Characterization of the Major Metabolites of Flavone Acetic Acid and Comparison of Their Disposition in Humans and Mice

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ABSTRACT

Flavone acetic acid represents a novel chemical structure currently undergoing clinical investigation. Broad spectrum activity has been observed in preclinical animal screens, but at doses close to toxic in mice. Phase I clinical trials have established that equivalent plasma drug levels can be achieved in humans, but to date Phase II trials have not demonstrated significant activity in a range of tumor types. Little is known about the drug's biotransformation, although metabolites have been implicated in proposed mechanisms of action. In this paper, we have purified the two major human metabolites present in urine (also the only two metabolites detected in plasma) and characterized their structure, chemical properties, activity, and pharmacokinetics. Metabolite 1 (M1) was a glucuronide conjugated to the 8-acetic acid grouping (M, 456), was chemically labile, and showed a strong tendency to undergo chemical rearrangement at mildly alkaline pH. Metabolite 2 (M2) was also a glucuronide (M, 456) but appeared to be an unusual isomer of M1. Both were noncytotoxic. In patients, biotransformation represented the predominant mechanism of drug clearance with as much as 80% of a low dose (0.5 g/m2) recovered in urine as M1 and M2 after only 6 h. At high dose (4.8 to 8.6 g/m2, 1- to 6-h infusion) the appearance of peak concentrations of metabolites in plasma and urine was delayed, apparently due to saturation of glucuronidation pathways. This resulted in an overall reduction in drug clearance by 3- to 4-fold. Mice cleared flavone acetic acid much more slowly than patients (289 ml/h/m2 after 600 mg/m2 i.p. versus 2.3 liters/h/m2 after 4.8 g/m2-1-h infusion) without producing M1 or M2. A different metabolite, exhibiting characteristics of a conjugate, was detected at low concentrations in plasma, tissues, and tumor. Extensive metabolism to inactive products followed by their rapid clearance may contribute to the lack of activity so far seen in humans.

INTRODUCTION

FAA1 [2-phenyl-8-(carboxymethyl)benzopyran-4-one, LM975, NSC 347512; Fig. 1] is a parent member of a potentially new class of anticancer drugs synthesized in France and based on the naturally occurring flavone ring system. It was selected for clinical evaluation because of high activity against murine colon 38, a tumor known to be refractory to many new compounds (1). The drug possesses unusual properties which distinguish it from conventional cytotoxic agents, and several novel mechanisms of action are currently under investigation (2). Greatest activity is seen with slowly growing, well-established, well-differentiated, solid animal tumors (MAC 26, colon 38, and Lewis lung) while little or no activity is seen against the same tumors growing i.p. (MAC 15A, Lewis lung) or with rapidly dividing hematological tumors (p 388, L1210) (1, 3, 4). The mode of its antitumor effect is unusual and has been likened to tumor necrosis factor in that massive hemorrhagic necrosis is induced, commencing a relatively short period of time after drug administration (5, 6). In contrast, high concentrations and long exposures are required in vitro for only modest cytotoxicity (3). FAA does not produce the normal spectrum of anticancer drug-induced toxicity and is nonmyelotoxic in both animals and humans (7, 8).

At least two proposed mechanisms of action have implied that a metabolite rather than the parent drug may be the active species. Bissey et al. (9) who demonstrated in vivo unique, nonrepairable single-strand DNA breaks in Gl's osteogenic sarcoma could see no obvious reactive center within the FAA molecule responsible for causing the damage. FAA stimulates natural killer cell activity in nonlymphoid tissue and synergizes with recombinant interleukin 2 in the treatment of murine renal cancer (10). Alone, FAA is unable to reproduce this effect in vitro (10), and while this may be due to the parent drug altering immune cascades not present in vitro, it may also be due to a lack of a key metabolite(s).

It is clear that critical concentrations of the drug or metabolite(s) are required at the site(s) of action. Zaharko et al. have been able to formalize this into the concept of a therapeutic window of plasma concentration (100 to 600 μg/ml) below which no activity is seen and above which acute death occurs (7). Prolonged exposure (>24 h) within the window results in delayed death. In our Phase I and pharmacokinetic study, we demonstrated that plasma drug concentrations well in excess of 100 μg/ml are clinically achievable without encountering unacceptable toxicity (8). Infusions were extended out to 6 h in order to maximize drug exposure within the therapeutic window. Two drug schedules, 4.8 g/m2 over 1 h and 8.6 g/m2 over 6 h, were taken into Phase II studies, which so far have indicated a lack of efficacy using either schedule. In this paper we report our findings of the biotransformation of FAA by humans and mice where we have purified the major human metabolites, characterized them with respect to structure, chemical properties, activity, and pharmacokinetics, and shown a major species difference.

