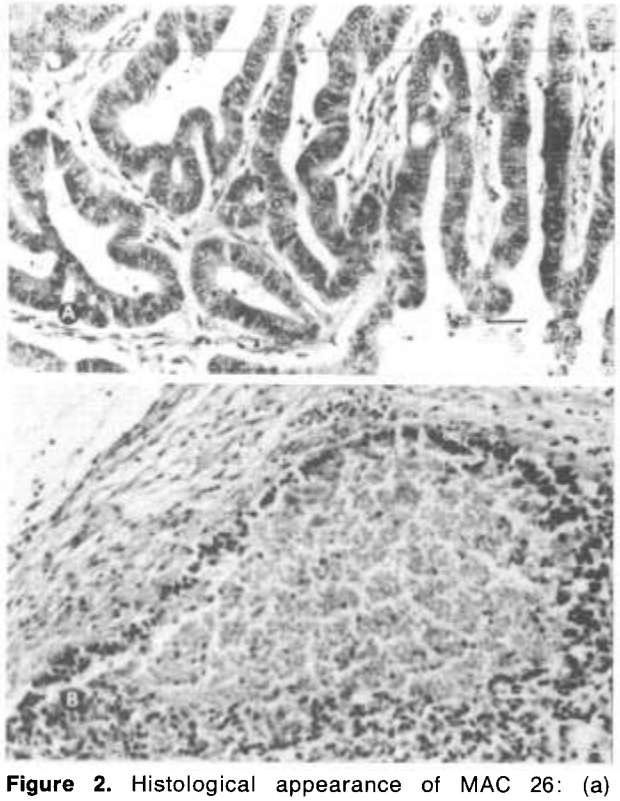

Figure 2. Histological appearance of MAC 26: (a) untreated and (b) 24 h after intraperitoneal treatment with FAA (200 mg/kg) (bar = 25 μm).

MAC 16.[13] MAC 16 is a slow growing transplantable colon adenocarcinoma which causes weight loss in tumor-bearing animals, with a small tumor mass (less than 1% of the host weight). Weight loss is directly related to tumor size and occurs without a reduction in host food intake.[14] The tumor is

Table 3. *In vivo* chemosensitivity of MAC 26TC to FAA

Tumor	Site	Dose (mg/kg)	Route	Day of treatment	Inhibition
MAC 26TC	s.c.	250	i.p.	2,9	100
	i.p.	250	i.p.	2,9	0
	i.v.	250	i.p.	2,9	0

resistant to a number of standard anti-cancer drugs but is highly responsive to FAA when the anti-tumor effects are accompanied by control of the host's cachexia.[13] The unique activity against this tumor suggested that the agent had a novel mechanism which may be dependent upon specific biological characteristics of tumors.

The anti-tumor activity of FAA in most tumors is also influenced by tumor site. FAA was shown to be inactive against early stage Lewis Lung carcinoma growing as small nodules in the lung whereas the compound was active against advanced subcutaneous Lewis Lung carcinoma[15] and a further study in this laboratory showed FAA to be highly active in subcutaneous transplantable adenocarcinoma in NMRI mice (MAC tumors) but ascitic or systemic disease induced using the same cell lines failed to respond to FAA.[16] Data showing these effects for MAC 26 TC tumors are presented in Table 3. We concluded at that time that these results were highly indicative of an effect in subcutaneous tumors which was dependent on specific biological properties of tumors in this site and that these properties may not exist in metastatic disease in man.[16]

Pharmacokinetic relationships

As a result of the pre-clinical solid tumor activity, LM 985 was selected for clinical trial. It did not cause myelosuppression or major organ toxicity, so there was a suggestion of a novel mechanism of action. LM 985 went into phase I clinical trial in the UK in 1985,[17] and the authors suggested that the hydrolysis product FAA may be the active principal and that substantially higher doses of FAA might be given without dose limiting hypotension. Pharmacokinetic studies of LM 985 in mice demonstrated rapid hydrolysis to FAA so it was clear that the acid was responsible for the dramatic anti-tumor effects seen in subcutaneous mouse tumors.[5,8]

Early on in the study of FAA, Zaharko *et al.*[18] introduced the concept of a therapeutic window of effective plasma concentrations and effective exposure times in attempts to maximize therapeutic effects and minimize toxic effects. They concluded that relatively high plasma concentrations (above 100 μg/ml) are needed for therapeutic activity with FAA and that lethality could result from two distinctly different causes. It was suggested that an acute lethality could result from an excessively high peak plasma concentration (above 600 μg/ml) and a delayed lethality could result from a too long exposure (over 24 h) at therapeutically effective plasma concentrations (100–600 μg/ml). They also noted that unexpected kinetic differences occurred between mouse, dog and man. Whereas usually with anti-tumor agents, plasma clearances are proportional to body surface area and hence faster in small species, quite the opposite is true with FAA, with mice exhibiting a slower plasma clearance relative to dog and man. Because of this suggestion of a therapeutic window and the clear indication from earlier studies that *in vitro* responses depended on high concentrations and long exposure times, several other groups started to examine the *in vivo* pharmacokinetics of FAA, partly with a view to understanding the mechanism of action but also in an attempt to aid clinical development.

