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# Influence of site on the chemosensitivity of transplantable murine colon tumours to flavone acetic acid (LM975, NSC 347512)*

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**Summary.** A number of experimental studies have demonstrated significant responses of s.c. solid tumours to flavone acetic acid (FAA). Clinical studies to date have been disappointing, with no objective responses being seen. The present study demonstrated that the tumour site is important for the anit-tumour action of FAA against two transplantable adenocarcinoma lines (MAC) in NMRI mice. Responses were achievable only when the tumours were implanted s.c. Ascitic or systemic tumours did not respond to FAA. Experimentally achievable plasma levels of FAA were not sufficient to induce significant cell kills in either MAC 15A or MAC 26 cell lines in vitro. A poor correlation exists between in vitro and in vivo responses, as the clonogenic assay could not predict the response of the solid MAC tumours grown s.c. The in vitro data indicated that the length of exposure to FAA was important, with long exposure times being necessary for cytotoxicity to develop, in these tumour cell lines. These studies imply that more than one mechanism is involved, and it is likely that the activity of FAA against s.c. tumours relies at least in part on a specific biological feature of tumours in this site. However, it may still be possible to achieve systemic tumour cell kill in vivo by increasing drug-exposure times.

## Introduction

Flavone acetic acid (FAA) is currently undergoing clinical evaluation in malignant disease. The rationale for the submission of this compound for clinical trial was its preclinical activity against a broad spectrum of murine transplantable solid tumours that tend to be refractory to conventional cytotoxic agents [7, 19]. Previous studies in this laboratory [1, 2] have demonstrated spectacular responses in established s.c. solid, transplantable colon tumours in mice (MAC tumours). Phase I studies [16] have indicated that the peak plasma concentrations associated with experimental activity in mice are achievable in humans, although no objective responses have been seen. Differences in the pharmacology of FAA between mouse and man therefore cannot be ruled out.

Preliminary studies in this laboratory have suggested that the tumour site may be important in the response to FAA [10] and that the establishment of a tumour vasculature may be necessary for the achievement of responses [3]. Previous in vitro studies using a variety of tumour cell lines [4, 21] have indicated that high drug concentrations and long exposure times are necessary for the development of direct cytotoxicity. The present study examines the influence of concentration and exposure time on the in vitro chemosensitivity to FAA of two transplantable colon adenocarcinoma lines derived from tumours with different growth characteristics. It also describes the influence of tumour site on the in vivo response of these tumours to FAA in an attempt to explain the lack of activity seen clinically. The relatively cytotoxic effects of FAA on an established human cell line, cultured as monolayers or spheroids, were investigated with the aim of assessing the influence of the three dimensional properties of a spheroid on chemosensitivity in vitro.

## Material and methods

*Animals.* Pure-strain 6- to 8-week-old NMRI mice from the inbred colony at the Clinical Oncology Unit, University of Bradford, were housed in cages in an air-conditioned room where regular, alternate 12 h cycles of light and darkness were maintained. Animals were supplied with a pellet diet (CRM Labsure, Croydon, England) and water ad libitum.

*Tumour system.* The development of several adenocarcinomata of the large bowel in NMRI mice from primary tumours induced by the prolonged administration of 1–2,dimethylhydrazine (DMH) has been described elsewhere [9]. The series was developed by the s.c. implantation of primary adenomatous polyps from DMH-treated mice and subsequent passaging in pure-strain mice by s.c. implantation. The panel comprises a series of colon adenocarcinomas with various growth characteristics and a histology ranging from poorly differentiated tumours to well-differentiated cystic and active mucin-producing tumours. Ascites tumours were derived from several of the solid tumours by the i.p. implantation of tumour fragments.

*In vivo studies.* MAC 26 tumours were transplanted into male mice by the s.c. implantation of tumour fragments (approx $1 \times 2$ mm) in the flank. MAC 15A ascites tumour cells were transplanted into male mice by the s.c., i.p. or i.v. inoculation of $1 \times 10^6$ tumour cells in 0.2 ml physiological saline.

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*In vitro studies.* The s.c. solid MAC 26 tumours were aseptically removed from the inguinal region of the mice. Ascitic MAC 15A cells were removed from the peritoneal cavity by aseptic peritoneal washes in saline (0.9% w/v). Cell lines were derived from the solid tumours by mechanical disaggregation and were routinely maintained as monolayer cultures in RPMI 1640 tissue-culture medium supplemented with 10% foetal calf serum, sodium pyruvate (1 m*M*), penicillin/streptomycin (50 IU $ml^{-1}$, 50 μg $ml^{-1}$) and buffered with HEPES (25 m*M*). MAC 15A cell lines were derived by the inoculation of "complete" RPMI 1640 medium with a small volume of peritoneal washings containing MAC 15A cells, and these were maintained as described above.