MATERIALS AND METHODS

Chemicals, Reagents, and Drugs. All ammonium acetate/acetate acid used was HPLC reagent grade (Fisons, Loughborough, England). Separexy 40-μm silica gel bonded with C18 for column chromatography was from Analytech International (supplied by Jones Chromatography, Henoged, Wales). Hesperidin was a kind gift from Aldrich Chemical Company (Gillingham, England), and hesperitin was from Sigma Chemical Company (Poole, England). Aryl-sulfatase (type V, low in β-glucuronidase) and β-glucuronidase (type VII) were from Sigma. FAA as a pure standard for HPLC analysis (free acid form) or Sigma as a pure standard for HPLC analysis (free acid form) or as formulated material for administration to mice or humans (sodium salt form) was provided by Lplha (Lyonnaise Industrielle Pharmaceutique, Lyon, France). Pure standards of M1 and M2 were obtained in our own laboratory after purification from urine as detailed below. Water was deionized and double distilled in a quartz glass still; other-
characterization of flavone acetic acid metabolites

Fig. 1. Molecular structure of FAA (NSC 347512, LM 975).

wise all other reagents, chemicals, and solvents were of the highest grade available commercially.

Drug Analysis Techniques. Apparatus and conditions for the determination by HPLC of the major metabolites of FAA in plasma and urine were essentially the same as recently described (11). With murine specimens, the internal standard was hesperetin instead of hesperidin; otherwise no change was made in our procedure. As the major metabolites of FAA are sensitive to chemical rearrangement and chemical degradation at neutral to alkaline pH, all samples were buffered and diluted with 10 mM ammonium acetate, pH 5.5. Below pH 5.5, insolubility problems are encountered with the parent drug. Tissue and tumor specimens were homogenized in 4 vol (w/v) of 10 mM ammonium acetate, pH 5.5, using a Polytron high speed blender prior to extraction. One- to 2-ml samples of plasma, urine, tissue, and tumor homogenates were extracted using a solid-phase preparation technique utilizing 500 mg of C₁₈-bonded silica gel in minicolumns (2.4-ml capacity) and a 10-place manifold operating under negative pressure (11). This technique has the advantage that it avoids strong chemical reagents which are harmful to the labile metabolites. For analysis, 20-μl samples were injected into the chromatograph, the HPLC mobile phase consisted of 80% 10 mM ammonium acetate, pH 5.5, in 20% propan-2-ol, and the column was 30 cm long x 3.8-mm internal diameter packed with C₁₈-μBondapak 10-μm silica gel particles (Waters Associates, Northwich, England). For preparative HPLC, 500-μl samples were injected into the chromatograph, the mobile phase was 82% 10 mM ammonium acetate, pH 5.0, in 18% propan-2-ol, and the column was 30 cm long x 7.8-mm internal diameter packed with C₁₈-μBondapak.

Metabolite Purification. The two major human metabolites of FAA were purified from urine on a semipreparative basis. One hundred ml of freshly collected urine were immediately diluted to 1 liter with 10 mM ammonium acetate, pH 5.5. FAA and the two metabolites were adsorbed onto 5 g of Separalyte packed into 1/16-in diameter, 25-ml volume columns installed in a 10-place manifold connected to a small vacuum pump. Under negative pressure, each column was initially solvated with 10 ml of methanol, washed with 20 ml of water, and conditioned with 20 ml of 10 mM ammonium acetate, pH 5.5, prior to loading on 20 ml of urine. Each column was then cleaned with 20 ml of water containing 10 mM hydrochloric acid. FAA could then be selectively eluted by exhaustive washing with chloroform and the two metabolites eluted in methanol. Methanol fractions were pooled and dried down under a stream of nitrogen to yield a solid powdery material rich in the two metabolites. Five hundred mg of this material were dissolved in 5 ml of 10 mM ammonium acetate, pH 4.0, and 5 ml of dimethyl sulfoxide and spun down to remove precipitated FAA. The two metabolites were then separated and purified to apparent homogeneity by preparative HPLC. Pure metabolites were isolated from collected fractions by passing through Separalyte, eluting in methanol, and drying down under a stream of nitrogen to finally yield an off-white crystalline substance for each metabolite.