Damia *et al.*[19] investigated the pharmacokinetics of FAA in BALB/c mice treated with intravenous doses of 100 or 300 mg/kg using a high performance liquid chromatography (HPLC) assay. They showed the kinetics of disappearance from plasma were monoexponential and dose dependent. In plasma and all tissues analyzed, the FAA AUC values were disproportionately greater after 300 than after 100 mg/kg. They found the highest drug concentrations in the liver and small intestine, intermediate concentrations in lung, heart and spleen, and the lowest concentration in brain. Less than 5% of the FAA dose was eliminated as unchanged drug in the stool. Total excretion of FAA as unchanged drug in the urine collected up to 96 h after drug treatment corresponded to 75 and 60% of the intravenous doses of 100 and 300 mg/kg, respectively. A minor fraction of the FAA dose corresponding to 1 and 6% of the two doses was eliminated in the urine as a glucoronide or sulfate. A later study by Chabot *et al.*[20] demonstrated, in mice bearing advanced stage subcutaneous colon 38 adenocarcinoma, the highest FAA concentrations were found in the gastrointestinal tract, mainly in the duodenum, suggesting an important biliary excretion of the drug. They also investigated possible causes of FAA non-linear pharmacokine-

tics. They attributed the non-linearity to a dose dependent decrease in both urinary and biliary clearances, concentration dependent serum protein binding, enterohepatic circulation and the instability of the FAA metabolite FAA acyl glucoronide under physiological conditions forming a futile cycle. These authors also suggested that their distribution data might offer possible tissue targets for anti-cancer efficacy and/or toxicity that could be useful in designing clinical trials. Cummins *et al.*[21] attempted to characterize the major metabolites of FAA and to compare their disposition in humans and mice. The study was undertaken because phase I clinical trials had by this time established that mouse equivalent plasma drug levels could be achieved in humans but phase II trials had not demonstrated any significant activity in a range of tumor types. These authors purified the two major human metabolites present in urine, also the only two metabolites detected in plasma, and characterized their structure, chemical properties, activity and pharmacokinetics. Metabolite 1 (M1) was a glucoronide conjugated to the eight acetic acid grouping, was chemically labile and showed a strong tendency to undergo chemical rearrangement at mildly alkaline pH. Metabolite 2 (M2) was also a glucoronide but appeared to be an unusual isomer of M1. The study demonstrated both of these metabolites to be non-cytotoxic. In patients, biotransformation represented the predominant mechanism of drug clearance with as much as 80% of a low dose (0.5 g/m^2) recovered in urine as metabolites M1 and M2 after only 6 h. At high dose (4.8–8.6 g/m^2, 1–6 h infusion) the appearance of peak concentrations of metabolites in plasma and urine were delayed, apparently due to saturation of glucoronidation pathways. This explains the overall reduction in drug clearance. In this study mice also cleared FAA much more slowly than patients without producing M1 or M2. A different metabolite exhibiting characteristics of a conjugate was detected at low concentrations in plasma, tissues and tumor. These latter observations on drug clearance confirmed the findings of Zaharko *et al.*[17] Because FAA had been shown to be very effective *in vivo* in solid tumors but to be not cytotoxic *in vitro*, Chabot *et al.*[22] attempted to activate FAA to cytotoxic species. These authors demonstrated that when mouse hepatocytes were co-cultured with ACT 116 cells, increased FAA cytotoxicity to 1 log cell kill at 30–100 μg/ml was seen. The addition of phenobarbital induced mouse liver supernatant S9000G also markedly increased FAA cytotoxicity to a 2 log cell kill at 300 μg/ml. The authors concluded that FAA can be activated to cytotoxic species that are more active than the parent compound; however, actual conversion to metabolites was not demonstrated nor were metabolite species identified. This study is at variance with other investigations which had shown that conjugation with glucoronic acid to yield inactive products represents the major pathway of biotransformation of FAA. This study by Cummings *et al.*[21] pointed out that the therapeutic window of plasma concentration is easily reached in humans but significant activity had not so far been seen. The authors suggested at that time it was possible that critical drug concentrations were still not being achieved close to or in tumor cells or other sites of action because of competition for metabolism, protein binding and urinary excretion of the active form of the drug. It is likely, however, that this is not the case. Studies from the UK[23] and Italy[24] have demonstrated that tumor FAA levels in patients are similar to those achieved in mouse studies, so the lack of activity in human tumors is unlikely to be due to reduced penetration of FAA.

In an attempt to explain the site dependent anti-tumor activity of FAA in an experimental system, Bibby *et al.*[25] examined tissue levels of FAA in tumor and non-tumor bearing animals. It was not possible to explain the resistance of lung deposits to FAA on the basis of altered pharmacokinetics so it appeared that the activity of FAA against subcutaneous tumors relied in part on specific features of tumors at this site.

Vasculature and perfusion

From the evidence described so far it appeared that direct cytotoxicity was not involved in the mechanism of action of FAA *in vivo* and it was more likely that there was an indirect, possibly host-mediated, mechanism. In an early study in this laboratory,[8] we had suggested that the establishment of a blood vasculature system may be an important factor in tumor responses to LM 985 *in vivo* and hence FAA. The importance of vasculature in the mechanism of action of FAA was further strengthened by a presentation in 1987 which showed a good correlation between tumor vascular composition and tumor weight inhibition in a subcutaneously implanted colon adenocarcinoma MAC 15A in mice.[26] In order to further elucidate the involvement of tumor vasculature in the mechanism of action of FAA the investigation subsequently followed histological changes and

effects on blood perfusion in a well-differentiated slow growing cystic adenocarcinoma MAC 26. Tumor blood volume was measured using an Evan's blue perfusion technique and a single intraperitoneal therapeutic dose of FAA (200 mg/kg), which caused 90% tumor volume inhibition, produced a 60% reduction in tumor blood volume. This was first demonstrated 4 h after treatment and persisted for at least 24 h. Parallel histology studies demonstrated massive tissue necrosis at 24 h with necrotic changes being seen as early as 2 h after treatment. At that time we offered the vascular mechanism as a possible explanation for the lack of response seen in the clinic where the biology of systemic disease may be very different from that of subcutaneous tumors in mice.[27]

