The relationship between tissue levels of flavone acetic acid (NSC 37512) and site dependent anti-tumour activity in murine colon tumours

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Summary Flavone acetic acid (FAA) is extremely active against subcutaneous transplatable tumours in mice, but disappointing there has been no demonstrable clinical activity. Previous studies have shown that lung tumour deposits are less responsive than the same cells implanted subcutaneously. The aim of this study is to examine the tissue disposition of FAA in an attempt to explain this site-dependent activity. The data show clearly that FAA clearance curves are influenced by the presence of MAC 15A tumours growing either subcutaneously or systemically. The decreased clearance of FAA from MAC 15A tumour bearing animals does not however explain the resistance of lung deposits. Neither can this be explained by differences in metabolism in these different sites. Cytotoxic metabolites have not been detected either in vitro or in vivo and their role in the mechanism of action of FAA is questionable.

Flavone acetic acid (2-phenyl-8-[carboxymethyl]-benzopyran-4-one NSC 37512) (FAA) is a compound that is capable of inducing significant responses in several murine solid tumours (Corbett et al., 1986; Flownman et al., 1986; Bibby et al., 1987b) although no objective responses have been observed in clinical trials (Kerr et al., 1987, 1989). The reasons for these discrepancies are unknown although differences in the mechanism of action of FAA between mouse and man may be significant. In murine tumour models, the action of FAA is characterised by the rapid shutdown of tumour vasculature, the appearance of haemorrhagic necrosis within 4 h of drug administration, and activity against advanced solid tumours that are normally resistant to conventional cytotoxic agents (Bibby et al., 1989a; Zwi et al., 1989). One further characteristic of FAA induced responses is that activity is dependent upon the site of tumour implantation (Bibby et al., 1989b; Giavazzi et al., 1988). Recent studies in this laboratory have demonstrated that murine colon tumours (MAC) grown subcutaneously respond to FAA (90% tumour inhibition) whereas the same cells grown systemically (as small spheroid-like nodules in the lung) or intraperitoneally (as ascites) do not respond (Bibby et al., 1989b). The reasons for these site dependent responses are unknown although differences in drug distribution between tumours with different histological characteristics and vascular supply may be a highly significant factor. The aim of this study therefore is to assess whether or not differences in FAA bioavailability can explain the spectrum of anti-tumour activity observed in the murine colon tumour model, MAC 15A. In addition, preliminary studies incorporating S-9 liver fractions or plasma from responding tumour bearing mice were conducted in vitro to further study the possibility of a cytotoxic metabolite being responsible for the anti-tumour activity of FAA as suggested by Chabot et al. (1989a).

Materials and methods

Animals

Pure strain NMRI mice (6–8 weeks of age) were used from our inbred colony. They received CRM diet (Labsure, Croxdon, England) and water ad libitum.

Test compounds

Clinically formulated FAA was a gift from Lipha (Lyon, France). It was dissolved in physiological saline at an appropriate concentration for a desired dose to be administered in 0.1 ml per 10 g body weight.

Tumour system

The development of several adenocarcinomas of the large bowel in NMRI mice from primary tumours induced by prolonged administration of 1,2-dimethyldihydrazine has been described previously (Double et al., 1975, Bibby et al., 1989a). MAC 15A ascitic tumour cells (1 × 107) were implanted via the intravenous (iv) and subcutaneous (sc) route. Tumour bearing animals were treated at day 12 with a dose of 200 mg kg⁻¹ FAA in line with protocols described by Bibby et al. (1989b).