Chemical and Enzymatic Hydrolysis of Human Urine. Incubations of human urine with a variety of chemical reagents and hydrolytic enzymes were performed in a final volume of 10 ml at 37°C in a shaking water bath, under normal lighting conditions. Each incubation contained 1 ml of urine to give a final concentration of 1000 μg/ml of FAA, 40 μg/ml of M₁, and 20 μg/ml of M₂, and experiments were performed in duplicate. Incubations at pH 5 were with 10 mM ammonium acetate, pH 9, with 10 mM sodium phosphate. Urine was also incubated with 1000 units of β-glucuronidase in 4 mM sodium phosphate, pH 6.8; 500 units of aryl-sulfatase in 10 mM ammonium acetate, pH 5.5; 1 M hydrochloric acid; and 1 M sodium hydroxide. At regular time intervals, 20 μl were removed for determination of FAA and metabolite concentrations by HPLC.

Molecular Spectroscopy. Mass spectral analyses were carried out using a VG Analytical 70-SEQ mass spectrometer (VG Analytical, Manchester, England). Fast atom bombardment spectra were obtained using xenon atoms at an energy of 8 kV and an atom gun current of 1 μA. Samples of FAA, M₁, and M₂ were dissolved in either glycerol or a thioglycerol matrix, and spectra were collected in the positive or negative ion modes at a scan speed of 10 s/decade. Spectra were processed by a VG 11/250 data system. All spectra were obtained using only the first two sectors (electrostatic and magnetic) of the triple sector instrument. UV absorption spectra were obtained during HPLC using a scanning high-speed multidiode array spectrophotometer.

Animals, Tumors, and Cell Lines. Pure strain NMRI mice were from the inbred colony based in the Clinical Oncology Unit, University of Bradford (Bradford, England). They were fed a standard laboratory diet (Labsure; Poole, England), allowed water ad libitum, and maintained under constant conditions of temperature and a 12-h dark/light cycle. The MAC series of solid tumors and in vitro continuous cell lines were established from primary tumors of the large bowel of NMRI mice induced by prolonged administration of 1,2-dimethylhydrazine (12).

MAC 26 is a well-differentiated solid tumor which retains some of the histological features of the organ from which it was derived. The tumor was transplanted into male mice by s.c. implantation of 1- to 2-mm fragments into the flank and has a doubling time of approximately 4 days. MAC 15A ascites tumor cells were established in culture, after being removed by aseptic peritoneal washout, in supplemented RPMI 1640.

In Vitro Chemosensitivity. Activity of the purified metabolites was assessed against MAC 15A using a clonogenic assay with continuous exposure (12). Single cell suspensions derived from primary monolayer cultures were exposed to increasing concentrations of M₁, M₂, and FAA (12.5 to 100 μg/ml) at 37°C in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin (50 IU/ml), and sodium pyruvate (50 μg/ml). Following treatment, cells were washed in Hanks’ balanced salt solution, and 0.5 × 10^⁵ viable cells were plated into 20-ml tissue culture flask containing 10 ml of complete RPMI 1640. After 5 to 7 days of incubation at 37°C, colonies of >50 cells were counted using an inverted microscope. Cytotoxic effects of treatment were expressed in terms of the percentage of survival against controls. Duplicate samples for each concentration were performed.

Pharmacokinetic Studies. Blood and urine samples collected during our recently published Phase I and pharmacokinetic study of FAA (8) were reevaluated to determine metabolite concentrations using the new analytical methodologies described above. All samples were stored at −70°C during the interim period.

Pharmacokinetic studies were also performed in NMRI mice bearing MAC 26 after a dose of 200 mg/kg i.p. This dose produces a growth delay of approximately 10 days, equivalent to 50% tumor volume inhibition. FAA was dissolved in phosphate-buffered 0.9% NaCl solution, pH 7.4, at an appropriate concentration for a desired dose to be administered in 0.1 ml/10 g of body weight. Three replicate mice were sacrificed at 2 min, 1 h, 2 h, 3 h, 6 h, 12 h, and 24 h. Blood, tumor, liver, heart, and kidney were removed; solid tissues were immediately frozen in liquid nitrogen, plasma was separated, and all were stored at −20°C until assayed. Tissues were allowed to thaw at room temperature and were thoroughly washed with buffered 0.9% NaCl solution prior to homogenization. Hearts were chopped into small pieces and washed again with 0.9% NaCl solution to further remove blood.