Around this time the Wayne State University Group in the US demonstrated an FAA induced reduction of tumor blood flow in Glasgow osteogenic sarcomas following treatment with FAA, and they suggested that this may also play an important role in its mechanism of action. The technique they used was *in vivo* nuclear magnetic resonance (NMR) spectroscopy using ^{2}H-NMR to monitor the washout of D_2O injected directly into the tumor both before and 4 h after treatment with 200 mg/kg FAA. Use of the fluorescent dye bis-benzamide Hoechst 33342 allows visualization of the functional vasculature of tumors in histological sections and in our laboratory the percentage vasculature of MAC 26 tumors was determined by measuring the volume enclosed by surrounding halos of Hoechst 33342 labelled endothelial cell nuclei. These studies showed that MAC 26 subcutaneous tumors have a good functional vasculature (Figure 3) and this is rapidly shut down following FAA treatment.[28]

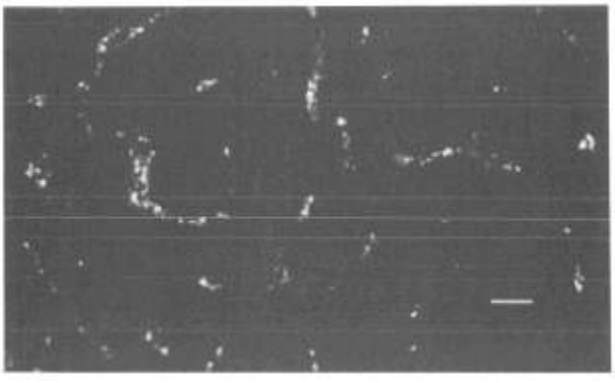

Figure 3. Functional vasculature of MAC 26. Frozen section 1 min following intravenous administration of Hoechst 33342 (bar = 25 μm).

Clinical phase I trials had shown that hypotension was a problem with FAA and, in view of the studies demonstrating vasculature to be important, it was necessary to establish whether hypotension was involved in the vascular shutdown mechanism. Blood pressure measurements following FAA treatment showed that at therapeutic doses FAA had no effect on systemic blood pressure in mice (Table 4). The vasodilator hydralazine produced a similar vascular shutdown in MAC 26 and other subcutaneous tumors but no significant anti-tumor activity. Hydralazine produced a drastic drop in blood pressure (Table 4) illustrating that in this case the vascular shutdown is as a result of the steal effect showing the FAA vascular shutdown to be due to a different mechanism.[29] Electron microscopy revealed that treatment with FAA resulted in epithelial cell separation and stromal damage as early as 4 h after treatment but no obvious endothelial cell damage.[30]

Since these earlier studies, a number of other laboratories have demonstrated vascular effects

Table 4. Influence of FAA or hydralazine on mean blood pressure and heart rate in mice

Treatment	Mean blood pressure			Heart rate		
	pre-dose (mm Hg)	post-dose (mm Hg)	change (%)	pre-dose (b.p.m.)	post-dose (b.p.m.)	change (%)
FAA 200 mg/kg	70	64[a]	−9	530	480[a]	−9
FAA 400 mg/kg	74	76[a]	+3	560	505[a]	−10
FAA 600 mg/kg	68	50[a]	−26	420	440[a]	+5
Hydralazine 5 mg/kg	78	42[a]	−46	530	620[a]	+17
Hydralazine 10 mg/kg	64	38[a]	−41	480	600[a]	+25
Hydralazine 20 mg/kg	68	32[b]	−53	620	530[b]	−15

[a]30 min post-dose.
[b]25 min post-dose

following FAA treatment. Hill *et al.*[31] used $^{86}RbCl$ extraction to measure relative blood flow in six experimental murine tumors and their data showed that the drug induced a drop in tumor perfusion within 6 h of treatment in all of the tumors examined and that this was correlated with growth delay measured. A study by Zwi *et al.*[32] using Hoechst 33342 confirmed the vascular shutdown demonstrated earlier, but they also used a double label fluorescence technique to show a significant decrease in blood flow in both subcutaneous colon 38 and intramuscular EMT-6/Ak tumors as early as 15 min after intravenous treatment with 1.2 mmol/kg FAA with progressively enlarging zones of perfusion failure.[32] The same group in 1990[33] used EMT-6 multicellular spheroids introduced into the peritoneal cavity of mice and allowed these to become vascularized, resulting in solid spherical tumors. This interesting study utilized the presence of both vascular and avascular components in each spheroid to study the role of vasculature in the anti-tumor action of FAA. Eighteen hours after treatment with FAA (0.8 mmol/kg) the vascularized core became necrotic and hemorrhagic while the outer avascular zone remained viable. They concluded that the results provided further evidence that FAA killed blood vessel dependent tumor cells by interrupting the tumor blood supply. The model system used would be extremely useful for evaluating compounds with a possible vascular mechanism of action. Peters *et al.*[34] demonstrated that tumor vessels are not uniformly affected by FAA. Either the vessels themselves differ in some way or the relationship between tumor vasculature, parenchyma and infiltrating host cells is non-uniform. These authors suggested that the disappointing results obtained using FAA in clinical trials should not discourage the use of this and other novel drugs as tools to investigate the complexities of tumor vasculature and parenchyma/stromal relationships. Honess and Bleehen[35] looked at the effect of FAA on blood flow in tumors and normal tissues in mice. They examined skin, muscle, lung, liver, spleen and kidney, and concluded that FAA affected blood flow in only one normal tissue assayed, i.e. spleen. The specificity of the vascular shutdown in tumors was further strengthened by a study of Mahadevan and Hart[36] who compared tumor microvasculature with new vasculature using an implanted sponge technique. They concluded that the effect of FAA was specific for tumor microvasculature and was not directed simply at new vessels, since a similar treatment in animals with implanted sponge induced granulation tissue had no effect on $t_{1/2}$ times when they used radioactive xenon (^{133}Xe clearance) to monitor local blood flow.