Measurement of drug concentrations in plasma and tissues

Sample collection: Blood samples from three mice at each time point were taken by cardiac puncture under ether anaesthesia, collected into heparinised tubes, centrifuged at 1000 g and 4°C for 10 min and the separated plasma was stored at −20°C until analysis. The rapidly dissected tissues and tumours were immediately frozen in liquid nitrogen and stored at −20°C. Samples were stored for less than 1 week during which time no degradation of FAA was observed. Each pharmacokinetic profile was repeated twice using three mice per time point. For the first run, 12 time points were studied ranging from 1 min to 18 h after the administration of FAA (200 mg kg⁻¹ ip). In repeat experiments, four time points (15 min, 1, 8 and 12 h) were studied. In the subcutaneous tumour study mean MAC 15A sc tumour weight was 0.55 g ± 0.21 (range 0.19 ± 0.92 g). In the case of MAC 15A iv tumours, mice were treated approximately 14 days after the iv inoculation of 1 × 106 cells at which time there were extensive tumour deposits in the lung. Measurement of FAA concentration in the lungs of MAC 15A iv tumour bearing mice was taken to represent tumour drug concentrations as dissection of normal from tumour tissue is not possible.

Sample extraction and chromatography

FAA was extracted from plasma and tissue homogenates (10% w/v in acetate buffer pH 4.0) using C18 Bond Elut cartridges (Analytichem International). Cartridges were activated by passing ethanol (1 ml) through, under vacuum (Vac Elut system – Analytichem International) and washed with acetate buffer (1 ml). Plasma samples (50 µl plasma, 50 µl internal standard [100 µg ml⁻¹ p-dimethylnitrobenzaldehyde and 100 µl acetate buffer] and tissue samples (100 µl homogenate plus 100 µl internal standard, 10 µg ml⁻¹) were applied to each cartridge. Following a further wash (1 ml acetate buffer), FAA was eluted in 500 µl ethanol. Extraction efficiency of FAA was greater than 90%.

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FAA was measured by reverse phase HPLC, details of which have been published elsewhere (Kerr et al., 1985; Double et al., 1986). Standard curves were prepared by the addition of FAA to buffered control mouse plasma (pH 4.0) and plotting the ratio of peak area of FAA to internal standard against drug concentration. The assay was sensitive to drug concentrations down to 10 ng ml$^{-1}$.

**Pharmacokinetic parameter determination**

All pharmacokinetic parameters were calculated using standard formulae (Gibaldi, 1984). The terminal half life ($t_1$) was calculated by least square linear regression analysis of the terminal log linear phase of the curve and the elimination constant ($K_e$) determined from the relationship $K_e = 0.693 / t_1$. The area under the curve (AUC) from time 0 to the last measured time point ($t_0$) was calculated using the trapezoid rule. The remaining area from $t_0$ to $\infty$ was calculated using the equation $C / K_e$ where $C$ equals the concentration at $t_0$. Total body clearance ($C_l$) was calculated as dose/AUC. Volume of distribution was calculated as dose/$C_0$ where $C_0$ is the concentration at time 0.

**Plasma protein binding**

Protein binding of FAA in plasma of non-tumour bearing and MAC 15A bearing mice was examined at 15 min and 8 h after FAA administration. Plasma samples were divided into two aliquots, one of which was extracted and analysed as described above the other being added to a Centrifree$^\text{TM}$ micropartition system (Amicon). Following centrifugation at 200 g for 20 min the ultrafiltrate was collected and FAA was extracted and analysed as described above.

**Preparation of S-9 liver fractions and chemosensitivity studies**

in vitro

S-9 microsomes were prepared from normal and phenobarbitone pre-treated mice (60 mg kg$^{-1}$ ip for 4 days) by the methods described by Chabot et al. (1989a). Mice were sacrificed by cervical dislocation and livers were aseptically excised and gently homogenised in four times their weight of cold RPMI 1640 tissue culture medium (pH 7.4) using a motor driven teflon/glass homogeniser and centrifuged at 9,000 g for 20 min at 4°C. One ml of this supernatant was added to a sterile universal tube containing 3 ml of MAC 15A cells in suspension. To this 0.5 ml FAA (1–5 mg ml$^{-1}$) and 0.5 ml of co-factors (glucose-6-phosphate, 33 mg ml$^{-1}$ NADP, 4 mg ml$^{-1}$; MgCl$_2$, 6H$_2$O, 6.6 mg ml$^{-1}$ and glucose-6-phosphate dehydrogenase, 1.66 units) were added and incubated at 37°C for 1 h. Following drug exposure, cytotoxic effects were assessed using a clonogenic assay, the details of which have been published elsewhere (Phillips et al., 1988). All assays were performed in triplicate and cells in the exponential phase of growth were used throughout. Controls containing S-9 preparations and co-factors were used as well as a positive control assay using cyclophosphamide (0.5 to 2 mg ml$^{-1}$ for 1 h).