RESULTS

Characterization of the Major Human Metabolites of FAA. Under optimized conditions the metabolites of FAA present in human urine were chromatographically resolved into up to four distinct peaks, consisting of two major components and two
minor components. The two major components were detected in every patient urine specimen analyzed, whereas the two minor components only appeared occasionally in urine specimens. A typical chromatogram of a direct injection of buffered human urine [10 mM ammonium acetate, pH 5.5:urine (9:1, v/v)] is shown in Fig. 2. Names were ascribed to the two major metabolites on the basis of chromatographic mobility. The earliest eluting peak and, therefore, the more water soluble of the two was designated M1, the later eluting peak being designated M2. Peaks 3 and 4 were only minor products representing much less than 1% of all excreted material in this particular patient. M1 and M2 were purified to apparent homogeneity by a two stage chromatography process involving adsorption and selective elution from Sepaplyte C2 bonded silica gel followed by preparative HPLC. Chromatograms of the purified metabolites are shown superimposed over a chromatogram of the starting material from which they were purified (Fig. 3). The metabolites, either in pure form or present in urine, were then subjected to a variety of molecular spectroscopic techniques, chemical treatments, and enzymatic hydrolyses in order to determine their chemical structure and properties (Table 1). Both metabolites were chemically labile and degraded back to the parent with 100% recovery as evidenced by an equivalent rise in the concentration of FAA with their fall in concentration (Table 1), suggesting that both are conjugates.

Decomposition occurred most rapidly at alkaline pH and even at pH 5.5 was significant. M1 was completely stable in acid throughout the time course of incubations, but M2 degraded by approximately 20% after 50 h. An interesting feature of the chemical degradation of M1 was that at pH 9 it quickly ($t_{1/2}$, 30 min) rearranged to an intermediate form which then decomposed back to the parent drug with the same half-life as M2 ($t_{1/2}$, 4 h). Limited chemical treatment of the purified metabolites confirmed the above findings. Addition of aryl-sulfatase to a pH 5.5 incubation mixture did not significantly alter their rate of breakdown. However, addition of β-glucuronidase accelerated the rate of breakdown of the two metabolites back to the parent, indicating that both are probably conjugates with glucuronic acid. Although, M2 appeared to be more resistant than M1 to β-glucuronidase treatment. UV absorption scans* of M1 and M2 revealed a chromophore (λmax in nm: M1, 253 and 300; M2, 253 and 300) almost identical to that of FAA (257 and 303 nm) and indicative of no major changes in the structure of the flavone ring system. Positive FAB mass spectra* yielded a molecular ion of $\text{MH}^+$ 457 for M1 equivalent to FAA (M, 280) plus glucuronic acid (M, 194) minus H₂O plus a proton with major fragments at $m/z$ 281 (FAA, MH$^+$) and $m/z$ 237 (M, 281-CO₂)$^+$. Negative FAB correspondingly gave ions at $m/z$ 455 (M-H)⁺, 280, 279, and 235. M2 behaved unusually in the mass spectrometer. When dissolved in thioglycerol and $m/z$ 555, 455, and 235. In negative FAB, the ions were at $m/z$ 573, 457, 281, and 237. In negative FAB, the ions were at $m/z$ 571, 455, and 235 in thioglycerol and $m/z$ 555, 455, and 235 in glycerol. This behavior would appear to be consistent with M2 (M, 456) plus either some abnormal matrix adducts or a contaminant. A later batch of M2 showed much lower levels of the higher mass ions. Methylation of M2 was shown by positive FAB to yield mono-, di-, and trimethylesters of FAA-glucuronide corresponding to $m/z$ 471, 485, and 499 but, significantly, no spurious high-molecular-weight species.

In vitro chemosensitivity of the two purified metabolites was assessed individually against MAC 15A cells in a continuous exposure clonogenic assay (Fig. 4). Both were completely without activity up to a concentration of 100 μg/ml, which is approximately twice their maximum plasma concentrations.

