Tumour concentrations of flavone acetic acid (FAA) in human melanoma: comparison with mouse data

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Summary  Flavone acetic acid (FAA) showed impressive effects against murine solid tumours but no activity in clinical studies. The mechanism of action in mice may involve damage to tumour vasculature or immunomodulation, and these effects may be species-specific. Alternatively, concentrations of FAA achieved in mouse tumours may be higher than in human tumours. It is important to resolve this issue since it raises important questions about the relevance of in vitro versus in vivo tumour screens and the development of FAA analogues. As part of a Cancer Research Campaign Phase II study of metastatic melanoma in which 8.4 g m⁻² FAA was given as a 6 h infusion, six tumour biopsies were obtained from four patients. FAA tumour concentrations were determined by HPLC and compared with subcutaneous murine solid tumours within the same analytical laboratory. Tumour/plasma percentages (range 26–61% mean ± SD, 43.2 ± 11.4%) were similar to those in mice, as was the area under the curve (AUC) extrapolated to infinity and the AUC above the putative activity threshold of 100 μg ml⁻¹. We conclude that the exposure of drug-refractory human melanoma tissue to FAA was comparable to that of sensitive mouse tumours. This suggests that reduced penetration of FAA into human tumours is unlikely to explain the lack of antitumour activity observed in clinical studies and that differences in mechanism of action are predominant.

Methods

Human studies

Patients  Nine patients (six female, three male, age range 28–69 years) were entered into the CRC Phase II study of FAA in malignant melanoma (Kerr et al., 1989). Metastatic disease was present in multiple sites including skin (seven soft tissues, four lymph nodes, three lung, two liver, peritoneum, brain, bone and a local recurrence. Liver and renal function were within normal limits. Patients received 8.6 g m⁻² FAA in a 6 h infusion with urine alkalinisation (sodium bicarbonate; 500 ml, 1.26%, 1 h before and after infusion of FAA). Dose reduction to 6.4 g m⁻² was applied in two patients following drug-induced hypotension during the first infusion of FAA. Treatment was repeated weekly to a maximum of six infusions. No evidence of tumour response was observed in any patient.

Plasma concentrations  Full pharmacokinetic profiles were obtained on 8/9 patients and a partial time course in the ninth.

Five ml of heparinised blood was collected at the start, the mid point and the end of the infusion (EOI) of FAA. Thereafter, further samples were collected at 5, 15, 30, 60, 90 min, 2, 3, 4, 6, 14 and 24 h after EOI. Plasma was stored at −20°C until analysis.

Tumour concentrations  With patient's informed consent, six tumour samples were obtained from 4/9 patients to determine FAA concentration. Excision of cutaneous or subcutaneous metastases was performed under local anaesthetic. Sampling times were at EOI in two cases, and at 10, 22 and 65 min and 12 h after EOI. The samples were subdivided and frozen at −70°C until analysis.

Mouse studies

Plasma and tumour concentrations  C3H/He mice bearing subcutaneously transplanted KHT sarcomas (range 300–400 mm³) were treated with 200 mg kg⁻¹ (600 mg m⁻²) FAA by intraperitoneal (ip) injection 10–12 days after subcutaneous inoculation. Groups of three mice were then sacrificed at 5, 15, 30 min, 1, 2, 4, 6, 8, 12 and 24 h after injection. C3H/He mice bearing transplanted RIF-1 and 16C...
tumours and BALB/c mice bearing EMT6 mammary tumours were treated with 250 mg kg$^{-1}$ (750 mg m$^{-2}$) FAA and sacrificed at 30 min for tumour and plasma FAA concentrations. These doses represent the highest therapeutic doses normally used in tumour bearing mice. Mice were exsanguinated under terminal diethyl ether anaesthesia and tumours were removed immediately after. Samples were stored as described above for human tissue. Experiments were independently replicated.

**FAA analysis** FAA concentrations were determined by a modification of the high performance liquid chromatography method of Cummings et al. (1988). Tumours were thawed, finely chopped and then homogenised rapidly on ice with 3–6% vol 10 mM ammonium acetate buffer, pH 5.3, using an all-glass homogeniser. After this the homogenate was treated as for plasma. Samples (100 μl) were mixed with the above buffer (200 μl) containing 4-(dimethylamino) benzoaldehyde (300 μg ml$^{-1}$) as internal standard. Aliquots (100 μl) were loaded onto C$_{18}$ cartridge columns (Sep-Pak, Waters Assoc., Milford, MA) previously washed with methanol (5 ml) and an ammonium acetate buffer (2 ml). After washing with buffer (1 ml) the samples were eluted with methanol (1 ml). Chromatography was carried out using modular equipment from Waters Assoc. Separation was achieved with a Novapak C$_{18}$ cartridge column (10 cm long; 8 mm i.d.; 4 mm bead size) and a mobile phase of 23% propanol in 10 mM ammonium acetate buffer, pH 5.3. Peak assignments were made on the basis of retention time and spectral properties and no interfering peaks were seen. Extraction efficiencies were 83% for spiked tumour homogenate, sensitivity was 3 μg ml$^{-1}$ and calibration curves were linear over the required range.

