Cancer Chemother Pharmacol (1989) 24: 15–22

Cancer Chemotherapy and Pharmacology

Pharmacodynamics and causes of dose-dependent pharmacokinetics of flavone-8-acetic acid (LM-975; NSC-347512) in mice*

Guy G. Chabot, Marie-Christine Bissery, Thomas H. Corbett, Kathleen Rutkowski, and Laurence H. Baker

Division of Hematology-Oncology, Wayne State University School of Medicine, Detroit, Michigan 48201, USA

Summary. Flavone acetic acid (FAA) is a novel antitumor agent with broad solid-tumor activity. However, this drug has shown a steep dose-response curve in preclinical trials, with a narrow sublethal window of efficacy. To investigate this threshold behavior, we studied various aspects of FAA pharmacology in mice after i.v. administration. Mice bearing advanced-stage s.c. colon 38 adenocarcinoma were treated at four dose levels (39, 65, 108, and 180 mg/kg), and only the highest dose produced significant antitumor activity, showing a steep dose-response curve. Using an HPLC assay, FAA pharmacokinetics in both plasma and tumors were found to be dose-dependent. As the dose increased, there was a decrease in both total body clearance and volume of distribution at steady state. The increase in tumor area under the curve (AUC) was more pronounced than the corresponding increase in plasma AUC, showing a better tumor exposure to FAA at high doses. The distribution of FAA in normal tissues showed a short-term retention in the liver and kidneys; low concentrations were observed in the heart, spleen, and brain, with some retention in the latter. The highest FAA concentrations were found in the gastrointestinal (GI) tract, mainly in the duodenum, suggesting an important biliary excretion of the drug. Various possible causes of FAA nonlinear pharmacokinetics were investigated. Serum protein binding was high (79%) and remained constant up to 100 μg/ml, but decreased thereafter at higher FAA concentrations, e.g., 76% at 500 μg/ml and 64% at 1,000 μg/ml. Urinary and biliary clearances were dose-dependent and decreased 5- and 9-fold, from the 39- to the 180-mg/kg dose levels, respectively. A direct assessment of FAA enterohepatic circulation using intercannulated mice showed that 27% of the plasma AUC was accounted for by enterohepatic circulation. FAA acyl glucuronide was identified as the major metabolite in mice and was found to contribute to the nonlinear pharmacokinetics due to its facile hydrolysis under physiological conditions, regenerating FAA. In conclusion, the steep FAA dose-response curve was found to be caused by dose-dependent pharmacokinetics in mice. The nonlinear pharmacokinetics of this drug was attributed to a dose-dependent decrease in both urinary and biliary clearances, concentration-dependent serum protein binding, enterohepatic circulation, and the instability of FAA acyl glucuronide under physiological conditions, forming a futile cycle. The distribution data also suggested possible tissue targets for anticancer efficacy and/or toxicity that could be useful in designing clinical studies.

Introduction

Flavone acetic acid (FAA; 2-phenyl-8-(carboxymethyl)-benzopyran-4-one; LM-975; NSC-347512) is a new anticancer drug with broad antitumor activity in murine models that has entered phase I clinical trials. FAA is active in colon adenocarcinoma 38 [2, 23] as well as a variety of other solid tumors of different tissue origin [16]. Unlike most of the clinically used anticancer agents, with FAA only modest activity is observed against either P388 or L1210 leukemia [2, 16, 23]. Although its mechanism of action is presently unknown, extensive irreparable DNA strand breaks were observed in tumor cells of Glasgow osteogenic sarcoma-bearing mice after FAA treatment, suggesting that DNA is one target macromolecule for this drug [5].

However, this drug is not without severe limitations. Its dose-response curve is steep, and the agent displays a threshold behavior, i.e., a narrow sublethal window of efficacy [16]. This complicates the safe administration of the drug and can limit its optimal use as an anticancer agent. To understand better and eventually control the steep dose-response behavior of this drug, we investigated various aspects of its pharmacology at various doses ranging from inefficacious to efficacious. A correlation was observed between the steep dose-response curve in mice and the dose-dependent pharmacokinetics of this drug. Various causes of its nonlinear pharmacokinetics were studied.

Materials and methods

Chemicals. FAA was synthesized by LIPHA (Lyonnaise Industrielle Pharmaceutique, Lyon, France) [7] and obtained from the Drug Evaluation Branch, National Cancer Institute. The drug was dissolved in 7.5% sodium bicarbon-

* This work was supported in part by the Wayne State University Ben Kasle Trust Fund for Cancer Research, by Public Health Service grants CA-43886 and CA-42449 from the National Cancer Institute, and by the Harper Medical Staff Trust Fund. Preliminary reports of this study were presented in abstract form [4, 8, 9].
Offprint requests to: Guy G. Chabot, Institut Gustave-Roussy, Pavillon de Recherche, 39 rue Camille Desmoulins, F-94805 Villejuif Cedex, France

ate and diluted with sterile distilled water to a final sodium bicarbonate concentration of 4%. The volume injected was 0.4 ml/mouse. All solvents were HPLC grade and chemicals were reagent grade.

Mice and tumors. C57Bl/6 and B6D2F$_1$ mice were bred in house from strains obtained from Jackson Laboratories (Bar Harbor, Me). Colon adenocarcinoma 38 was passaged in the mouse strain of origin (C57Bl/6) and transplanted into the same strain for therapy trials [11–14]. The animals received bilateral s.c. implants of 30- to 60-mg tumor fragments on day 0.

Efficacy trial. The techniques of chemotherapy and data analysis have been described elsewhere [13, 15]. Briefly, tumors were allowed to grow to 150- to 480-mg size before the mice were injected i.v. with the various doses of FAA on days 21, 25, and 29. The tumors were measured with a caliper three times weekly. Tumor weights (mg) were calculated from a bidimensional measurement using the following formula: $(a \times b^2)/2$, where a and b are the tumor length and width (mm), respectively. A cure was defined as a total disappearance of the tumor over a 90-day period.