**Influence of FAA containing mouse plasma on cytotoxicity in vitro**

Blood samples from normal mice and mice bearing subcutaneous MAC 15A tumours were collected by cardiac puncture 1 h and 4 h after the ip administration of a therapeutic dose of FAA (200 mg kg$^{-1}$). Blood was immediately centrifuged and plasma samples (1 ml) added directly to a pellet of MAC 15A cells and incubated at 37°C for 1 h. Following exposure to plasma samples the cells were washed and chemosensitivity was assessed as described previously. For control cultures FAA, at peak plasma drug concentrations (0.6 mg ml$^{-1}$) was added to fresh mouse plasma.

**Results**

FAA was administered to tumour bearing and non-tumour bearing mice at 200 mg kg$^{-1}$ ip, and the resulting plasma and tissue concentrations of FAA at various times thereafter are presented in Figures 1 and 2. In all cases the elimination of FAA was biphasic. The presence of MAC 15A tumours grown iv or sc has a significant effect upon the pharmacokinetic behaviour of FAA compared to non-tumour bearing mice. In both MAC 15A iv and sc tumour bearing mice the total body clearance of FAA is reduced in all tissues compared with non tumour bearing mice resulting in significant

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**Figure 1**  Means concentration of FAA ± 1 s.d. in plasma and tissues of non-tumour bearing NMRI mice (△) and mice implanted with MAC 15A tumour cells by the iv (○) and sc (△) route 12 days earlier. FAA was given at a single ip dose of 200 mg kg$^{-1}$. Values presented are the means of three mice per time point.
plasma drug concentrations (PeakTB/PeakNTB = 0.91 and 1.12 respectively, Table II).

The relationship between peak concentrations or AUC values of FAA in tumour samples and anti-tumour activity is presented in Table III. For the purposes of comparison, three additional MAC tumours (MAC 15Aip, MAC 16 and 26) have been included, details of pharmacokinetic exposures at the tumour site have been published elsewhere (Bibby et al., 1987b; Bibby et al., 1988; Bibby et al., 1989a). There is a poor correlation between pharmacokinetic parameters at the tumour site and the final outcome of chemotherapy in vivo. In both the resistant MAC 15A ip and iv tumour lines AUC and peak levels of FAA are higher than these achieved in the very responsive MAC 16 tumour (Table III). Plasma protein binding of FAA is presented in Table IV. There are no significant differences in plasma protein binding between non-tumour and MAC 15A iv and iv tumour bearing mice with values ranging from 68.7% to 85.6%.

The cytotoxic activity of FAA in vitro was not enhanced by the inclusion of S-9 or phenobarbitone induced S-9 liver fractions in the incubation mixture (Figure 3). The positive control compound, cyclophosphamide is activated by the S-9 liver preparation. No cell kill was observed in MAC 15A cells exposed to plasma from responding tumour bearing mice.

**Discussion**

Two main conclusions can be drawn from the results presented in this study; first, the presence of MAC 15A tumours has a significant influence upon the pharmacokinetic behaviour of FAA and second, that the site-dependent response of MAC 15A tumours cannot be explained on the basis of poor drug bioavailability.