**Pharmacokinetic analysis** Pharmacokinetic parameters were calculated by nonlinear regression analysis using Subroutine V-COSAD of the Harwell Subroutine Library. Parameters were derived from standard equations (Wagner, 1975). Area under the curve (AUC) above 100 μg ml$^{-1}$ was calculated using the trapezoidal method.

**Tumour blood flow assessment** Relative tumour perfusion was assayed in mice bearing KHT sarcomas after ip injection of 200 μCi $^{86}$Rubidium (Sapirstein, 1958). Briefly, approximately 8 μCi $^{86}$RbCl were injected i.v. and the mouse was killed 60 sec later. Tumours were excised, weighed and counted in a Wallace 1282 Compu gamma counter. Groups of 12–16 mice were used for each time point. The percentage of injected counts per gram of tumour was calculated, and means and standard errors were calculated for each group and subsequently expressed as a percentage of the mean of the control group.

**Results**

Plasma pharmacokinetic parameters in the eight patients with full time courses are shown in Table I. Mean plasma FAA concentrations at the end of infusion and after 30 m were 378 and 306 μg ml$^{-1}$. The average elimination half-life (t$\beta$) was 5.4 h and the AUC$_{0-\infty}$ was 3612 μg ml$^{-1}$ h. Pharmacokinetics were closely comparable to those reported in the Phase I studies (Kerr et al., 1987). The mean AUC above the postulated activity threshold (Zaharko et al., 1986) of 100 μg ml$^{-1}$ was 1865 μg ml$^{-1}$ h. A typical time course is illustrated in Figure 1.

FAA concentrations from the six melanoma tumour samples are shown in Table II. Analysis of divided tumours demonstrated excellent reproducibility. Average tumour concentrations achieved between the EOI and 65 min thereafter ranged between 122 and 183 μg FAA per gram. The sample taken 12 h after the EOI showed a mean tumour concentration of 20.1 μg g$^{-1}$. The tumour to plasma percentages for the samples from EOI to 65 min were closely grouped with a mean of 47.5 (± 7.9, SD)%. The value at 12 h was 25.7% in the single specimen analysed. The overall tumour to plasma percentage including the 12 h point was 43.9 (± 11.4, SD)%.

Concentrations were determined in plasma and tumour in mice bearing four different subcutaneous transplanted mouse tumours 30 m after 250 mg kg$^{-1}$ (750 mg m$^{-2}$) ip. Mean tumour levels ranged from 276–332 μg g$^{-1}$ and the tumour/ plasma percentages were 48.6 ± 12.6 for EMT6, 62.7 ± 11.4 for RIF-1, 56.7 ± 5.9 for 16C and 58.5 ± 30.3 for KHT (± SD, n = 5–8). Detailed pharmacokinetic parameters were determined (Figure 2a and Table III). Maximal tumour concentrations were achieved at 1 h (442.3 μg g$^{-1}$) with an
elimination $t_1$ of 5.8 h. At 1–6 h the tumour/plasma % was constant with a mean of 73%. Thereafter, the tumour concentration fell more slowly than the plasma concentration, such that tumour exceeded plasma at these late times. Tumour/plasma percentages at 12 and 24 h after injection were 452% and 852% respectively. Similar results were obtained in a repeat experiment using 200 mg kg$^{-1}$ (600 mg m$^{-2}$) FAA (data not shown).

Parallel studies of KHT tumours after 200 mg kg$^{-1}$ (600 mg m$^{-2}$) ip FAA showed a rapid decline in the relative tumour blood flow. A nadir of 5% of control was observed at 6 h, followed by a slow recovery to 20% at 24 h (Figure 2B). No significant alteration occurred in a range of normal tissues (data not shown). Doses of 200 and 250 mg kg$^{-1}$ (600–750 mg m$^{-2}$) FAA ip resulted in regrowth delays of around 3–5 days in KHT sarcomas, compatible with the previously reported efficacy of FAA in murine tumours (Corbett et al., 1986; Plowman et al., 1986; Bibby et al., 1988).

In contrast to plasma (Cummings et al., 1988; Cummings & Smyth, 1989), no metabolites of FAA were detected in either human or mouse tumours.

### Table II  Tumour and plasma concentrations of FAA in human melanoma

| Pt no. | Time after EOI (h) | Tumour (mean) (μg g$^{-1}$) | plasma (mean) (μg ml$^{-1}$) | Tumour/plasma (%) |
|-------|-------------------|-----------------------------|-----------------------------|------------------|
| 8     | 0                 | 196.8                       | 183.4                       | 50.5             |
|       |                   | 157.3                       |                             | 40.4             |
|       |                   | 196.6                       |                             | 50.3             |
| 7     | 0                 | 114.1                       | 131.8                       | 40.2             |
|       |                   | 123.0                       |                             | 43.4             |
|       |                   | 122.3                       |                             | 43.1             |
|       |                   | 165.4                       |                             | 58.4             |
|       |                   | 134.3                       |                             | 47.4             |
| 9     | 10 min            | 183.3                       | 178.7                       | 42.7             |
|       |                   | 174.1                       |                             | 40.6             |
| 6     | 22 min            | 125.1                       | 120.5                       | 43.1             |
|       |                   | 116.5                       |                             | 40.1             |
|       |                   | 119.9                       |                             | 41.3             |
| 8     | 65 min            | 169.0                       | 176.3                       | 58.3             |
|       |                   | 183.6                       |                             | 63.4             |
| 8     | 12 h              | 14.5                        | 20.1                        | 18.5             |
|       |                   | 20.1                        |                             | 25.7             |
|       |                   | 23.6                        |                             | 30.1             |
|       |                   | 22.4                        |                             | 28.7             |