Collection of biological specimens. Blood (20 µl) obtained from the tail vein was added to normal saline (180 µl), vortexed and centrifuged at 13,000 *g* for 5 min, and immediately frozen until analysis. Tissues under study were harvested, blotted free of blood, weighed, and frozen until analysis. The gastrointestinal (GI) tracts (or segments thereof) and their content were harvested from the cardia to the rectum. Bile samples were collected in bile-cannulated mice (gallbladder cannulation) using an exteriorized polyethylene tube (PE-10). Urine and feces were collected in glass metabolic cages that allowed the separation of these excreta with minimal cross-contamination. The urine samples were collected on ice and acidified immediately on collection by the addition of 0.1 ml 86% phosphoric acid to the collection tube. Due to the light sensitivity of FAA, all biological specimens were processed in subdued light. All samples were immediately frozen at −20° C until HPLC analysis.

HPLC analysis. Plasma and tissue homogenates (200 µl or appropriate dilutions that fall within the calibration range) were deproteinized with trichloroacetic acid (40 µl 5% trichloroacetic acid) and extracted twice with diethyl ether (2 ml). The ether phase was evaporated to dryness under a nitrogen stream and reconstituted with 200 µl HPLC mobile phase. The sample (25 µl) was injected onto a C_{18} reverse-phase column (MicroBondapak, Waters Associates) protected by a precolumn. FAA was eluted with a mobile phase composed of acetonitrile, water, and acetic acid (40:60:2) at a flow rate of 1 ml/min (Waters pump M-45). Under these conditions, FAA eluted at approximately 8 min and was detected in the UV spectrum at 300 nm (Spectroflow 773, Kratos). The calibration curves were linear from 0 to 10 µg/ml, with the correlation coefficient near unity. Peak heights were used for quantification. Drug extraction from biological samples was 77% ± 3%.

Pharmacokinetic parameter determination. Pharmacokinetic parameters were determined using the nonlinear regression program PCNONLIN (Statistical Consultants, Inc., Lexington, Ky). The rapid i.v. injection data were best fitted to a linear two-compartment model. The equation for this model is:

$$C_p = A\,e^{-\alpha \times t} + B\,e^{-\beta \times t},$$

where C_p is the plasma concentration at time t and A and B are the ordinate intercepts of the α and β phases, respectively. The other pharmacokinetic parameters were calculated using standard formulae [17]. Briefly, the plasma concentration at time 0 (C_0) was calculated as A + B; the half-lives ($T_{1/2}$) of the α and β phases were calculated by dividing 0.693 by α or β, respectively; the volumes of distribution were calculated as:

V_c (central compartment) = Dose/C_0, Vd_β = Dose/B, Vd_{area} = Dose/(AUC × β), and Vd_{ss} (steady-state) = (Dose × $(A/\alpha^2 + B/\beta^2)$)/$(AUC)^2$;

the total body clearance (Cl_T) was calculated as the Dose/AUC. The AUC from time 0 to infinity was calculated by the model-independent trapezoidal method. The urinary and biliary clearances were obtained from the slope of the amount of FAA excreted as a function of time vs the plasma AUC for the same time intervals.

Serum protein binding. Mouse and human blood were harvested without anticoagulant and allowed to clot at room temperature for 30 min; the serum was obtained by centrifugation at 13,000 *g* for 5 min. FAA binding to fresh serum protein was determined in vitro using the dialysis method. FAA was added to 1 ml serum in glass tubes at the following concentrations: 10, 50, 100, 500, and 1,000 µg/ml. After agitation for 30 s with a vortex, the serum was transferred into dialysis tubing (molecular weight cutoff, 15,000) and incubated at 37° C for 24 h in 30 ml phosphate-buffered saline (pH 7.4) with continuous shaking. Ultrafiltrates were checked for protein leaks (Multistix, Ames Division, Miles Laboratories). Appropriate controls without serum were run simultaneously. FAA concentrations were determined in both serum and ultrafiltrates by HPLC as described above.

Bile-intercannulated two-mouse system. Two donor mice were anesthetized with diethyl ether and their gallbladders were cannulated using a polyethylene tube (PE-10). The unattached end of the cannula was then inserted into the duodenum of a recipient mouse. The donor mouse received 180 mg/kg FAA i.v. In this system, FAA and its metabolites excreted in the bile of the donor mouse are delivered to the duodenum of the recipient mouse. Blood samples were harvested by the tail-vein method in both donor and recipient mice and FAA levels were assayed by HPLC as described above. Bioavailability was calculated as the percentage ratio of the plasma AUC of the recipient mouse to that of the donor mouse.

FAA acyl glucuronide determination and stability. FAA acyl glucuronide was isolated and purified from mouse urine using a semipreparative reverse-phase column (MicroBondapak C_{18}, 7.8 × 300 mm). The chromatographic conditions were similar to those described above except that the flow rate was 3 ml/min and the volume of injection, 300 µl. The peak corresponding to the UV-detectable

Table 1. Treatment of advanced stage colon adenocarcinoma 38 with FAA in mice[a]

Dose[b] (mg/kg)	Total dose (mg/kg)	Mean body wt. change, days 19–32 (g/mouse)	Median tumor burden per mouse on day 32, in mg (range)	T/C day 32 (%)	Time for median tumor burden to reach 1 g (days)	Tumor growth delay T-C (days)	Total $\log_{10}$ kill	Cure day 90
Control	–	+3.9	3111 (2533–3441)	–	25	–	–	–
180	540	+0.8	180 (75–320)	5.8	66	41	2.9	1/5[c]
108	324	+3.1	1521 (1234–3016)	48.9	29.5	4.5	0.3	0/5
65	195	+1.2	3163 (2078–3317)	101.6	Inactive based on T/C			
39	117	+1.1	2864 (2495–2883)	92.1	Inactive based on T/C			