* Copies of UV absorption spectra and mass spectra of M1 and M2 have been deposited with the Editorial Office of Cancer Research and are available on request.
CHARACTERIZATION OF FLAVONE ACETIC ACID METABOLITES

Table 1 Influence of chemical treatment and enzyme hydrolysis of human urine on the concentrations of FAA and its two major metabolites

One ml of patient urine (containing a final concentration of: 100 µg/ml of FAA; 40 µg/ml of M2; and 20 µg/ml of M1) was incubated at 37°C in a final volume of 10 ml with the different chemical reagents or enzymes described below. β-Glucuronidase (1000 units) was made up in 10 mM sodium phosphate, pH 6.8; aryl-sulfatase (500 units, low in β-glucuronidase) made up in 10 mM ammonium acetate, pH 5.5. The incubations at pH 5 were with 10 mM ammonium acetate and at pH 9 with 10 mM sodium phosphate. At regular time intervals, 20 µl aliquots were removed to determine drug and metabolite concentrations by HPLC.

Table 2 Twenty-four-h cumulative urinary excretion of FAA and its two major metabolites in humans

Urine was collected in 6-h aliquots and immediately buffered to 10 mM ammonium acetate, pH 5.5. Drug and metabolite concentrations were determined by HPLC after solid-phase extraction (as described in "Materials and Methods").

Fig. 4. Percentage of survival of MAC 15A cells after continuous exposure to FAA or its purified metabolites M1 and M2. Survival was assessed by clonogenic assay, the purified metabolites were completely without activity up to 100 µg/ml, and the IC50 of FAA was 29 µg/ml.

achieved clinically (see Table 3). FAA had an IC50 of 29 µg/ml.

Urinary Excretion and Pharmacokinetics of FAA and Its Major Metabolites in Humans. Twenty-four h cumulative urinary excretion of FAA and its major metabolites M1 and M2 was followed in our Phase I cancer patients after either a low (0.5 g/m² 1-h infusion) or high dose (8.6 g/m² 6-h infusion) of drug. Results are in Table 2, and a typical time course of excretion is shown in Fig. 5. At the low dose, the bulk of administered drug was recovered in the urine as metabolites M1 and M2 (Table 2), principally during the first 6-h period after drug administration (Fig. 5). At the high dose, more drug was eliminated as FAA, the appearance of the metabolites was delayed up to the 18-h mark, and their cumulative excretion was significantly reduced.

M1 and M2 were the only metabolites detected in plasma. Their pharmacokinetics are summarized in Table 3 along with FAA. The half-lives of M1 and M2 were always 2 to 3 times longer than that of the parent drug. Plasma metabolite concentrations increased quickly at the low dose and decayed quickly before entering a long terminal phase, closely mirroring the rise and fall in plasma concentrations of the parent drug and consistent with their rapid appearance in urine in large amounts (Fig. 5). Regardless of the length of infusion, at the high dose, plasma metabolite concentrations were slower in rising to their peak levels (occurring 4 to 8 h after the start of infusions) and slower in falling before entering a long terminal phase, consistent with their delayed appearance in urine. As infusion lengths were extended from 1 to 3 to 6 h and even although the dose was increased from 4.8 to 6.4 to 8.6 g/m², peak concentrations of the parent drug fell progressively. In contrast, peak concentrations of the metabolites rose progressively, almost approaching 50 µg/ml at 8.6 g/m² 6-h infusion.

Pharmacokinetics, Tissue and Tumor Concentrations of FAA, and Its Major Metabolites in Mice. Neither of the two major human metabolites was detected in mice. However, low concentrations of a single biotransformed product were measured which had an HPLC retention time intermediate between that of human M1 and M2 [4.8 min versus 4.1 min (M1) and 5.1 min (M2)]. It was designated M-M1 to distinguish it from the human metabolites. Like the human metabolites, M-M1 was labile and apparently degraded back to the parent drug, suggesting that it may be a conjugate. We attempted to purify it from our samples using the same procedures detailed earlier for the human metabolites but were unsuccessful due to its lability and low concentration. M-M1 exhibited pharmacokinetic behavior different from the human metabolites in that it was produced in comparatively smaller quantities on an AUC basis and had a shorter half-life than the parent drug (9.8 h versus 19.1 h) rather than a longer one (see Table 3). In fact, 93% of circulating FAA in mice was unchanged drug, whereas in humans, metabolites could account for up to 40% of circulating material and up to 80% of material excreted in the urine.