*Three samples taken from one patient after three different infusions.

### Table III  Pharmacokinetic parameters in mice with KHT tumours receiving 200 mg kg$^{-1}$ (600 mg m$^{-2}$) ip FAA

|                         | Peak (μg ml$^{-1}$) | Co (μg ml$^{-1}$) | $t_1$ (h) | AUC$_{0-h}$ (μg ml$^{-1}$ h) | AUC$_{0-24}$ (μg ml$^{-1}$ h) |
|-------------------------|---------------------|-------------------|-----------|-----------------------------|-------------------------------|
| Plasma                  | 965.3               | 734.6             | 2.8       | 2985                        | 1816                          |
| Tumour                  | 431.0               | 348.3             | 5.8       | 2790                        | 9834                          |

### Discussion

One possible explanation for the marked discrepancy between the impressive effects of FAA against transplanted murine solid tumours (Corbett et al., 1986; Plowman et al., 1986; Bibby et al., 1988) and the absence of activity in human studies (Kerr et al., 1987; 1989; Kaye et al., 1990) is a difference in tumour drug exposure between mice and humans. This study has shown a close parallel between the plasma exposures in the two species. Mean AUC$_{0-h}$ was 3612 μg ml$^{-1}$ h in the patients and 2985 μg ml$^{-1}$ h mice.

![Figure 2](image) FAA concentration versus time following ip injection of 250 mg kg$^{-1}$ (750 mg m$^{-2}$) in mice bearing KHT sarcomas. Open circles: plasma concentration (± 1SD) μg ml$^{-1}$; triangles: tumour concentrations (± 1SD) μg g$^{-1}$. A parallel experiment to show effects on tumour blood flow by rubidium extraction after ip injection of 200 mg kg$^{-1}$ (600 mg m$^{-2}$) FAA.
Similarly, above the postulated threshold of 100 μg ml⁻¹ (Zaharko et al., 1986) the plasma drug exposures were highly comparable, being 1865 and 1816 μg ml⁻¹ h in humans and mice respectively. Tumour/plasma percentages were found to be marginally higher in the case of mouse tumours (48–63%) but of similar magnitude to that observed in humans (mean 47.5%) at early time points. The peak (30 min) tumour concentrations for mouse tumours (300 μg g⁻¹) were higher by a factor of 2 compared with the melanoma deposits over the first hour after EOI (150 μg g⁻¹). After 6 h, there was a reproducible and marked elevation in the tumour/plasma percentages for the KHT tumour. This was not seen in the single melanoma biopsy obtained at 12 h, where the value was 25%. The elevation in the tumour/plasma percentages for the KHT tumour coincided with the abrupt fall in tumour blood flow, as noted previously in sensitive mouse solid tumours (Corbett et al., 1986; Plowman et al., 1986; Bibby et al., 1988). Thus, it seems possible that the rise in tumour/plasma percentages was due to the trapping of FAA in KHT tumours as a result of the reduction of blood flow. Although it was only possible to obtain a single late time point in the human tumour study, the relatively low value there suggested that this trapping effect may not be seen in man.

It is particularly interesting to compare the exposures to FAA in the mouse and human tumours. The AUC∞-∞ for the murine KHT tumour was 2790 μg g⁻¹ h. This is similar to that of 1733 μg g⁻¹ h for the human melanomas estimated as the product of the average plasma AUC∞-∞ and the mean tumour/plasma ratio of 0.48. The corresponding values above the putative activity threshold showed even closer agreement at 983 μg g⁻¹ h for the mouse KHT tumour and 895 ± 377 (SD) μg g⁻¹ h for human melanomas. Thus the mouse and the predicted human FAA exposures are very similar. We have to emphasise however that we have only a single human melanoma value for time points beyond 1 h and further data for later times would be useful to confirm our prediction.

The mouse plasma and tumour exposures obtained in the present study are similar to those reported previously (Damia et al., 1988; Chabot et al., 1989). For example, Damia et al. (1988) observed a plasma AUC∞-∞ (2 SE) of 2021 ± 166 μg ml⁻¹ h and a tissue/plasma % for the mouse PAN/03 tumour of 57% after 200 mg kg⁻¹ (600 mg m⁻²) FAA. The Mario Negri group also reported recently on the breast, colon, and head and neck melanoma. Invest. New Drugs, 8, 195.

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