[a] Tumor fragments (30–60 mg) were implanted s.c. by trocar into the axillary region of C57Bl/6 mice on day 0. On day 20, mice carried an average of 317 mg tumor burden (range, 150–610 mg). Tumor transplant generation 91. The tumor-volume doubling time was 4.25 days

[b] FAA was prepared in 4% sodium bicarbonate in distilled water and injected i.v. (0.4 ml/mouse). The average mouse weight was 24 g. Drug treatments were given on days 21, 25, and 29 at the four indicated dose levels; there were five mice per dose level

[c] Three partial regressions, one complete regression, and one cure

(300 nm) major FAA metabolite was collected and the mobile phase was evaporated at room temperature under low pressure. The isolated and purified metabolite was subjected to mild alkaline treatment (0.01 *N* NaOH at room temperature). β-Glucuronidase digestion (25 units from abalone entrails; Sigma, St. Louis, Mo) was carried out in a 0.1 *M* citrate-phosphate buffer at pH 3.8 and 37° C for 24 h. The acyl glucuronide hydrolysis and regeneration of FAA were monitored by HPLC using the analytical system described above. The UV spectrum analysis was carried out in the mobile phase on a Varian DMS-100 spectrophotometer. The stability studies at different pH values were carried out in citrate-phosphate buffer (0.1 *M*) for pH 3–7, phosphate buffer for pH 8, and NaOH solutions for pH 9 and 10.

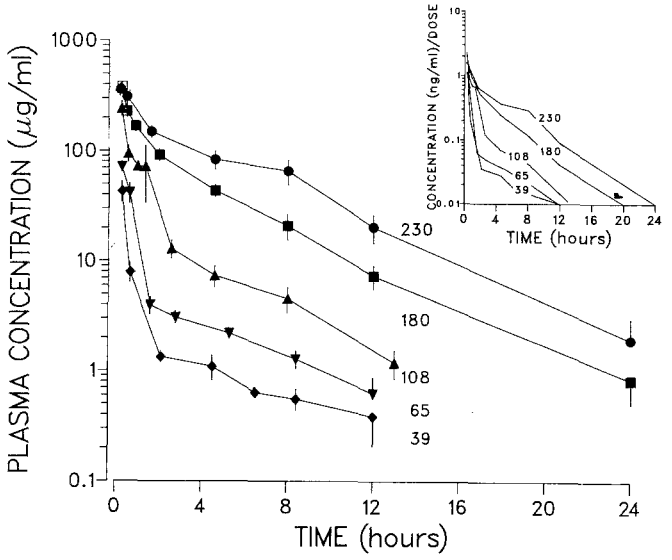

Fig. 1. FAA plasma pharmacokinetics in mice after an i.v. bolus injection at the indicated dose levels (mg/kg). FAA levels were assessed by HPLC as described in *Materials and methods*. The *inset* indicates the nonsuperposition of the curves when the plasma concentrations are divided by the dose given. $n = 7$ data sets for 39–108 mg/kg; $n = 22$ for 180 mg/kg; $n = 10$ for 230 mg/kg. *Error bars*, SE

Statistics. All data are expressed as the mean ± SE. Statistical difference at the 5% confidence level was assessed by one-way analysis of variance (ANOVA) and the Student's Newman-Keul test.

Results

FAA antitumor effects in colon 38-bearing mice.

Mice bearing advanced stage s.c. colon 38 adenocarcinoma were treated i.v. with FAA at four dose levels (Table 1). FAA was clearly active at 180 mg/kg with a T/C of 5.8% and 3/5 partial regressions, 1 complete regression, and 1 cure; this dose produced a stupor and/or somnolence that lasted 30–60 min. The three lower dose levels were inactive, with a T/C of >42%. A similarly steep dose-response relationship has been described for all trials carried out with this agent [16, 23]. To understand better this threshold behavior, we then studied FAA pharmacokinetics in plasma, tumors, and normal tissues.

Plasma pharmacokinetics

FAA was injected as an i.v. bolus at the same doses used for the above antitumor efficacy trial plus an additional dose of 230 mg/kg, which is a lethal dose for approximately 20% of the mice [16]. The resulting FAA plasma concentrations at different times thereafter are shown in Fig. 1. FAA plasma elimination was biphasic for all dose levels. At the highest dose, a shoulder on the elimination phase was observed at 8 h; this shoulder was followed by a terminal linear phase. The superposition method (i.e., a plot of the plasma concentrations divided by the dose given) was applied to assess the linearity of FAA pharmacokinetics (Fig. 1, *inset*). It was observed that the curves were not superposable, indicating that FAA pharmacokinetics was dose-dependent (i.e., nonlinear).

The pharmacokinetic parameters for each dose level are presented in Table 2. The distribution α phase was affected by an increase in FAA dose, as shown by the decreased slope of the α phase and the consequent increase in $T_{½\alpha}$. The terminal elimination β phase and its $T_{½\beta}$ did not change markedly with dose. The volume of the central compartment (Vc) increased with dose, suggesting a saturation phenomenon at higher doses. All other volumes of distribution (Vd_{area}, Vd_{β} and Vd_{ss}) decreased markedly

Table 2. FAA plasma and tumor pharmacokinetics in mice[a]