There have been suggestions that clotting mechanisms are implicated in the mechanism of FAA induced vascular shutdown. Rubin *et al.*[37] suggested that FAA prevented the growth of transplantable tumors in mice primarily by altering the platelet function needed for establishment of tumor growth because FAA inhibited platelet adhesion (demonstrated by the effect of FAA on ristocetin induced platelet agglutination). Studies by Murray *et al.*[38] in mice showed that following a dose of 300 mg/kg of FAA the clotting times were significantly reduced at 15–30 min in both tumor and non-tumor bearing mice of both CBA and WH strain. Detailed studies on coagulation in the CBA strain (with or without CaNT tumors) indicate that in tumor bearing animals the initial decrease in clotting time is followed 4–6 h later by an increase in clotting time, thrombin time and fibrin degradation product levels. Platelet counts of tumor bearing mice also decreased significantly over this period. The authors concluded that all the data from these coagulation tests on mice with CaNT tumors were consistent with the hypothesis that intravascular coagulation occurs following treatment with FAA and that vascular occlusion in tumors as a result of FAA induced coagulopathy may contribute to tumor regression. This same group[39] later demonstrated that in human umbilical vein endothelial cells, FAA at 800 μg/ml for 4 h resulted in a 3- to 11-fold increase in pro-coagulant activity. They showed that this increase was due to enhanced tissue factor expression on the endothelial cell surface. From this study the authors concluded that they believed the vascular effect of FAA in murine solid tumors was a major component of its anti-tumor action, but in addition they hypothesized that vascular occlusion results from a combination of events; changes in endothelial barrier function leading to increased vascular permeability and increased endothelial pro-coagulant activity potentiation by TNF-α, both observed *in vitro* with human endothelial cells.

Thomsen *et al.*[40] showed that FAA and structurally related analogs increase plasma nitrite plus nitrate (NO_2^-/NO_3^-) levels in mice. They demonstrated that the presence of palpable subcutaneous colon 38 tumors significantly enhanced the levels and a clear relationship was found between increased plasma NO_2^-/NO_3^- levels and tumor growth delays induced by FAA and active xanthanone-4-acetic acid (XAA) analogs. The

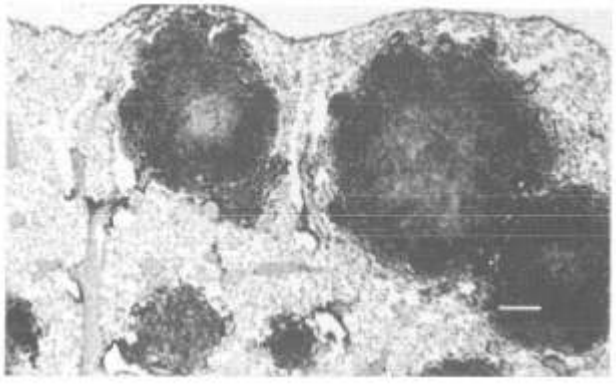

Figure 4. MAC 15A nodules in the lung following intravenous injection of MAC 15A cells. Hematoxylin & eosin (bar = 250 μm).

inactive analog 8-methyl-XAA failed to increase plasma N_2^-/NO_3^- levels. The authors suggested that nitric oxide may contribute to tumor cell death by two mechanisms, i.e. alteration of blood flow leading to tumor ischemia and direct tumor cell killing.

As described earlier,[16] studies in this laboratory had demonstrated the importance of site on the chemosensitivity of transplantable murine colon tumors to FAA. One of the tumors employed in this study was the MAC 15A and the target for systemic therapy is shown in Figure 4. Utilization of the Hoechst 33342 method for visualizing functional vasculature demonstrates subcutaneous MAC 15A cells to have a reasonable blood vascular supply (Figure 5). However when the same cells are injected intravenously or intrasplenically and tumors are allowed to develop in the lungs or liver there is no evidence of neovascularization within the body of the tumors. Sequential frozen sections, one stained with hematoxylin & eosin and the other with Hoechst 33342, are shown for a lung tumor deposit in Figure 6 and a liver tumor deposit in Figure 7. Since only subcutaneous tumors respond

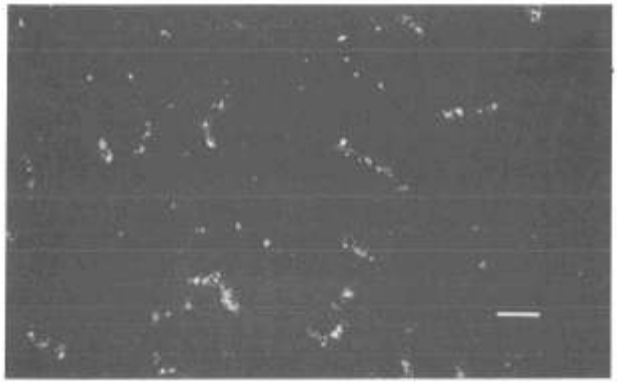

Figure 5. Functional vasculature of a subcutaneous MAC 15A tumour visualized with Hoechst 33342 (bar = 25 μm).