Plasma, tissue, and tumor concentrations of FAA and M-M1...
Blood samples were collected for up to 24 h after drug administration, and plasma was separated and stored at −20°C. Prior to extraction, plasma samples were buffered with ammonium acetate (10 mM, pH 5.5). Drug and metabolite concentrations were then determined by HPLC after solid-phase extraction as described in "Materials and Methods."

### Table 3 Pharmacokinetics of FAA and its two major metabolites in humans

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<tr>
<th>Dose level (g/m²)</th>
<th>Length of infusion (h)</th>
<th>No. of patients</th>
<th>Compound</th>
<th>Peak plasma concentration (µg/ml)</th>
<th>Time to peak (h)</th>
<th>Terminal half-life* (h)</th>
<th>AUC* (µg/ml x h)</th>
<th>Clearance* (liters/h/m²)</th>
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<tr>
<td>0.5</td>
<td>1</td>
<td>3</td>
<td>FAA</td>
<td>42 ± 9.0*</td>
<td>1.0</td>
<td>2.9 ± 1</td>
<td>79 ± 23</td>
<td>7.1 ± 2.1</td>
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<td></td>
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<td>M1</td>
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<td>1.3 ± 0.4</td>
<td>12.8 ± 6</td>
<td>24 ± 5</td>
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<td>12.3 ± 5</td>
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* Half-life calculated from the terminal portion of plasma concentration/time profiles by nonlinear regression using the ELSFIT computer program.

* AUC calculated by the trapezoidal rule between 0 and 24 h.

* Clearance calculated as dose divided by AUC.

* Mean ± SD.

Fig. 5. A typical time course of urinary excretion of FAA and its metabolites M1 and M2 after either a low dose (0.5 g/m²) as a 1-h infusion or high dose (8.6 g/m²) as a 6-h infusion. Urine was collected in 6-h aliquots, and drug and metabolite concentrations were determined by HPLC after solid-phase extraction as described in "Materials and Methods."

Discussion

We have attempted to identify the major metabolites of FAA and compare both quantitatively and qualitatively pathways of biotransformation in humans and mice. In doing so we have turned up an apparent anomaly. Two major more water-soluble metabolites are present in human urine and can be purified to yield two separate entities, yet in almost every respect the two metabolites are identical. Both have the same molecular weight and share identical UV absorption spectra. Both are chemically labile over a wide pH range degrading back to FAA and are hydrolyzed by β-glucuronidase but not aryl-sulfatase. The differences observed, although appearing small, must nevertheless be significant HPLC retention time represented the major difference (4.1 min for M1, 5.1 min for M2, and 6.02 min for FAA), and it was this property which was exploited in order to purify the two metabolites. Others have recognized that more water-soluble products of FAA are present in urine, some have suggested they are glucuronides but, to the best of our knowledge, none has reported resolving them into several species (13, 14). Indeed, during many preliminary experiments, we were unable to separate out the metabolites until we found that pH, buffer ionic strength, and choice of buffer were all critically important (11). Therefore, this difference in HPLC retention time is not as large as it might seem. Apart from this, M1 and
M2 differed only subtly in chemical properties. At pH 9, M1 quickly transformed into new entity(ies) which were quite distinct from both M2 and the parent drug but which remained intact conjugates. M2 did not exhibit this behavior. Glucuronic acid is always bonded to aglycones at the hydroxyl on C-1 of the sugar ring in an enzymatic reaction catalyzed by UDP-glucuronyltransferase. Ester glucuronides, however, are known to undergo isomerization in which aglycones originally attached to C-1 migrate under mild alkaline conditions (pH 7 to 9) successively and in an unidirectional reaction from C-1 to C-2, C-2 to C-3, and C-3 to C-4 (15). The C-2, C-3, and C-4 isomers are not cleavable by β-glucuronidase, although they are still hydrolyzed by mild alkali. These intramolecular rearrangements have been reported for many classes of drugs and suspected for many more, including benzoic acid, which is analogous to the A ring of FAA (16). The observation that M1 undergoes rearrangement to distinct products suggests that M2 is unlikely to be one of these isomers. It is also important as it highlights the need to buffer samples in order to avoid introducing metabolite artifacts, thus overestimating the number of genuine metabolites present. In fact, it is possible that Peaks 3 and 4 (Fig. 2) are two such in situ catabolites, bearing in mind that present clinical protocols recommend alkalization and that urinary pHS in the region of 8 to 9 are uncommon. M2 differed from M1 in two ways: (a) it showed extra high mass ions during mass spectrometry; and (b) it tailed significantly during HPLC (11). Looking at the structure of FAA (Fig. 1), it is difficult to see how two separate sites could exist for direct addition of a molecule of glucuronic acid. However, two sites may exist: the obvious acetic acid grouping attached to C-8 and the sugar ring in an enzymic reaction catalyzed by UDP-glucuronyltransferase. Adduction formation and peak tailing could be explained by the presence of the additional charge on the molecule. M1 would then most likely be the 10-O-glucuronide capable of undergoing isomerization. However, at this stage it is impossible to completely rule out M2 being one of the positional isomers of M1.