MAC cell lines do not readily form spheroids; thus, HT-29 human colon cancer cells [13] were used for spheroid studies. These cells, which were originally derived from a human primary adenocarcinoma of the colon, were a gift from the Department of Pathology, University of Leeds, and were maintained as monolayers as described above. Spheroid formation was initiated by the seeding of approx $10^5$ cells into 75-$cm^2$ tissue-culture flasks that had previously been base-coated with 1% agar. Spheroids (250 μm in diameter) were harvested approx. 10 days after the initial seeding of cell suspensions and chemosensitivity was assessed as described below.

*Test compounds.* FAA was a gift from Lipha (Lyon) via Prof. S. B. Kaye, University of Glasgow; 5-fluorouracil (5-FU) was generously donated by Roche, UK; tauromustine (TCNU) was a gift from Leo Laboratories (Helsingborg, Sweden); and chlorambucil was donated by Dr. D. E. V. Wilman, Institute of Cancer Research (Sutton, UK). For in vivo experiments, FAA, 5-FU and TCNU were dissolved in physiological saline and chlorambucil was dissolved in 10% ethanol/arachis oil. Appropriate concentrations were used for a desired dose to be given in 0.1 ml/10 g body weight.

*Chemosensitivity.* In vivo studies: The differing morphology and growth characteristics of the two tumour lines necessitated the use of different chemotherapy and assessment protocols. As MAC 26 is a relatively slow-growing, well-differentiated tumour (tumour-volume doubling time, 4.5 days), chemotherapy was begun when the tumours had reached a size that could accurately be measured (tumour volume, approx. 40 $mm^3$), viz., approx. 18 days after implantation. Chemosensitivity was assessed twice weekly by caliper measurements of the tumour. Tumour volumes were determined by the formula $a^2 \times b/2$, where a is the smaller and b is the larger diameter of the tumour [14]. The percentage of volume inhibition was determined when tumour regrowth occurred. For MAC 15A ascitic tumour cells implanted via the i.p. and i.v. routes, anti-tumour activity was determined by comparison of the life span in treated and control groups. Deaths were recorded and the median survival times (MST) determined. The percentage of tumour-cell inhibition was calculated from a survival-inoculum curve. MAC 15A tumours grown in the s.c. site were weighed and the effects of chemotherapy estimated from the percentage of weight inhibition of tumours 14 days after implantation. Groups of ten animals were used in each case.

*In vitro studies:* The colony-forming ability of tumour cells surviving drug treatment was assessed using a slightly modified version of the Hamburger and Salmon [15] clonogenic assay. No soft agar was used in this assay, as fibroblastic contamination was minimal and colony integrity was maintained. Previous studies in this laboratory [18] have shown that a linear relationship exists between the number of cells plated and the number of colonies formed using this methodology. Single-cell suspensions derived from monolayer cultures (0.25% trypsin) were exposed to a range of experimentally achievable drug concentrations in complete RPMI 1640 and incubated at 37° C for various intervals. Following drug exposure, the cells were washed twice in Hanks' balanced salt solution (HBSS) and $2-5 \times 10^4$ viable cells (trypan blue exclusion) were plated into 25 $cm^2$ plastic flasks containing 10 ml complete RPMI 1640. After 5–7 days of incubation at 37° C, colonies of $\geqslant 50$ cells were counted using an inverted microscope and plating efficiencies were calculated for each drug exposure. Cytotoxic effects of drug treatment were expressed in terms of the percentage of survival, taking control plating efficiencies to represent 100% survival. Each assay was carried out in triplicate. Chemosensitivity studies were restricted to cultures passaged fewer than ten times (except HT-29 cells), and cells in the exponential phase of growth were used throughout.

*Spheroids.* Multi-cellular spheroids approx. 250 μm in diameter were exposed to various concentrations of FAA. Following drug exposure, spheroids were washed in HBSS before being dissociated into a single-cell suspension by trypsin (2.5%). The resulting cell suspensions were washed in HBSS and assessed for clonogenic properties as described above. Comparative studies on the effects of FAA on spheroids vs monolayers of HT-29 cells were run simultaneously.

*In vivo sensitivity of in-vitro-derived MAC 26 cells.* MAC 26/TC cells derived as previously described and established in tissue culture were reimplanted in NMRI mice by the i.p., i.v. and s.c. routes. Chemosensitivity was assessed by the same method previously described for MAC 15A cells.