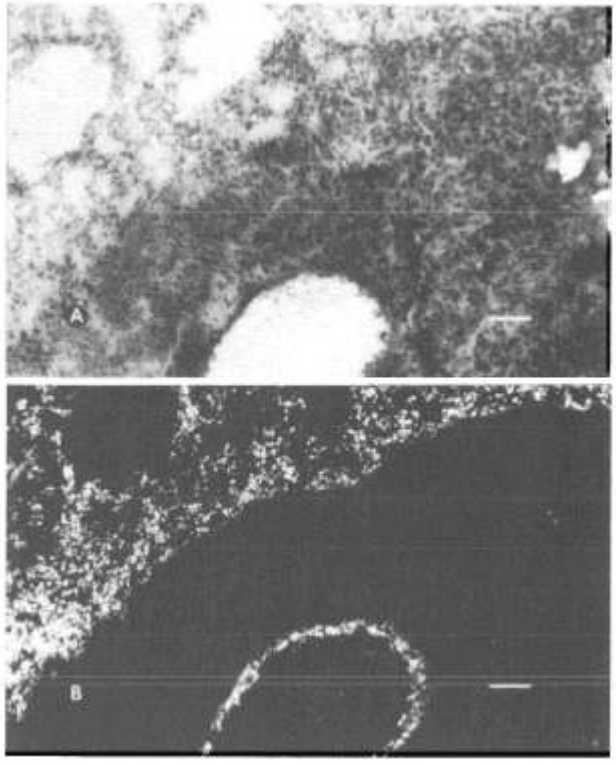

Figure 6. Frozen section through a large MAC 15A lung deposit: (a) hematoxylin & eosin and (b) Hoechst 33342 (bar = 25 μm).

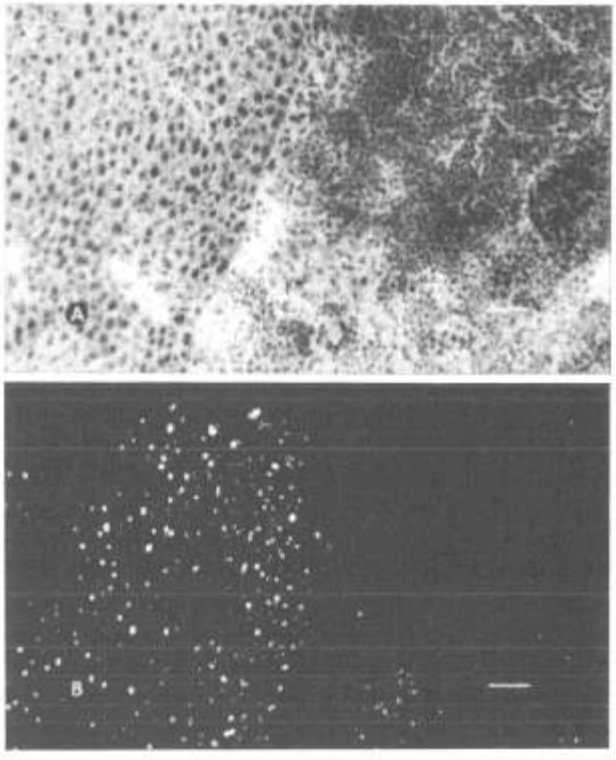

Figure 7. Frozen section through a large MAC 15A liver deposit: (a) hematoxylin & eosin and (b) Hoechst 33342 (bar = 25 μm).

to FAA this study presents very clear evidence for the requirement for new tumor vessels for responses to occur.

Immune effects

Relatively early on in the study of FAA, Ching and Baguley[41] were interested in identifying a possible host mediated mechanism of toxicity. In an experimental study to examine possible immune mechanisms they showed that FAA stimulates natural killer (NK) activity in spleen cells in mice. The activity of FAA was measured using a ^{51}Cr-release assay employing the YAC-1 lymphoma line as a target cell population and they found that FAA is as potent a stimulator of NK activity as the interferon (IFN) inducer polyinosinic–polycytidylic acid (poly I–C). The highest NK activity was observed after 24 h and the authors approximate the kinetics of induction of NK activity to the kinetics of induction of hemorrhagic necrosis of the colon 38 tumor. Wiltrout *et al.*[42] showed that FAA potently augments NK activity in the spleen, liver, lungs and peritoneum of mice in a dose dependent manner after intravenous or intraperitoneal administration. The augmented activity peaked by 24 h after FAA injection and returned to normal after 6 days. Combined treatment of established murine renal cell carcinoma with FAA and recombinant interleukin-2 (or IL-2) results in up to 80% long-term survival whereas FAA or rIL-2 alone was unable to induce any long-term survivors. The optimal dose of rIL-2 required for use with FAA was between 10 000 and 30 000 U/day. Further studies demonstrated that the regimen of FAA plus rIL-2 administration that was effective in treating established murine renal cancer also induced a more potent augmentation of NK activity than did either compound alone. Subsequent studies revealed that the therapeutic effectiveness of FAA plus rIL-2 was significantly reduced when tumor bearing mice were treated with anti-asialo GM1 serum. These authors suggested that because pre-clinical models had usefully predicted clinical utility with other treatment approaches using rIL-2, the results of this study suggested that FAA and rIL-2 should be expeditiously evaluated for the treatment of cancer in humans.

A preliminary study by Urba *et al.*[43] described the ability of FAA to augment NK cell activity in the peripheral blood of humans. They measured NK cell activity and IFN production in six patients receiving FAA for treatment of a variety of malignancies. NK cell activity was significantly increased in three out of six patients receiving FAA (6.4 g/m) by 3 h intravenous infusion. There was no evidence of induction IFN-γ but three of four patients tested had evidence of induction of type I IFN as measured in a virus neutralization assay. A further study[44] demonstrated that *in vivo* administration of FAA induces, in a dose dependent manner, high levels of serum IFN within 4 h in BALB/c, C57BL/6 and BALB/c nude mice. Antibody neutralization studies indicated that FAA induced IFN of the α/β type while molecular hybridization studies demonstrated that FAA rapidly stimulated the production of IFN-α mRNA in splenic leukocytes. *In vivo* administration of anti-IFN-α/β antibodies to FAA treated mice inhibited the FAA induced augmentation of splenic NK cell activity at 4 h. The authors suggested that this was evidence that FAA mediates its anti-tumor effects indirectly by immunomodulation as well as directly by anti-proliferative or cytotoxic activity.