The reasons for altered pharmacokinetics in MAC 15A tumour bearing mice relative to non-tumour bearing mice are unknown although it is clearly not due to differences in

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**Table I** Plasma and tissue distribution of FAA following the ip administration of FAA (200 mg kg⁻¹)

|                      | Cₘₐₓ (µg g⁻¹) | Tₚₚₚ (h) | T₁ (h) | Kᵣ (h⁻¹) | Vₛ (L kg⁻¹) | Clᵣ (L h⁻¹) | AUC₀₋∞ (µg h g⁻¹) |
|----------------------|---------------|-----------|--------|-----------|-------------|-------------|------------------|
| **Non tumour bearing mice** |               |           |        |           |             |             |                  |
| Plasma               | 511           | 0.25      | 3.5    | 0.198     | 0.357       | 0.138       | 1443             |
| Kidney               | 586           | 0.25      | 3.1    | 0.224     | 0.307       | 0.097       | 2058             |
| Liver                | 136           | 0.16      | 2.7    | 0.256     | 1.25        | 0.401       | 499              |
| Lung                 | 243           | 0.25      | 2.8    | 0.247     | 0.77        | 0.203       | 984              |
| Lung                 | 383           | 0.25      | 2.7    | 0.256     | 0.661       | 0.277       | 720              |
| Lung                 | 113           | 0.25      | 1.3    | 0.203     | 1.49        | 0.898       | 1455             |
| Lung                 | 167           | 0.25      | 1.5    | 0.462     | 1.05        | 0.358       | 558              |

**MAC 15A iv**

|                      | Cₘₐₓ (µg g⁻¹) | Tₚₚₚ (h) | T₁ (h) | Kᵣ (h⁻¹) | Vₛ (L kg⁻¹) | Clᵣ (L h⁻¹) | AUC₀₋∞ (µg h g⁻¹) |
|----------------------|---------------|-----------|--------|-----------|-------------|-------------|------------------|
| Plasma               | 520           | 0.25      | 8.6    | 0.080     | 0.37        | 0.056       | 3526             |
| Kidney               | 474           | 0.25      | 8.5    | 0.081     | 0.39        | 0.041       | 4878             |
| Liver                | 183           | 0.25      | 10.5   | 0.066     | 1.01        | 0.144       | 1384             |
| Liver                | 157           | 0.25      | 8.9    | 0.077     | 1.18        | 0.118       | 1697             |
| Lung                 | 222           | 0.166     | 9.9    | 0.070     | 0.81        | 0.112       | 1770             |
| Lung                 | 290           | 0.25      | 7.5    | 0.093     | 0.65        | 0.061       | 3237             |
| Lung                 | 137           | 0.083     | 6.9    | 0.101     | 1.38        | 0.271       | 740              |
| Lung                 | 119           | 0.25      | 6.4    | 0.107     | 1.54        | 0.287       | 696              |

**MAC 15A sc**

|                      | Cₘₐₓ (µg g⁻¹) | Tₚₚₚ (h) | T₁ (h) | Kᵣ (h⁻¹) | Vₛ (L kg⁻¹) | Clᵣ (L h⁻¹) | AUC₀₋∞ (µg h g⁻¹) |
|----------------------|---------------|-----------|--------|-----------|-------------|-------------|------------------|
| Plasma               | 610           | 0.25      | 9.2    | 0.075     | 0.31        | 0.037       | 5536             |
| Kidney               | 536           | 0.25      | 8.7    | 0.079     | 0.35        | 0.052       | 3859             |
| Liver                | 155           | 0.25      | 12.6   | 0.055     | 1.14        | 0.112       | 1789             |
| Liver                | 239           | 0.25      | 7.3    | 0.094     | 0.82        | 0.098       | 2036             |
| Lung                 | 375           | 0.08      | 5.8    | 0.119     | 0.51        | 0.072       | 2776             |
| Lung                 | 361           | 0.25      | 5.4    | 0.129     | 0.53        | 0.078       | 2573             |
| Lung                 | 169           | 0.25      | 6.9    | 0.101     | 1.11        | 0.198       | 1009             |
| Tumour               | 192           | 0.25      | 6.9    | 0.101     | 1.01        | 0.162       | 1236             |
| Tumour               | 136           | 1         | 12.6   | 0.055     | 0.95        | 0.114       | 1784             |
| Tumour               | 151           | 1         | 6.9    | 0.101     | 0.83        | 0.159       | 1251             |