## Results

### *In vitro studies*

Dose-response curves following the exposure of MAC 15A and MAC 26 cells to FAA for various intervals are presented in Fig. 1 and 2, respectively. Chromatographic analysis of culture media during drug exposures showed that FAA is stable in vitro over a 24-h incubation at 37° C. Increasing both the duration of drug exposure and the drug concentration improves clonogenic cell kill. For a given drug concentration, an increase in the duration of drug exposure increases cell kill; similarly, for a given exposure time (with the exception of 1-h drug exposures), an increase in drug concentration improves clonogenic cell kill.

The responses of MAC 15A and MAC 26 cell lines following a 1-h exposure to FAA are similar (Figs. 1 and 2). However, as the duration of drug exposure increases, MAC 26 cells are more sensitive to FAA than MAC 15A cells. To compare the effects of exposure time on the re-

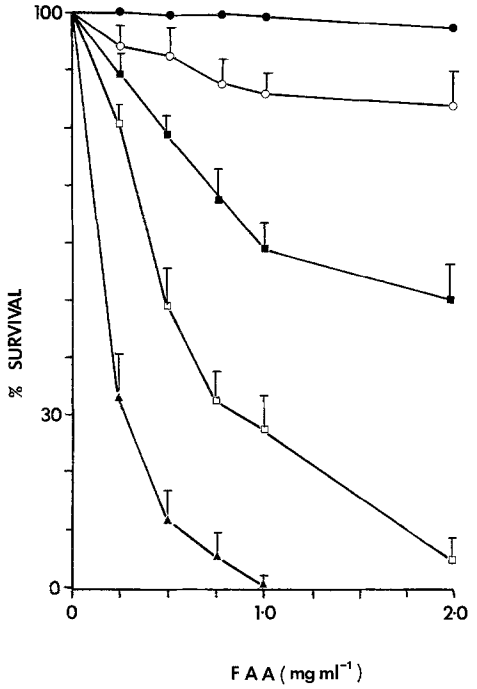


Fig. 1. MAC 15A dose-response curves following a 1-h (●——●), 2-h (○——○), 4-h (■——■), 8-h (□——□) and 24-h (▲——▲) exposure to FAA. Values presented are the means ± SD of three samples

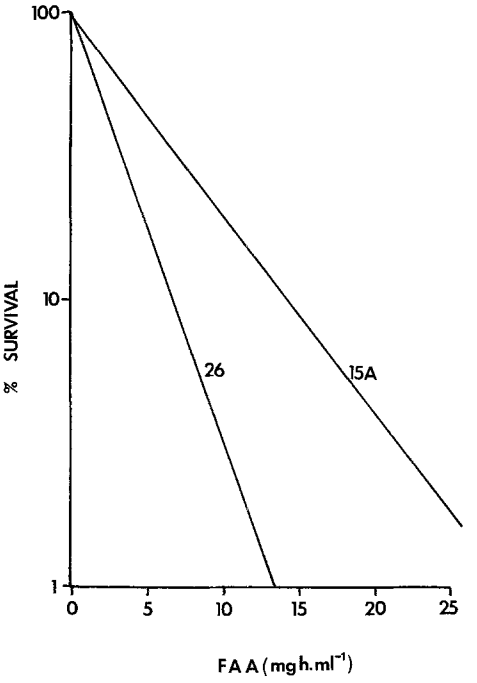


Fig. 3. The relationship between the total in vitro drug C × t exposure and the clonogenic cell survival of MAC 26 and MAC 15A cell lines

sponse of these two cell lines to FAA, the data in Figs. 1 and 2 are presented in an alternative way. The total drug concentration × time (C × t) exposure in MAC cell lines in vitro was calculated from all possible combinations of drug concentration and exposure times used in vitro and

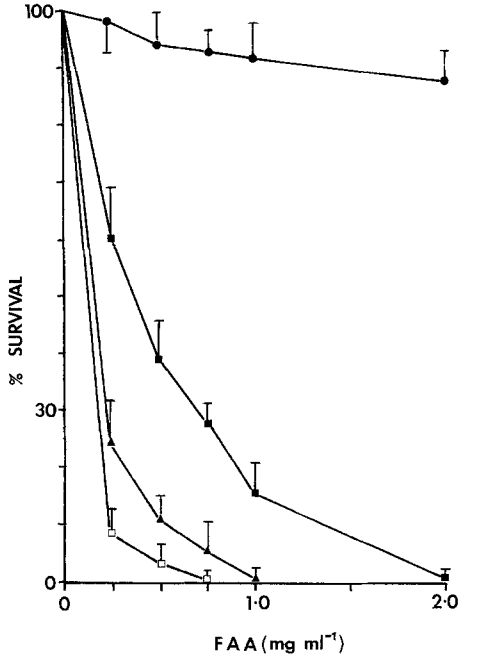


Fig. 2. MAC 26 dose-response curves following a 1-h (●——●), 3-h (■——■), 6-h (▲——▲) and 24-h (□——□) exposure to FAA. Values presented are the means ± SD of three samples