Ching and Baguley[45] attempted to assess the role of NK cells in FAA induced tumor necrosis. They again showed that NK cell activity in the spleen was augmented 4 h after FAA treatment, and when spleen cells were cultured with IL-2 to induce the production of lymphokine activated killer (LAK) cells, higher levels of LAK cell activity were generated by spleen cells from FAA treated animals than spleen cells from untreated control mice. Response to FAA by spleen cells from mice bearing the colon 38 tumor was compared with that of non-tumor-bearers. Activity against NK sensitive YAC-1 tumor targets was augmented to a similar degree but no activity against NK resistant P815 targets was detected. They went on to show that FAA induced hemorrhagic necrosis in the P815 tumor grown as a subcutaneous solid tumor and also hemorrhagic necrosis was induced by FAA in colon 38 tumors growing in mice which had been depleted of NK activity by treatment with anti-asialo GM1 antibody. They concluded that although NK activity could be involved in the long-term host response to the tumor, it did not appear to be a major determinant of FAA induced hemorrhagic necrosis. The same authors,[46] in an attempt to elucidate the clearly complex immune effects of FAA, attempted to characterize tumor infiltrating leukocytes (TIL) in the colon-38 tumor by measuring their levels of cytotoxic effector activity before and after FAA treatment. Between 1 and 2 $\times$ 10^5 TIL were isolated per gram of tumor and these were made up of mainly small lymphocytes and macrophages. Spontaneous activ-

ity against YAC-1 and P815 targets was tested in the 4 h ^{51}Cr-release assay for lymphoid cytotoxic effector cells. High levels of activity were exhibited by TIL against both P815, which is resistant to NK cells, and to NK sensitive YAC-1 cells. They showed that, in contrast, splenic cell populations contained only NK cell activity. Within 1 h of intraperitoneal administration of FAA (330 mg/kg) cytotoxic effector cell activity of the TIL population was dramatically depressed, remaining low during the time which extensive tumor necrosis became evident. In contrast splenic NK activity was unchanged at 1 h and elevated at 4 h. The authors concluded that this decrease in lymphoid killer activity of the TIL population following treatment argued against the primary involvement in these effector cells mediating the anti-tumor action of FAA.

The hemorrhagic necrosis produced in mouse tumors by FAA has been likened to the effects seen with TNF-α where massive hemorrhagic necrosis occurs starting as early as 2–4 h after administration.[9] Since TNF is a product of activated macrophages, Ching and Baguley[47] examined the influence of FAA on the tumoricidal activity of peritoneal macrophages *in vitro*. Lysis of tumor targets was measured using the standard 18 h ^{51}Cr-release assay for activated macrophages. Lytic activity increased with increasing concentrations of FAA up to 100 μg/ml before reaching a plateau. At 80 μg/ml FAA, 3-fold fewer peritoneal exudate cells were necessary to obtain the same level of activity as in control cultures without FAA. The lytic activity was mediated by Thy-1 negative and glass adherent cells in the peritoneal exudate population, and was inhibited by dexamethasone. The activity of peritoneal exudate cells against several different tumor targets (P815 mastocytoma, YAC-1 lymphoma, P388 lymphoma and a Lewis lung carcinoma cell line) were all enhanced by FAA. The authors concluded that the results showed that FAA could enhance the lytic potential of peritoneal macrophages *in vitro* to kill a range of tumor cells.

It appeared then that FAA had a broad immune modulatory activity stimulating both lymphoid and macrophage cytotoxic effector cells. As a result of these observations on the immune system, Ghosh *et al.*[48] measured NK cell activity and lymphokine activated killer (LAK) cell cytotoxicity in patients receiving rIL-2 and FAA for treatment of progressing metastatic melanoma. NK activity was increased in 23 of 26 patients and LAK activity induced in 13 of 26 patients. However, levels of cytotoxicity in that study were not significantly greater than previous investigation using rIL-2 alone. LAK cell precursors, demonstrated by *in vitro* incubation of pre-treatment lymphocytes with IL-2 and subsequent cytotoxicity, were no different in the patients compared with normal controls. The authors analyzed the cell surface phenotypes but failed to reveal any significant change in the cell populations examined, including IL-2R and Leu 19. They were surprised by these latter observations because *in vitro* treatment with IL-2 results in large increases in IL-2R positive cells and Leu 19 cells so they concluded that FAA might suppress these cells. The study showed no significant correlation between clinical outcome and NK or LAK cell induction although the patient with the highest NK value did show a complete response.

Working from a baseline that FAA acts as an immunomodulator by augmenting NK activity in both humans and rodents after *in vivo* administration, and that FAA synergizes with IL-2 for the treatment of murine renal cancer, Mace *et al.*[49] initiated studies to investigate whether the *in vivo* administration of FAA would alter the expression of cytokine mRNA in leukocytes. They used splenic leukocytes or liver non-parenchymal cells from untreated and FAA treated mice as a source of their RNA for Northern blot analysis. IFN-α and IFN-γ mRNA in the spleen was up-regulated within 1.5 h after treatment, with peak induction occurring by about 2 h. An up-regulation of TNF-α mRNA was detected in the spleen by 0.5 h after treatment with peak induction occurring by 1–1.5 h. Induction of TNF-α mRNA was also detected in hepatic non-parenchymal cells. They did not detect up-regulation of splenic mRNA for TNF-β, or IL-1α or β, or IL-2 after FAA administration. IFN and TNF activities were detectable in the serum by bioassay immediately following the appearance of mRNA in FAA treated mice. They showed that the up-regulation by FAA of cytokine mRNA and the corresponding serum protein was strictly dose-dependent with substantial induction of both mRNA and proteins occurring only at FAA doses of 150 mg/kg or higher. This dose range has been shown to be the minimum required for immunomodulatory and immunotherapeutic effects. The evidence that FAA acts as a potent inducer of at least three cytokines *in vivo* suggested that the immunomodulatory and immunotherapeutic effects of FAA may be partially mediated by these induced cytokines.