*Units for plasma levels are µg ml⁻¹; **Units for plasma AUC values are µg h ml⁻¹; **N.B. Lung tissues contain extensive tumour deposits at the time of chemotherapy. For each tissue results from two independent experiments are presented. Kᵣ = Elimination constant; T₁ = Terminal half life; C₀ = Peak concentration ± s.d.; Tₚₚₚ = Time of peak concentration; Clᵣ = Total body clearance; Vₛ = Apparent volume of distribution; AUC₀₋∞ = Area under the curve.
Table II  Summary of peak plasma and plasma AUC values in tumour bearing and non tumour bearing mice

|            | Peak\(^a\) plasma values (\(\mu g\) ml\(^{-1}\)) | Ratio \(\mu g\) peak\(^b\) to peak\(^c\) | Plasma\(^a\) AUC\(_{0-\infty}\) (\(\mu g\ h\mu l\(^{-1}\)) | Ratio AUC\(_{TB}\) to AUC\(_{NTB}\) |
|------------|-----------------------------------------------|------------------------------------|-----------------------------------------------|------------------------------------|
| MAC 15A iv | 497                                           | 0.91                               | 4202                                          | 2.4                                |
| MAC 15A sc | 573                                           | 1.04                               | 4608                                          | 2.6                                |
| Non tumour | 549                                           | 1.00                               | 1751                                          | 1.0                                |

\(^a\)Mean values for two experiments with three mice per point in each experiment. \(^b\)TB = Tumour bearing; NTB = Non tumour bearing.

Table III  Relationship between tumour pharmacokinetic parameters and anti-tumour activity

| Dose and route (mg kg\(^{-1}\)) | Peak\(^a\) conc (\(\mu g\) g\(^{-1}\)) | Plasma\(^a\) AUC\(_{0-\infty}\) h.g\(^{-1}\) | % Tumour\(^a\) inhibition in vivo |
|---------------------------------|--------------------------------------|-----------------------------------------------|----------------------------------|
| MAC 15A iv\(^d\)               | 200 ip                                | 128                                           | 718                               | 0                                 |
| MAC 15A sc                     | 200 ip                                | 140                                           | 1517                             | 90                                |
| MAC 15A ip\(^e\)               | 200 ip                                | 3100                                          | 3210                             | 0                                 |
| MAC 16\(^f\)                   | 200 ip                                | 41                                            | 500                              | 100                               |
| MAC 26\(^g\)                   | 200 ip                                | 130                                           | 1260                             | 82                                |

\(^a\)Units for peritoneal washings are \(\mu g\) ml\(^{-1}\); \(^b\)Units for peritoneal washings are \(\mu g\) ml\(^{-1}\); \(^c\)Tumour inhibition assessed by: (1) MAC 15A ip and iv: survival times; (2) MAC 15A sc: tumour weight; (3) MAC 16 and 26: growth delay; \(^d\)Peak and AUC values for FAA represent those achieved in lung tissues which contain extensive tumour deposits at the time of chemotherapy; \(^e\)Bibby et al., 1987b; \(^f\)Bibby et al., 1988; \(^g\)Bibby et al., 1989a; All drug values presented are the mean of two experiments with three mice per point in each experiment.