Pharmacokinetic parameter	Dose (mg/kg)				
	39	65	108	180	230
Plasma:					
C_0 (μg/ml)	137 ±6[b]	172 ±42	384 ± 84	459 ±82	441 ± 44
A (μg/ml)	136 ±6	167 ±56	361 ±104	354 ±96	259 ± 41
α phase (h^{-1})	4.7 ±0.1	3.1 ± 0.8	2.3 ± 0.7	1.6 ± 0.5	1.4 ± 0.6
$T_{1/2\alpha}$ (h)	0.15±0.004	0.22± 0.05	0.30± 0.09	0.44± 0.14	0.49± 0.20
B (μg/ml)	1.8 ±0.2	4.5 ± 1.3	23.1 ± 7	104 ±16	181 ± 38
β phase (h^{-1})	0.14±0.02	0.16± 0.03	0.22± 0.04	0.21± 0.01	0.18± 0.03
$T_{1/2\beta}$ (h)	5.1 ±0.7	4.4 ± 1	3.1 ± 0.7	3.4 ± 0.2	3.8 ± 0.6
V_c (l/kg)	0.28±0.01	0.38± 0.12	0.28± 0.08	0.39± 0.09	0.52± 0.05
Vd_{area} (l/kg)	7.77±0.9	4.85± 0.4	2.0 ± 0.2	1.59± 0.2	1.06± 0.2
Vd_β (l/kg)	21.7 ±0.3	14.4 ± 3	4.7 ± 3	1.7 ± 0.2	1.3 ± 0.2
Vd_{ss} (l/kg)	3.2 ±0.7	1.9 ± 0.3[c]	1.1 ± 0.1[c]	1.2 ± 0.2[c]	0.8 ± 0.1[c]
Cl_T (l/h per kg)	1.09±0.12	0.78± 0.07[c]	0.47± 0.03[d]	0.32± 0.04[d]	0.22± 0.04[d]
K_{10} (h^{-1})	3.3 ±1	2.1 ± 0.5	1.5 ± 0.4	0.6 ± 0.1	0.4 ± 0.04
K_{12} (h^{-1})	1.4 ±0.1	0.9 ± 0.3	0.7 ± 0.3	0.6 ± 0.3	0.5 ± 0.3
K_{21} (h^{-1})	0.2 ±0.02	0.2 ± 0.06	0.3 ± 0.2	0.5 ± 0.1	0.7 ± 0.3
AUC (μg/ml × h)	38 ±4	87 ± 8	305 ± 49	735 ±84	1320 ±174
Increase in dose[e]	1	1.67	2.77	4.62	5.90
Increase in AUC[f]	1	2.29	8.03	19.34	34.74
Normalized AUC[g]	0.98±0.09	1.34± 0.12	2.82± 0.46	4.08± 0.47[d]	5.74± 0.76[h]
Tumors:					
AUC (μg/g × h)	4.7 ±0.2	19.6 ± 4	53.7 ± 4	233 ±67	739 ±255
Increase in AUC[i]	1	4.2	11.4	49.6	157.2

[a] Mice received FAA as a rapid i.v. injection at the indicated dose
[b] Mean ± SE. For plasma data, $n = 7$ data sets for the 39- to 108-mg/kg dose levels, $n = 22$ for 180 mg/kg, and $n = 10$ for 230 mg/kg. For tumors, $n = 4$ tumors per time point
[c] $P < 0.05$ vs 39 mg/kg
[d] $P < 0.05$ vs 39 and 65 mg/kg
[e] Ratio of a given dose divided by the lowest dose used (39 mg/kg)
[f] Ratio of the AUC at a given dose divided by 38 (AUC at 39 mg/kg)
[g] (AUC divided by dose) × 1,000
[h] $P < 0.05$ vs all other groups
[i] Ratio of the AUC at a given dose divided by 4.7 (AUC at 39 mg/kg)

with increased dose. The total body clearance (Cl_T) also decreased significantly with increased dose. The AUC was increased 35-fold for an increase in dose of only 5.9-fold. The microconstants indicated that the apparent first-order elimination-rate constant (K_{10}) from the central compartment and the transfer rate constant from the central to the peripheral compartment (K_{12}) decreased markedly with dose. However, the transfer rate constant from the peripheral to the central compartment (K_{21}) increased with dose, suggesting an increased contribution from the peripheral compartment to the plasma levels as the dose was escalated.

Tumor pharmacokinetics

FAA tumor levels were also determined after FAA i.v. administration (Fig. 2). The two highest efficacious doses yielded FAA levels in tumors above 10 μg/g for 8 h at the 180 mg/kg dose and for about 24 h for the 230 mg/kg dose. As previously noted for the plasma, the tumor AUC also increased more than proportionally to the increase in FAA dose, as shown by the nonsuperposition of the curves corrected for the dose (Fig. 2, *inset*). However, the increase in tumor AUC was more pronounced than the corresponding increase in plasma AUC (Table 2), indicating a more than proportional increase in FAA tumor exposure at high

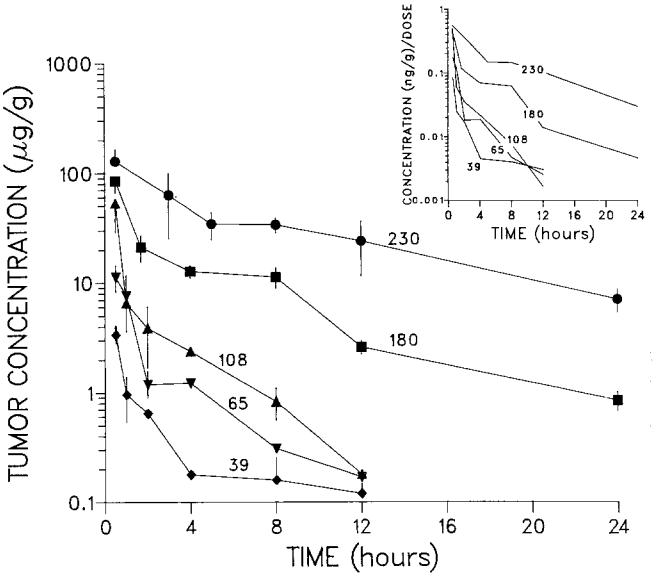

Fig. 2. FAA tumor pharmacokinetics in mice after an i.v. bolus injection at the indicated dose levels (mg/kg). The *inset* indicates the nonsuperposition of the curves when the tumor concentrations are divided by the dose given. $n = 4$ tumor levels per time point. *Error bars*, SE