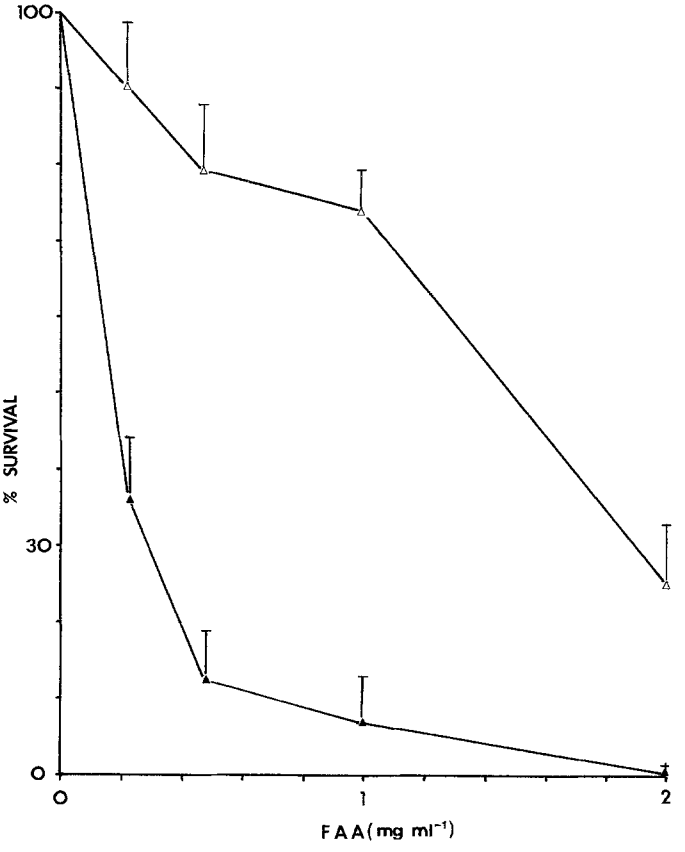


Fig. 4. The response of HT-29 multicellular spheroids (△——△) and monolayer cultures (▲——▲) following a 24-h exposure to FAA. Values presented are the means ± SD of three samples

the results were plotted against the percentage of clonogenic cell survival. Graphic representations of the relationship between cell survival and in vitro drug C × t exposures are presented in Fig. 3. In this figure, the line of best fit was determined by the linear regression analysis of semilogarithmically transformed data. The coefficients of variation for MAC 26 and MAC 15A curves were 0.85 and 0.89, respectively (individual data points were omitted from these curves in the interest of clarity). Figure 3 clearly shows that MAC 26 cells are inherently (approx. 2 times) more sensitive to FAA than MAC 15A cells; furthermore, increasing the drug C × t exposure in vitro increases clonogenic cell kill.

The response of HT-29 colon carcinoma cells exposed to FAA as multicellular spheroids or monolayer cultures is described in Fig. 4. Spheroids were (approx. 4 times) more resistant to FAA than the same cells cultured as monolayers, although fluorescence studies demonstrated that FAA penetrated to the centre of the spheroids. Histologically, HT-29 spheroids resemble MAC 15A (i.v.) lung nodules (Figs. 5 and 6). Both the tumour and the spheroid contain peripheral proliferating cells, deeper lying, non-proliferating cells and necrotic centres.

*In vivo studies*

The effects of FAA treatment on s.c. MAC 26 tumours (Fig. 7) are presented in Table 1. On these treatment schedules this tumour was highly sensitive to FAA, with 100% remission being seen at the maximum tolerated dose, which was given twice at 7-day intervals.