Table IV  Plasma protein binding of FAA

| Dose (mg kg\(^{-1}\)) | Time (min) | Expt. A. binding\(^e\) | Expt. B. binding\(^e\) |
|-----------------------|------------|------------------------|------------------------|
| Non tumour bearing    |            |                        |                        |
| 200 ip                | 15         | 75.2±6.1               | 78.4±4.8               |
| 480                   | 70.8±7.1   | 68.7±7.5               |                        |
| MAC 15A sc            | 200 ip     | 74.2±5.4               | 76.6±6.1               |
| 480                   | 81.4±3.6   | 85.6±9.1               |                        |
| MAC 15A iv            | 200 ip     | 78.6±4.9               | 85.2±4.7               |

\(^a\)Values presented are the means±standard deviations from two independent experiments (three mice per point in each experiment).

plasma protein binding (Table IV). It also seems unlikely that this phenomenon is a direct result of the dose dependent, non-linear characteristic of FAA pharmacokinetics (Damia et al., 1988; Chabot et al., 1989b) as the increase in plasma \(\text{AUC}_{0-\infty}\) in tumour bearing mice is not accompanied by an increase in peak concentrations (Table II). Whether or not saturation of tubular secretion is involved in this case remains an open question. A much more likely explanation is a combination of host factors such as depression of metabolic capacity, impaired kidney function etc caused by an aggressively growing tumour (Donelli et al., 1984).

Whatever the mechanism, the relevance of these changes to anti-tumour activity appears to be limited, as increases in plasma and tissue \(\text{AUC}_{0-\infty}\) values for FAA occur in both the resistant MAC 15A iv and the sensitive MAC 15A sc models (Table I). In addition, such significant increases in plasma and tissue AUC values for FAA were not observed in MAC 26 and MAC 16 tumours, both of which are sensitive to FAA (Cummings et al., 1989; Bibby et al., 1988). In terms of tolerance however, these observations may be relevant particularly as Zaharko et al. (1986) have shown that increased lethality results from too long an exposure to therapeutically effective concentrations of FAA. Further detailed studies on the relationship between AUC and tolerance in normal and tumour-bearing mice are under way.

The relationship between tumour levels (both peak concentrations and \(\text{AUC}_{0-\infty}\) values) and anti-tumour activity in vivo (Table III) clearly demonstrates that the lack of activity against MAC 15A ip and iv tumours is unlikely to be the result of limited drug penetration. Concentrations of FAA in the lungs of unresponsive MAC 15A iv tumour bearing mice (\(\text{AUC}_{0-\infty}<718 \mu g\) h.g\(^{-1}\)) for example are similar to those achieved in the very responsive MAC 16 tumour (500 \(\mu g\) h.g\(^{-1}\)). Furthermore, additional studies including Phase I clinical trials have shown that plasma concentrations of FAA associated with activity in mice are achievable in humans which strongly suggests that poor penetration of FAA into human tumours is unlikely to explain the lack of activity observed in clinical studies (Maughan et al., 1989; Kerr et al., 1987).

Two metabolites of FAA were detected in vivo. The first metabolite eluted was present at low levels (7% of circulating FAA) in plasma samples but was barely detectable in other tissues. Similar findings have been reported in NMRI mice (Cummings et al., 1989) and Balb/c mice (Damia et al., 1988). The second metabolite could only be detected in liver samples. Attempts to identify cytotoxic metabolites in vivo or to activate FAA to a cytotoxic species in vitro failed to substantiate the results presented by Chabot et al. (1989a). In this and previous studies (Damia et al., 1988), no metabolites arising from the inclusion of S-9 liver fractions in the incubation 'mix' in vitro were detected by HPLC. Furthermore, as no metabolites could be detected in tumour tissues, their role in the anti-tumour activity of FAA remains questionable.

In conclusion, the results of this study have demonstrated that the site dependent activity of FAA cannot be fully explained on the basis of differences in drug and/or metabolite bioavailability or on altered pharmacokinetic profiles caused by the presence of large tumour loads. The reasons for the site dependent activity of FAA remain unclear although it is likely that the activity of FAA against sc tumours relies in part on the specific vascular feature of tumours at this site.

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