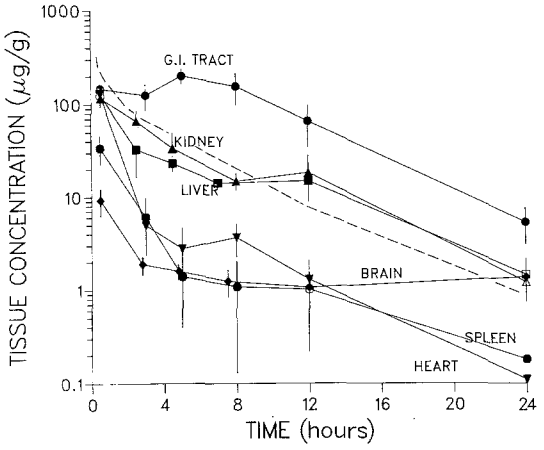

Fig. 3. FAA pharmacokinetics in normal tissues after an i.v. injection of 180 mg/kg. The *dashed line* represents the plasma levels at the same dose for comparison. $n = 3-5$ per time point. *Error bars*, SE

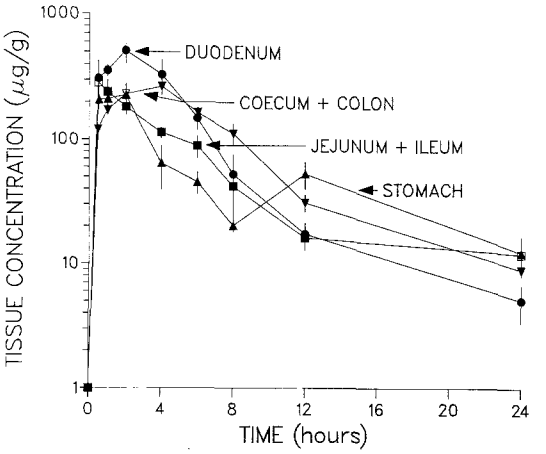

Fig. 4. FAA pharmacokinetics in the GI tract. Mice received FAA i.v. at 180 mg/kg, and at the indicated times the GI tract was harvested with its content and FAA levels were assayed. $n = 3$ mice per time point. *Error bars*, SE

doses compared with the corresponding increase in plasma AUC. Antitumor efficacy was reached at a minimal plasma AUC of 735 μg/ml × h, which yielded a corresponding tumor AUC of 233 μg/g × h.

Normal tissues pharmacokinetics

After i.v. injection of 180 mg/kg, FAA concentrations were determined at various times in the GI tract, kidney, liver, brain, spleen, and heart (Fig. 3). The highest levels were found in the GI tract, where concentrations greater than 100 μg/g were maintained for about 10 h. The liver and kidney levels were slightly lower than the plasma levels for the first 8 h, but at later time points there was a short-term retention in these tissues as compared with plasma. Although low FAA concentrations were observed in the heart, brain, and spleen, we noted the persistence of low levels in brain tissue, with no measurable decay from 4 to 24 h. The corresponding AUCs for these tissues are listed in Table 3.

Table 3. FAA normal tissue pharmacokinetics in mice[a]

Tissue/fluid	AUC (μg/g × h)
GI tract total	1,951 ± 341
GI tract differential:	
stomach	1,490 ± 103
duodenum	2,522 ± 336
ileum and jejunum	1,455 ± 30
colon and coecum	2,117 ± 43
(Plasma)[b]	(735 ± 84)
Liver	600 ± 135
Kidney	483 ± 22
(Tumor)[b]	(233 ± 67)
Heart	227 ± 52
Brain	84 ± 13
Spleen	80 ± 30

[a] $B6D2F_1$ mice received 180 mg/kg FAA as an i.v. bolus; $n = 3-5$ per tissue

[b] These values are taken from Table 2 and are presented here as a reference

Differential GI distribution

The higher FAA concentrations in the GI tract, as compared with those in other tissues tested, suggested biliary secretion of this drug. To assess exposure of the different parts of the GI tract to FAA, we determined drug concentrations in various segments after i.v. injection of 180 mg/kg (Fig. 4). High concentrations were observed in all segments as early as 0.5 h after FAA administration. Drug levels in the stomach were lower than in other parts of the GI tract for the first 8 h after i.v. injection, although concentrations increased markedly at 12 h. The highest initial levels were observed in the duodenum, which is compatible

Table 4. FAA excretion and clearance in urine and bile[a]

Dose (mg/kg)	Percentage excreted		Clearance (ml/h)	
	Urine	Bile	Urine	Bile
39	55 ± 4.5[b]	68 ± 3.5	11.9 ± 1.2	13.7 ± 0.7
180	36 ± 3.9	24 ± 3.9	2.3 ± 0.2	1.6 ± 0.3

[a] FAA was injected i.v. in $B6D2F_1$ mice at the indicated dose, with urine and bile collected for 24 h

[b] Mean ± SE; $n = 4$

Table 5. Evaluation of FAA enterohepatic circulation in the bile-intercannulated two-mouse system[a]

Experiment number	Donor mouse AUC	Receiver mouse AUC	Total AUC	Percentage of AUC_r/AUC_t[b]
1	756	286	1,042	27
2	733	266	999	27

[a] The donor $B6D2F_1$ mouse received FAA i.v. (180 mg/kg) and its bile was diverted to the duodenum of a second mouse (*receiver mouse*). Blood samples from both mice were collected by the tail-vein method and FAA concentrations were determined by HPLC. The AUCs (μg/ml × h) were determined by the trapezoidal method

[b] Percentage of AUC_r (receiver)/AUC_t (total)

with biliary secretion of the drug after i.v. administration. The next segments (jejunum and ileum) showed lower levels, which was indicative of FAA reabsorption. Drug levels in the coecum and colon were higher than in the other GI segments during the 4–12 h interval after FAA administration.