Inoculation of tissue-culture cells (MAC 26TC) derived from MAC 26 tumours provides the opportunity to study the chemosensitivity of the same cells at various sites. Localised tumours were produced by s.c. inoculation

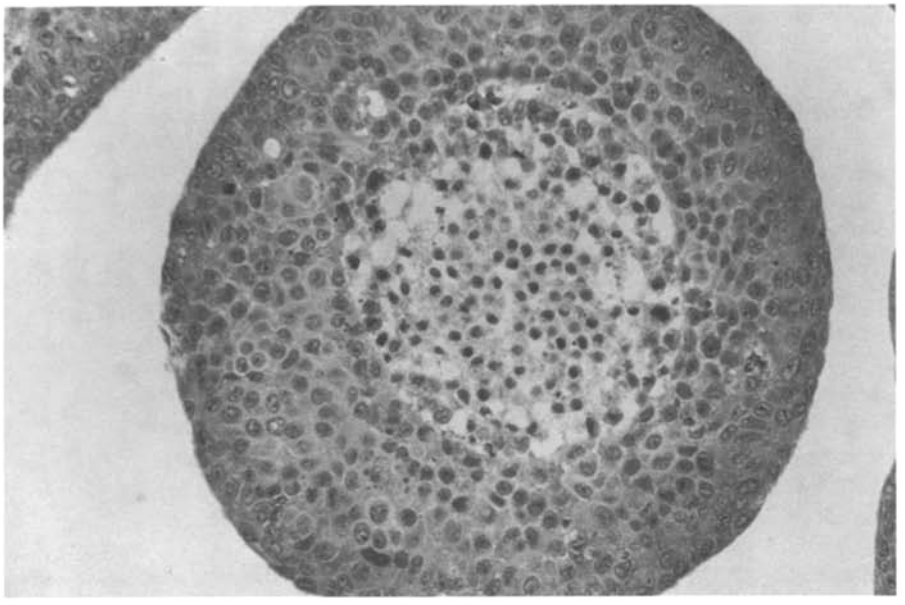

**Fig. 5.** Section through the centre of a multicellular spheroid (HT-29) stained with H&E

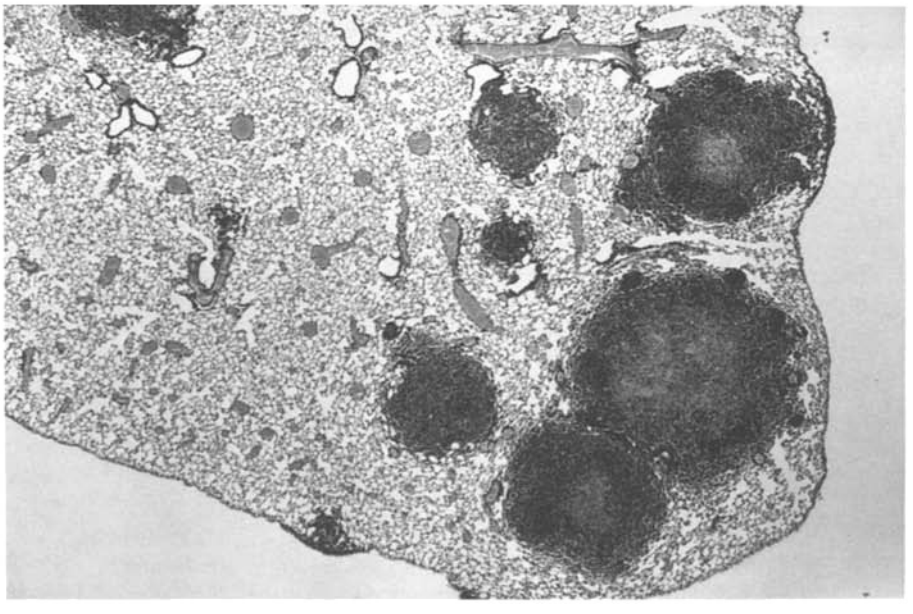

**Fig. 6.** Section through a mouse lung demonstrating spheroid-like lung nodules following i.v. inoculation of MAC 15A cells