Mahadevan *et al.*[50] went on to further evaluate the role of TNF in FAA induced tumor vascular shutdown. They showed that FAA induced

vascular shutdown in colon 26 tumors was virtually abolished in tumor bearing mice pre-treated with an antiserum against TNF, while no such effect was observed in controls pre-treated with non-immune serum. Furthermore they showed that *in vitro* FAA was seen to induce TNF secretion from murine peritoneal cells and splenocytes. This evidence is very suggestive that FAA induced tumor vascular shutdown in the colon 26 tumor is mediated by TNF.

Interest in the immunological effects of FAA continued and a preliminary study in this laboratory[51] examined the influence of immune status on FAA activity by comparing responses of two transplantable colon tumors, MAC 16 and 26, in normal NMRI mice with those growing in thymectomized NMRI mice and nude mice. At a dose of 200 mg/kg on day 2 and day 9 both tumor types were highly responsive to FAA in their normal NMRI host (greater than 50% cures) but neither tumor exhibited significant growth delay in thymectomized NMRI or nude hosts. Histological examination of treated tumors revealed significant areas of hemorrhagic necrosis in all three hosts, suggesting a clear immunological component in the mechanism of FAA which is necessary to progress hemorrhagic necrosis to measurable anti-tumor responses. Similar results have been seen with a third tumor, MAC 15A (Table 5).

Pratesi *et al.*[52] investigated the activity of FAA against colon 26 murine tumors grown subcutaneously in euthymic and athymic mice. FAA was active in euthymic but not in athymic mice. They further demonstrated that NK cell activity was increased in both mouse strains indicating a lack of major involvement of this lymphocyte population in FAA efficacy. In euthymic mice, tumor-specific T cells were activated and after *in vivo* depletion of lymphocyte sub-populations L3T4 and Lyt-2, tumor inhibition by FAA was abrogated. Anti-tumor efficacy of FAA was also reduced when the treatment was followed by injection of anti-TNF-α antibodies thus confirming the work of Mahadevan *et al.*[50] These authors also suggested that when anti-TNF-α antibodies were given after FAA treatment, the toxicity was greatly reduced.

Further studies partly carried out in this laboratory[53] examined the activity of FAA against two human colon tumor xenografts, HT-29 and COBA. There was no measurable anti-tumor activity against either tumor, and pharmacokinetic studies in HT-29-bearing mice showed that the lack of response could not be explained on the basis of limited drug bioavailability as measured plasma and tumor levels of FAA were similar to those seen in responding murine colon tumors. Another study in this laboratory examined the vasculature of mouse tumors and a human tumor, HT-29, in nude mice. Histological examination of the HT-29 unresponsive tumors revealed no obvious hemorrhagic necrosis or vascular shutdown. On the other hand, the three MAC tumors described earlier, which were also poorly responsive in nude hosts, showed significant changes in mean percentage functional vasculature in these hosts following FAA treatment.[54]

A recent key paper from Futami *et al.*[55] has compared the expression of cytokine genes in mouse splenic leukocytes with human peripheral blood leukocytes. They investigated the ability of FAA treatment to directly induce cytokine mRNA expression in total mouse splenic leukocytes and selected leukocyte subsets as well as in total human peripheral blood leukocytes. Analysis of RNA isolated from FAA treated mouse splenic leukocytes demonstrated that treatment with 100 μg/ml or greater of FAA induced expression of TNF-α mRNA by 1 h, and induced maximal expression of TNF-α, IFN-α and IFN-γ mRNA within 3 h. The expression of all cytokine genes was diminished by

Table 5. The anti-tumor activity of FAA against MAC tumors grown (subcutaneously) in both NMRI and nude mice

	Tumor description	Dose (mg/kg)	Schedule (days)[a]	% T/C	
				NMRI	nude
MAC 15A	rapidly growing, poorly differentiated solid tumor grown subcutaneously	200	2,9	10	96
MAC 16	slow growing, moderate to well differentiated cachexic solid tumor grown subcutaneously	200	21,28	0	100
MAC 26	slow growing, well differentiated solid tumor grown subcutaneously	200	21,28	18	100

[a]Days after tumor transplantation.

6 h. Biological activity was detected in supernatants of mouse splenic or peripheral blood leukocytes after treatment with FAA. The authors suggested that these results correlated well with the previously reported induction of cytokine mRNA genes and biological activity by FAA *in vivo*. In contrast though, FAA did not induce detectable mRNA expression or cytokine protein expression by human peripheral blood leukocytes under similar conditions. The results demonstrate that FAA can directly stimulate cytokine gene expression in mouse but not in human leukocytes. They went on to further characterize the mouse leukocyte subsets. The authors concluded from the paper that their results suggested that the failure of FAA to induce profound immune modulation or therapeutic responses may relate to an inherent difference in sensitivity to FAA between human and mouse cells. They further suggested that flavonoid compounds or analogs that stimulate cytokine gene expression in human cells might be therapeutically active in cancer patients.