Studies of possible causes of dose-dependent pharmacokinetics

To understand better FAA dose-dependent pharmacokinetics, we investigated various causes that could explain the nonlinearity:

1. *Urinary, biliary, and fecal excretion.* As shown in Table 4, FAA urinary excretion was dose-dependent and the percentage eliminated by this route decreased with increased dose. The urinary clearance decreased about 5-fold from the 39- to the 180-mg/kg dose levels. FAA biliary excretion was also dose-dependent, as shown by a higher percentage of excretion at low (68%) compared with high doses (24%) (Table 4). The biliary clearance was reduced about 9-fold from the low- to the high-dose levels. FAA fecal excretion (24 h) was low (1.20%±0.1%) and did not show any dose dependency.

2. *Serum protein binding.* By the dialysis method, FAA binding to mouse serum protein was assessed in vitro (37° C, pH 7.4) to be 79%±0.6% for drug concentrations of 10, 50, and 100 μg/ml. However, at 500 and 1,000 μg/ml, FAA binding decreased to 76%±0.7% and 64%±2%, respectively. We also compared FAA binding to either mouse or human serum (at 100 μg/ml): drug binding to mouse proteins was lower (79%±0.6%) than to human proteins (95%±0.4%).

3. *Plasma pharmacokinetics in bile-cannulated mice.* To evaluate the contribution of enterohepatic circulation to FAA plasma levels, we used a bile-intercannulated two-mouse system in which the bile from a donor mouse that was injected i.v. with 180 mg/kg FAA was diverted to the duodenum of a receiver mouse. In this model, the relative importance of enterohepatic circulation to FAA plasma levels can be assessed. As presented in Table 5, the enterohepatic drug circulation in the receiver mouse accounted for 27% of the total AUC.

4. *Contribution of FAA metabolism to the dose-dependent pharmacokinetics.* (a) *Identification of the major FAA metabolite in mice:* The major FAA metabolite was isolated and purified from mouse urine; it was identified as the acyl glucuronide of the drug by its hydrolysis under mild alkaline conditions that regenerated FAA, by its susceptibility to β-glucuronidase hydrolysis, which also regenerated FAA, and by the superposition of the UV spectra of FAA and its acyl glucuronide.

(b) *Stability of FAA acyl glucuronide as a function of pH:* The stability of FAA acyl glucuronide was investigated in vitro (at 37° C and various pH) because its possible instability could contribute to FAA nonlinear pharmacokinetics. The glucuronide was more stable at acidic pH than at either neutral physiological or alkaline pH, as shown in Fig. 5. At neutral or alkaline pH values, it was unstable,

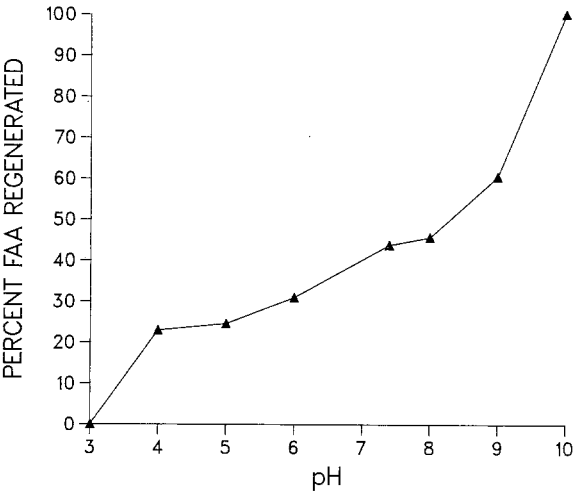

Fig. 5. FAA acyl glucuronide stability at different pH values for 20 h at 37° C. The metabolite was isolated from mouse urine, purified by HPLC, and dissolved in buffers at the indicated pH. Hydrolysis of the glucuronide was monitored by HPLC by measuring both the disappearance of the metabolite and the appearance of FAA

forming new peaks (as detected on the HPLC chromatogram) that were resistant to β-glucuronidase but could be hydrolyzed by mild alkaline treatment to regenerate FAA. The regeneration of FAA from its major metabolite accounted for 44%±2% at pH 7.4 for a 20-h incubation and was similar in either buffer or plasma. However, the initial hydrolysis rate of FAA acyl glucuronide at physiological pH was faster in mouse plasma (19% at 4 h) than in phosphate buffer (11% at 4 h).

(c) *Percentage of FAA acyl glucuronide as a function of dose:* FAA acyl glucuronide formation was dose-dependent and increased with dose: at 39 mg/kg, the urinary excretion (24 h) was 1.6%±0.2% compared with 16.3%±2.1% at 180 mg/kg (expressed as a percentage of the FAA dose).

Discussion

FAA is a new antitumor agent that has entered phase I clinical trials due to its high antitumor activity observed in mice [2, 16, 23]. Although promising for clinical use, this drug is not without severe limitations since its dose-response curve is exceptionally steep [16]. Furthermore, splitting the dose of the agent into low or multiple daily injections markedly decreases efficacy and increases the total dose required to reach toxicity [16, 23]. Since this threshold behavior can limit the optimal use of this drug in humans, we investigated various aspects of FAA pharmacology that could explain its behavior and perhaps offer clues that could help to circumvent the problem.

In our studies, the steep FAA dose-response curve was found to be due to dose-dependent pharmacokinetics. As the dose was increased, we observed a disproportionate increase in both plasma and tumor AUCs reaching efficacy and toxicity in a sudden fashion above a threshold dose of 180 mg/kg. The tumor AUC increased more rapidly than the corresponding increase in plasma AUC, explaining the good antitumor activity obtained only at high doses. This

nonproportional increase in tumor vs plasma AUC could be due to the saturable protein binding at high doses that could force the drug to diffuse out of the blood compartment and distribute into peripheral tissues, including the tumor. We observed that high concentrations of FAA (500–1,000 μg/ml) caused a decrease in protein binding and hence could increase the free drug available to the tumor. These high plasma concentrations could be reached immediately after a rapid i.v. injection. Another possible explanation for drug retention by the tumor tissue at high doses could be due to the hemorrhage and necrosis observed at these dose levels [5], preventing FAA washout from the tumor.