Glucuronide metabolites are almost without exception pharmacologically inactive (16), and we have confirmed that FAA glucuronides are nontoxic to tumor cells responsive to the parent drug. Thus, their formation in humans would seem to represent a true pathway of drug inactivation. NMRI mice do not metabolize FAA in the same way as humans. M1 and M2 are not produced, but small quantities (7% of circulating material) of a novel species with the characteristics of a conjugate are detected. Similar findings have been reported with BALB/c mice, where the single conjugate accounted for 6% of a dose of 300 mg/m² (18).

There was no evidence of other pathways of biotransformation occurring apart from conjugation. Flavonoids are readily oxidizable, resulting in an opening up of the γ-pyrene ring (C ring) at 0-1 (19), and it is possible that such a reaction could occur with FAA and escape our attention.

Drugs in the molecular weight range of FAA (M, 280) are normally cleared faster in small animal species than humans (7). Zaharko et al. (7) have reported a clearance of 240 ml/h/m² for mice (after 600 mg/m²), which is in good agreement with our value of 289 ml/h/m² (Table 3; Ref. 8). By following both plasma pharmacokinetics and urinary excretion of FAA and its metabolites in humans at several dose schedules, we have been able to study a number of the factors which could be responsible for the elevated clearance. We found that FAA is extensively metabolized to products normally cleared faster in small animal species than humans (7).
3- to 4-fold fall in drug clearance. Nevertheless, clearance remained high, and M1 and M2 were still produced in gram quantities, illustrating the body's large capacity to glucuronidate drugs (20). Lack of this pathway of biotransformation in mice is likely to be contributing to the greatly reduced rate of clearance seen in this species. The metabolite produced by mice was only taken up into tissue and (even more so) tumor in a small amount and for a short period of time, in comparison to FAA. Therefore, it seems unlikely that it is exerting a major cytotoxic effect on the tumor, although other sites of action cannot be ruled out. These results await confirmation with purified metabolite(s).

It has been reported that FAA cytotoxicity to human colon tumor cells is enhanced upon addition of murine liver 9000 × g supernatants (S-9) to incubation medium (21). The implication here is that FAA is converted to more cytotoxic metabolites, although actual conversion to metabolites was not demonstrated nor were metabolite species identified. In nature, flavonoids act as catalysts in electron transport reactions, stimulating the flow of reducing equivalents to molecular oxygen, which in the above incubations would result in increased production of toxic oxygen radicals and could therefore account for the observed effect. One of the earliest events that occurs in FAA-treated mice is a dramatic reduction in blood flow to the tumor (60 to 80% after 2 to 4 h) (6, 22) corresponding with the first signs of tissue necrosis. In the MAC 26 tumor, which responds in this dramatic manner to FAA (6), concentrations of the parent drug remained higher than other tissues and plasma from which it was cleared more rapidly from 3 to 24 h. This effect could well be a direct consequence of these alterations in tumor blood flow.

Clearance, protein binding (13), and metabolism are all elevated in humans as compared with mice, indicating a greatly different dynamic situation. Once a therapeutic window of plasma FAA concentration (100 to 600 μg/ml) is reached in mice, good activity in a broad spectrum of tumors is seen. In drug-responsive murine MAC 26, we have demonstrated that drug uptake into the tumor is high and comparable with that of well-perfused organs like heart, kidney, and liver. The therapeutic window of plasma concentration is easily reached in humans; nevertheless significant activity has so far not been seen. It is possible that critical drug concentrations are still not being achieved, close to or in tumor cells or other sites of action because of competition from metabolism, protein binding, and urinary excretion for the active form of the drug.

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Characterization of the Major Metabolites of Flavone Acetic Acid and Comparison of Their Disposition in Humans and Mice

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