Combination therapy

As a result of the dramatic vascular effects of FAA described, a number of studies have addressed the possibility of combination of FAA with other therapies. The interaction of FAA with radiation was studied in murine KHT sarcoma.[56] FAA produced a rapid increase in tumor hypoxia with a dose of 200 mg/kg inducing close to 100% radiobiological hypoxia when given 1 h prior to irradiation. Complete tumor hypoxia was still apparent 18 h after treatment with FAA. The authors showed that doses below 100 mg/kg did not produce the hypoxia. A previous study by Sun and Brown[57] had shown that FAA markedly potentiated the cytotoxicity of 3-amino-1,2,4-benzotriazine 1,4-di-*N*-oxide (SR 4233) in the SCCVII and RIF-1 tumors. Brown[58] went on to discuss the rationale of manipulating blood flow with vasoactive drugs, amongst which he included FAA, in targeting bioreductive drugs to tumors. The overall conclusion of his review was that although it may not be necessary to use vasoactive agents to increase tumor hypoxia, if such agents produce only a modest increase in tumor hypoxia, then the killing by the hypoxic cytotoxic will be magnified many fold and greater tumor cell killing would be produced than could have been obtained in fully oxygenated tumors. Having shown that TNF and FAA can induce hypoxia in the KHT sarcoma as described earlier, Edwards *et al.*[59] went on to investigate whether TNF and FAA could potentiate the anti-tumor activity of SR 4233 and 1-(2-nitro-1-imidazolyl)-3-(1-aziridinyl)-2-propanol (RSU 1069) or mitomycin-C (MMC) in the KHT and RIF-1 sarcomas. The authors demonstrated the FAA potentiated SR 4233 in both tumors, thus confirming the results of Sun and Brown[57] using the SCCVII tumor model. However FAA failed to enhance the activity of RSU 1069 or MMC in either of the tumors. TNF potentiated both RSU 1069 and SR 4233 to a similar extent in the KHT tumor and they observed some potentiation for MMC. The authors had already shown with other methods of hypoxia induction, such as tumor clamping and administration of hydralazine, that the greatest potentiation was seen with RSU 1069.[60] These results show that the potentiation of the bioreductive drugs examined is not simply due to hypoxia. The vascular shutdown with FAA is likely to have more profound microenvironmental effects which will alter also the molecular enzymology responsible for activation of the bioreductive agent.

Horsman *et al.*[61] examined the effect of combining FAA and hyperthermia on the growth of a C3H mammary carcinoma in mice. They examined the effect of FAA (200 mg/kg, i.p.) on the response of a C3H tumor to hyperthermia (42.5°C) for 1 h but also on blood perfusion using an ^{86}Rb-extraction technique. FAA substantially increased the response of the tumor to hyperthermia and the effect was dependent on the FAA dose used and the time interval between the two modalities, and furthermore the enhancement correlated with the ability of FAA to decrease tumor blood flow.

In view of the earlier described observation that experimental colon tumors failed to respond in nude hosts even though vascular shutdown and hemorrhagic necrosis still occurred,[54] in this laboratory we attempted to exploit the vascular damage occurring in nude mice by combining FAA with an investigational clinically active nitrosourea, Tauromustine (TCNU).[62] The study showed that the damage to the tumor produced in nude hosts by FAA was enhanced by combination with TCNU. Both TCNU and FAA as single agents produce only minor growth delays against the MAC 26 tumor, whereas administration of a well tolerated dose of TCNU (20 mg/kg), 1 min before FAA (200 mg/kg), produced significant tumor regression and a 15 day regrowth delay. All of these studies suggest that if the vascular effects seen in experimental tumors could be produced in clinical disease, combination

with other therapy could significantly increase the overall anti-tumor effects.

Conclusions

There is still an urgent need to develop drugs which are active in common solid cancers but which are not limited in their usage by life threatening host toxicity. The identification of FAA led for the first time to an agent with very broad, perhaps nearly universal solid tumor activity in mice, and as described earlier, a considerable amount of effort has gone into characterizing the activity in an attempt to identify a mechanism of action. The spectacular anti-tumor activity seen in mouse systems was not accompanied by conventional toxicities associated with anti-cancer drug therapy, although a short report by Zwi *et al.*[63] described necrosis and thrombosis in non-tumor tissues at therapeutic doses, indicating the necrotizing action of FAA was not entirely specific for tumor tissues.

As a result of the pre-clinical solid tumor activity FAA and its ester have been evaluated in clinical trials. They did not cause myelosuppression or major organ toxicity but unfortunately there has been no indication of anti-tumor activity. The activity of FAA in murine tumors depends on a number of factors:[64] (i) achievement of adequate drug concentrations within the therapeutic window, (ii) establishment of an adequate blood vasculature to the tumor and (iii) a competent immune system. The immune effects of FAA are complex and still undergoing evaluation. The observations[55] that FAA was unable to induce detectable mRNA expression or cytokine protein expression in human peripheral blood leukocytes is clearly important and must be relevant to the lack of response by human tumors. With regard to the vascular studies, it is clear that most of these investigations have examined the effects of FAA in subcutaneous transplantable tumors in mice. It is highly unlikely that the vasculature of these tumors is similar to that seen in clinical tumors in systemic sites and these, after all, are the target for chemotherapy. Studies with this compound have taught us lessons about pre-clinical evaluation and it is clear that in order to realize the potential of anti-cancer agents, there is a need to consider both direct and indirect effects in a drug development programme. Experience with FAA has taught us that we must use model systems which are appropriate for clinical disease. In this respect it is important to realize that many of the currently used model systems may be totally inappropriate for the evaluation and development of novel agents that may well have an 'indirect biological action' rather than a cytotoxic one. Finally, since the experimental activity of FAA is so dramatic it may be possible, if only the complex mechanism of action could be fully elucidated, to use that information to develop analogs whereby the tumor destruction seen in solid tumors in mice could be produced in the common solid cancers in man.

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