FAA pharmacokinetics in normal tissues indicated some interesting distribution features that could be useful in the design of clinical trials. The high concentrations found in the intestines may be toxic to the intestinal epithelium. Moreover, the high concentrations attained in the liver and kidney, followed by a short-term retention in these organs, indicate that they are potential targets for this drug. FAA-induced liver and kidney toxicities have been observed in dogs [21], and short-term accumulation in the liver and kidney has also been observed with other flavonoids [18]. The acute CNS toxicity, described as sleepiness in mice, was observed only at high efficacious doses, suggesting that FAA crosses the blood-brain barrier at these doses; drug passage into the brain was indeed observed shortly after i.v. injection, as FAA brain concentrations attained 10 μg/g 15 min after administration. Although drug levels in the brain were low at later time points, they persisted beyond 24 h, indicating some retention in this tissue. This acute CNS toxicity, which can be dose-limiting, has also been observed in rats and dogs [21], and FAA transfer into the CSF has been reported in the latter species [22]. These preclinical observations may indicate a potential toxicity site in humans, but if clinically manageable, FAA transfer into the CNS could be useful for treating tumors in this tissue.

Of interest was the finding of higher FAA levels in the coecum and colon than in the jejunum, which was probably due to the regeneration of free FAA from its acyl glucuronide by the β-glucuronidase of the bacteria present in this intestinal segment. The low fecal excretion of free FAA indicates extensive catabolism of the drug by the intestinal flora and/or its reabsorption.

Various factors that could explain the dose-dependent pharmacokinetics of FAA were investigated. The dose-dependent pharmacokinetics was found to be caused by dose-related decreases in both biliary and urinary clearance, concentration-dependent protein binding, enterohepatic cycling, and the instability of the major metabolite of the drug. The capacity-limited FAA excretion in the liver and kidney may be attributed to drug-related local inhibitory effects, since decreases in biliary and renal clearance were paralleled by drug retention in these tissues. Nonlinear pharmacokinetics has also been observed with other drugs containing a carboxylic group, e.g., salicylates and various penicillins [1].

FAA was shown to be highly bound to serum proteins, and a concentration-dependent decrease in binding was observed only at high concentrations (500–1,000 μg/ml). As mentioned above, this decreased binding at high FAA plasma concentrations (reached a few minutes after an i.v. bolus) probably plays a role in drug distribution at high doses, allowing more free drug to diffuse to tissues than at lower doses. A different distribution pattern was indeed observed as a function of FAA dose in the target tumor tissue. The difference in FAA protein binding in mouse vs human sera, which has also been reported by Bibby et al. [3], may partly account for the higher human plasma concentrations thus far attained in clinical studies [19] without undue toxicity to the host, compared with the mouse plasma concentrations attained in our studies at the murine maximum tolerated dose.

The contribution of enterohepatic circulation to the plasma pharmacokinetics of FAA was studied because the high biliary excretion of this drug led to high intestinal content. Enterohepatic circulation was also suggested by lower FAA levels in the jejunum and ileum than in the duodeum (indicating FAA reabsorption) and the formation of more FAA acyl glucuronide at higher doses (indicating a recirculation of the drug through the liver). Since enterohepatic circulation accounted for 27% of the total AUC, it therefore contributes to the prolongation of the residence time of this drug and probably contributes significantly to the maintenance of therapeutic concentrations that may be important for FAA antitumor effects in vivo. Enterohepatic circulation has also been reported for other flavonoids [18]. These observations also underline the probable influence of food and GI transit time on the bioavailability of FAA in mice (and possibly other species) even after an i.v. injection.

Another factor found to contribute significantly to FAA dose-dependent pharmacokinetics was the instability of the major drug metabolite in mice, identified as FAA acyl glucuronide. Since the percentage of formation of the metabolite increased with drug dose, its spontaneous hydrolysis under physiological conditions contributes to FAA plasma levels in a dose-dependent fashion. This instability is therefore of therapeutic importance, since free drug is regenerated by this process and this cycle provides a reservoir from which FAA can continuously be liberated. Similar, so-called futile cycles have been reported for other acyl glucuronides [20]. In addition to nonenzymatic hydrolysis, the acyl glucuronide may also be hydrolyzed by the β-glucuronidase found in many tissues such as the liver, kidney, spleen, intestinal tract, endocrine, and reproductive organs [25]. The formation of glucuronide conjugates of flavonoids has also been reported in several other species, including man [18]. The resistance of FAA acyl glucuronide previously exposed to neutral or alkaline conditions to β-glucuronidase suggests that these acyl glucuronides are not in the 1-β-configuration but are isomers at other positions in the glucuronic acid moiety. Intramolecular migrations of the aglycone to other hydroxyl groups of the glucuronic acid, leading to β-glucuronidase-resistant species, have been reported for other acyl glucuronides [6, 10].

These pharmacokinetic studies indicate that high FAA plasma levels, generating high FAA tumor levels for a sufficient period of time, are required for anticancer efficacy. Because of the dose-dependent pharmacokinetics of this drug, these conditions are reached in a sudden fashion near the 180-mg/kg dose level, and since this dose is close to the murine maximum tolerated bolus dose, FAA is difficult to handle without encountering toxicity. Nonlinear pharmacokinetics was also recently reported for FAA in humans [24]; therefore, either pharmacologic modulation

studies of the FAA excretion pattern or the development of FAA analogs with linear pharmacokinetics should be undertaken.

In summary, the steep FAA dose-response curve was found to be caused by dose-dependent pharmacokinetics in mice. The nonlinear pharmacokinetics was mainly attributed to dose-dependent decreases in both biliary and urinary clearance, concentration-dependent protein binding, enterohepatic circulation, and the instability of FAA acyl glucuronide under physiological conditions.