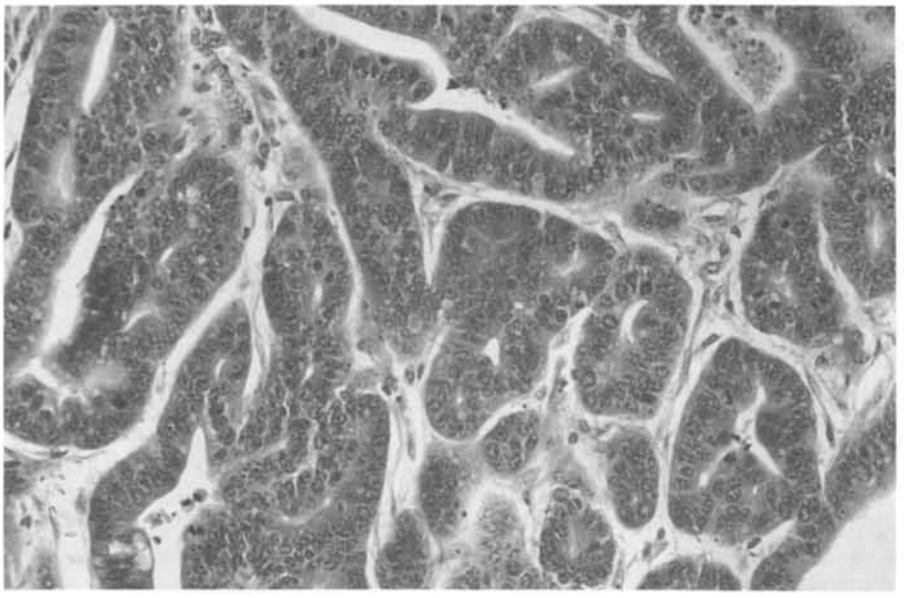

**Fig. 7.** MAC 26 tumour – routine s.c. passage stained with H&E

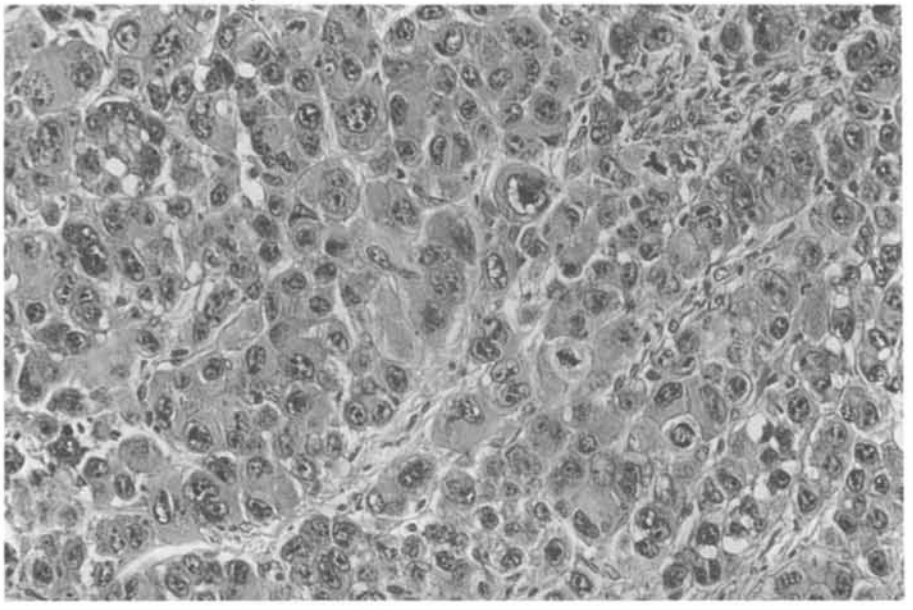

**Fig. 8.** Anaplastic MAC 26TC tumour produced by s.c. inoculation of tissue-culture-derived cells from the MAC 26 tumour (H&E)

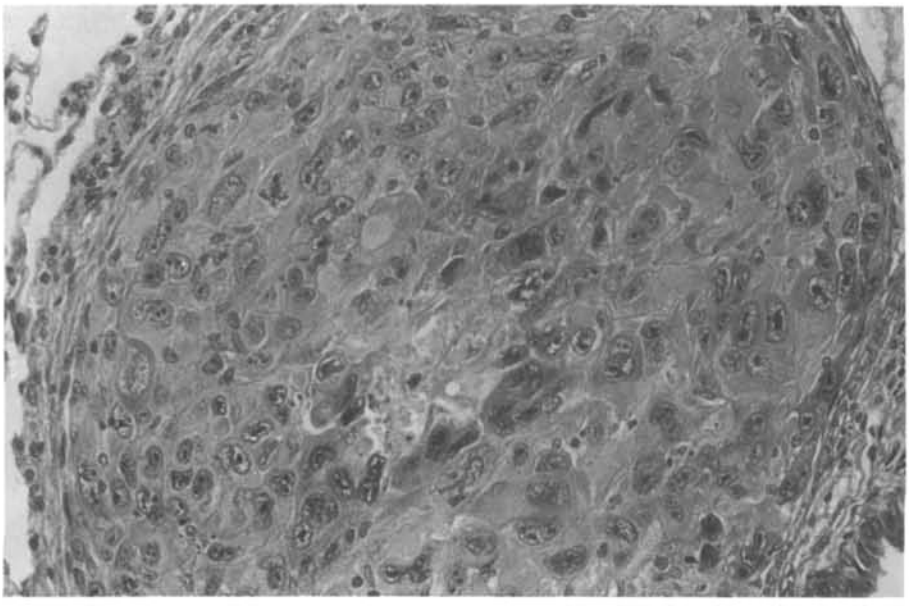

**Fig. 9.** Spheroid-like lung nodules produced by i.v. inoculation of tissue-culture-derived cells from the MAC 26 tumour (H&E)

(Fig. 8), spheroid-like lung nodules (Fig. 9), by i.v. inoculation via the tail vein and ascites tumours, by i.p. inoculation. The chemosensitivity of these tumours is presented in Table 1. The treatment of s.c. tumours by a day 2, day 9 schedule resulted in 100% remission, although s.c. tumour morphology was very different from that of the original MAC 26 tumours. No anti-tumour effects were seen in ascites or systemic tumours on this schedule, with median survival times (MST) being identical for control and treated animals (10 days for i.p. tumour cells, 32 days for i.v. tumour cells).