References

1. American Hospital Formulary Service Drug Information (1985) American Society of Hospital Pharmacists, Bethesda, Md, pp 190, 200, 201, 386, 731
2. Atassi G, Briet P, Berthelon JJ, Collonges F (1985) Synthesis and antitumor activity of some 8-substituted-4-oxo-4*H*-1-benzopyrans. Eur J Med Chem Chim Ther 20: 393
3. Bibby MC, Double JA, Phillips RM, Loadman PM (1987) Factors involved in the anti-cancer activity of the investigational agents IM985 (flavone acetic acid ester) and LM975 (flavone acetic acid). Br J Cancer 55: 159–163
4. Bissery MC, Chabot GG, Corbett TH, Rutkowski K (1986) Flavone acetic acid (FAA; NSC-347512): nonlinearity of area under the concentration X time curves with changing dosage. Proc Am Assoc Cancer Res 27: 282
5. Bissery MC, Valeriote FA, Chabot GG, Crissman JD, Yost C, Corbett TH (1988) Flavone acetic acid (NSC-347512)-induced DNA damage in Glasgow osteogenic sarcoma in vivo. Cancer Res 48: 1279
6. Blanckaert N, Compernolle F, Leroy P, Van Houtte R, Fevery J, Heirwegh KPM (1978) The fate of bilirubin-IX alpha glucuronide in cholestasis and during storage in vitro. Intramolecular rearrangement to positional isomers of glucuronic acid. Biochem J 171: 203
7. Briet P, Berthelon JJ, Collonges F (1982) (4-oxo-4*H*-1(1)-benzopyran-8-yl)-alkanoic acids, their salts and derivatives, and medicines containing them. European Patent Application EP--80943 AL, p 34
8. Chabot GG, Bissery MC, Corbett TH, Rutkowski K (1986) Flavone acetic acid (FAA; NSC-347512) pharmacokinetics in mice. Proc Am Assoc Cancer Res 27: 407
9. Chabot GG, Bissery MC, Corbett TH, Rutkowski K (1987) Causes of the nonlinear pharmacokinetics of flavone acetic acid (FAA, NSC 347512) in mice. Proc Am Assoc Cancer Res 28: 436
10. Compernolle F, Van Hees GP, Blanckaert N, Heirwegh KPM (1978) Glucuronic acid conjugates of bilirubin-IX alpha in normal bile compared with post-obstructive bile. Transformation of the 1-O-acylglucuronide into 2-, 3-, and 4-O-acylglucuronides. Biochem J 171: 185
11. Corbett TH, Griswold DP Jr, Roberts BJ, Peckham JC, Schabel FM Jr (1975) Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Res 35: 2434
12. Corbett TH, Griswold DP Jr, Roberts BJ, Peckham JC, Schabel FM Jr (1977) Evaluation of single agents and combinations of chemotherapeutics agents in mouse colon carcinomas. Cancer 40: 2660
13. Corbett TH, Leopold WR, Dykes DJ, Roberts BJ, Griswold DP Jr, Schabel FM Jr (1982) Toxicity and anticancer activity of a new triazine antifolate (NSC 127755). Cancer Res 42: 1701
14. Corbett TH, Roberts BJ, Trader MW, Laster WR Jr, Griswold DP Jr, Schabel FM Jr (1982) Response of transplantable tumors of mice to anthracenedione derivatives alone and in combination with clinically useful agents. Cancer Treat Rep 66: 1187
15. Corbett TH, Roberts BJ, Leopold WR, Peckham JC, Wilkoff LJ, Griswold DP Jr, Schabel FM Jr (1984) Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in C57Bl/6 mice. Cancer Res 44: 717
16. Corbett TH, Bissery MC, Wozniak A, Plowman J, Polin L, Tapazoglou E, Dieckman J, Valeriote F (1986) Activity of flavone acetic acid (NSC-347512) against solid tumors of mice. Invest New Drugs 4: 207
17. Gibaldi M, Perrier D (1982) Multicompartment models. In: Swarbrick J (ed) Drugs and the pharmaceutical sciences. Vol 15. Pharmacokinetics. Marcel Dekker, New York, pp 45–111
18. Hackett AM (1986) The metabolism of flavonoid compounds in mammals. In: Cody V, Middleton E Jr, Harborne JB (eds). Plant flavonoids in biology and medicine: biochemical, pharmacological, and structure-activity relationships. Alan R. Liss, New York, pp 177–194
19. Kerr D, Kaye SB, Cassidy J, Bradley C, Harding M, Rankin E, Adams L, Young T, Forrest G, Soukop M (1987) A phase I trial of flavone acetic acid. Proc Am Assoc Cancer Res 28: 224
20. Meffin PJ, Zilm DM, Veenendaal JR (1983) Reduced clofibric acid clearance in renal dysfunction is due to a futile cycle. J Pharmacol Exp Ther 227: 732
21. O'Dwyer PJ, Shoemaker D, Zaharko DS, Grieshaber C, Plowman J, Corbett T, Valeriote F, King SA, Cradock J, Hoth DF, Leyland-Jones B (1987) Flavone acetic acid (LM-975, NSC-347512), a novel antitumor agent. Cancer Chemother Pharmacol 19: 6
22. Osterman CL, Lu K, Raber MN, Newman RA (1986) Preclinical pharmacology of 4-oxo-2-phenyl-4*H*-1-benzopyran-8-acetic acid (Flavone acetic acid, NSC-347512) in dogs. Proc Am Assoc Cancer Res 27: 405
23. Plowman J, Narayanan VL, Dykes D, Szarvasi E, Briet P, Yoder OC, Paull DK (1986) Flavone acetic acid (NSC-347512), a novel agent with preclinical antitumor activity against the colon adenocarcinoma 38 in mice. Cancer Treat Rep 70: 631
24. Staubus AE, Lyon ME, Grever MR, Wilson HE Jr, Malspeis L (1987) Dose-dependent pharmacokinetics of flavone acetic acid (NSC 347512) in humans. Proc Am Assoc Cancer Res 28: 227
25. Testa B, Jenner P (1976) Drug-metabolizing enzyme systems. In: Swarbrick J (ed) Drug metabolism, chemical and biochemical aspects. Marcel Dekker, New York Basel, pp 273–328

Received April 11, 1988/Accepted October 18, 1988