The *in vivo* chemosensitivity of MAC 15A cells grown in various sites is presented in Table 2. Spheroid-like lung colonies (Fig. 6) were also formed following the i.v. inoculation of MAC 15A cells. The chemosensitivity to three other agents is also presented; in none of these cases was activity in the s.c. site superior to that in ascites or systemic tumours. FAA is active against MAC 15A tumours grown s.c. but not against ascitic or systemic tumours; FAA activity was not even demonstrated in systemic tumours when the route of administration was altered to i.v. or when tumours were allowed a longer time to establish (12 days).

## Discussion

The results of this study clearly demonstrate the site-dependent sensitivity to FAA of transplantable colon tumours in mice. Significant anti-tumour effects occurred in s.c. tumours, but ascitic and systemic (largely lung deposits) tumours failed to respond. This observation is very interesting in the light of the dramatic experimental activity of FAA against solid tumours [2, 7, 19] and its lack of clinical activity to date [16]. These latter data are suggestive of possible species differences in the anti-tumour action of FAA, and certainly there are differences in clearance and metabolism between mouse and man [8]. Evidence has also been reported of the activation of FAA to cytotoxic metabolites in vitro by the addition of phenobarbitol-induced mouse liver supernatant [5]. However, the site-dependent action of FAA demonstrated in the present study suggests that the experimental anti-tumour action of the drug may be due to specific properties of s.c. tumours.

In vitro studies showed that increasing the drug C × t exposure in MAC cell lines increases clonogenic cell kill (Fig. 3). Although previous studies [7, 19] have failed to show an improvement in activity against s.c. tumours, methods aimed at increasing drug C × t exposures in vivo (e.g. continuous infusion or split scheduling) may improve systemic tumour responses, provided that toxicity does not cancel out any improvement in the therapeutic index. The present studies also showed that MAC 26 cells are inherently more responsive than MAC 15A cells when prolonged (>3-h) exposures are used, whereas little or no difference is seen with 1-h exposures (Figs. 1–3). These results are broadly reflected in the response of MAC tumours in vivo, in that these tumours (growing s.c.) are more responsive to FAA than MAC 15A tumours grown at the same site (Tables 1 and 2).

Although direct comparison of C × t values achieved in vitro with those obtained in vivo is difficult due to the different shapes of the respective curves, experimentally achievable plasma (2.52 mg h $ml^{-1}$) and peritoneal levels (3.21 mg h $ml^{-1}$) of FAA following the i.p. administration of 300 mg $kg^{-1}$ FAA [2] seem highly unlikely to be sufficient to induce significant cell kills in vitro. Whereas these results correlate with the poor response of MAC 26TC and MAC 15A tumours grown i.v. or i.p., they do not correlate with the extremely good response of MAC tumours grown s.c. Furthermore, in vitro studies could not predict the improved response of MAC tumours grown s.c. when chemotherapy was given by a different schedule (e.g. day 9 treatments vs day 2 treatment; Table 2). Therefore, the clonogenic assay could not forecast the site-dependent response of MAC tumours or the schedule dependence of MAC tumours grown s.c.

These results suggest that some mechanism other than direct cytotoxicity may be responsible for FAA-induced

**Table 1.** In vivo chemosensitivity of MAC 26 to FAA

| Tumour | Site | Dose (mg $kg^{-1}$) | Route | Day of treatment | Inhibition (%) |
|---|---|---|---|---|---|
| MAC 26 | s.c. | 200 | i.p. | 21 | 82 |
| | s.c. | 200 | i.p. | 21, 28 | >99 |
| | s.c. | 300 | i.p. | 21 | 84 |
| | s.c. | 300 | i.p. | 21, 28 | 100 |
| 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 |

**Table 2.** In vivo chemosensitivity of MAC 15 A to various chemotherapeutic agents

| Agent | Dose (mg $kg^{-1}$) | Day of treatment | Route | Inhibition (%) of tumours implanted at different sites | | |
|---|---|---|---|---|---|---|
| | | | | i.p.[a] | i.v.[a] | s.c.[b] |
| 5-FU | 120[c] | 2 | i.p. | 65 | 60 | 50 |
| Chlorambucil | 30[c] | 2 | i.p. | 75 | 70 | 25 |
| TCNU | 30[c] | 2 | i.p. | 99 | 100 | 95 |
| FAA | 250[c] | 2 | i.p. | 0 | 0 | 40 |
| FAA | 250 | 9 | i.p. | 0 | 0 | 70 |
| FAA | 250 | 2, 9 | i.p. | 0 | 0 | 90 |
| FAA | 250 | 2, 9 | i.v. | – | 0 | – |
| FAA | 250 | 5, 12 | i.p. | – | 0 | – |

[a] Calculated from survival-inoculum curves
[b] Calculated from tumour weights
[c] Maximum tolerated doses
5-FU, 5-fluorouracil; TCNU, touromustine

activity against MAC 15A tumours growing s.c. This suggestion is further strengthened by the observation that HT-29 spheroids are more resistant to FAA than the same cells cultured as monolayers. Whereas an exact explanation for the resistance of spheroids is not known, it is clear that the three-dimensional properties of the spheroid (i.e. pH, $pO_2$, nutrient, proliferation gradients have an adverse effect on FAA-induced cytotoxicity. Preliminary fluorescence microscopy studies in this laboratory have shown that FAA can penetrate spheroids (unpublished observations), thereby suggesting that drug-penetration barriers cannot likely explain drug resistance in spheroids, although concentrations have not yet been measured. Nevertheless, the drug levels that induce significant cell kills in spheroids are much higher than those that produce a comparable response in monolayer cultures ($ID_{70}$ of 45.6 mg h $ml^{-1}$ and 6.48 mg h $ml^{-1}$, respectively; Fig. 4).

A similar result in the systemic MAC 15A and MAC 26 tumours, which are structurally similar to HT-29 spheroids in cross section, may account for the resistance of these tumours in vivo, as the drug levels required to produce a response would not be experimentally achievable. By the same token, the drug levels required to produce a response by direct cytotoxicity in MAC 26 and MAC 15A tumours growing s.c. ($ID_{70}$ values of 4.5 mg h $ml^{-1}$ and 10.0 mg h $ml^{-1}$, respectively; Fig. 3) would also be unachievable in vivo. The difference between in vivo and in vitro responses suggests that the in vivo activity against s.c. tumours may be due to an indirect action of FAA that may be related to tumour architectoric.

Preliminary studies in this laboratory [3] have demonstrated that tumour vasculature may be important, as the vascular composition increases with time after the s.c. inoculation of MAC 15A tumour cells. The chemotherapeutic response of MAC 15A tumours to FAA improves with time after its implantation in the s.c. site. Of course, tumour vasculature is important for drug delivery to s.c. tumours but may itself be a target for chemotherapeutic attack [17]. Preliminary studies in this laboratory [11] and by Evelhoch et al. [12] have demonstrated that FAA alters the blood flow in experimental s.c. tumours. Further, ongoing studies in this laboratory using a fluorescent dye (Hoechst 33342) have demonstrated differences in vasculature in tumours growing in different sites in NMRI mice (unpublished observations).

The anti-tumour studies on MAC 15A tumours derived from the i.v. inoculation of cells indicate that drug penetration is not a problem with chlorambucil, 5-FU or TCNU, producing comparable responses in ascitic and systemic tumours.

Numerous possible mechanisms of action for FAA have been suggested in the literature in an attempt to explain the experimental solid-tumour activity demonstrated for this compound. Smith et al. [22] have suggested that FAA and tumour necrosis factor (TNF) may work similarly because of the haemorrhagic necrosis produced in colon 26 and colon 38 tumours. Rubin et al. [20] have suggested that anti-tumour effects in mice may be related to altered platelet function following FAA treatment. Wiltrout [23] and Ching and Baguley [6] have suggested that natural killer (NK) cells may be involved in the mechanism of action, as FAA activates NK-cell activity in mice. However, the site-dependent activity described in this paper is difficult to explain by any of these mechanisms.

In conclusion, FAA is highly active in s.c. transplantable adenocarcinomata in NMRI mice (MAC tumours), but it is only active in vitro following long exposure times. The in vivo effects of long exposure to low levels are difficult to measure in mice due to problems with drug treatment. Ascitic or systemic disease induced using the same cell lines failed to respond to FAA. The results, highly indicative of an effect in s.c. tumours, are dependent on specific biological properties of tumours in this site that may not exist in metastatic disease in man.

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