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Reactivity of flavone acetic acid and its acyl glucuronide

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Flavone-8-acetic acid (FAA; LM-975; NSC-34712) is a novel anticancer agent with unprecedented broad antitumor activity in murine models [1–3]. This drug is now undergoing clinical trials. Because metabolism is implicated in the cytotoxic activity of this drug *in vivo* and *in vitro* [4], we are presently studying FAA biotransformation. In the course of these studies, it was noted that FAA acyl glucuronide (FAA-G), the major metabolite of FAA in mouse and man [5, 6], was unstable.

In this study, we observed that FAA-G and FAA can react *in vitro* with nucleophilic molecules. These data suggest that FAA-G and FAA could react *in vivo* with nucleophilic species, and this chemical reactivity could play a direct, or indirect, role in the anticancer activity of FAA.

Materials and Methods

Chemicals. FAA sodium salt was kindly provided by Dr P. Briet (LIPHA, Lyon, France). Diazald® (*N*-methyl-*N*-nitroso-*p*-toluene-sulfonamide) was obtained from Aldrich-Chemie, Germany. Reduced glutathione was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Cesium iodide (Ultrapur, Normatom®), was obtained from Prolabo, France. Glycerol was 99.5% spectrophotometric grade (Janssen Chimica, Belgium).

Mice treatment and urine collection. Four B6D2F1 mice (28 g) received 180 mg/kg of FAA dissolved in water (0.5 mL) i.v., and urine was collected overnight on ice in a tube containing formic acid.

FAA acyl glucuronide (FAA-G) purification. Acidified urine (pH 2) was extracted twice with ethyl acetate (10 mL). The organic phase was evaporated under a nitrogen stream, the dry residue was redissolved in 1 mL of water, and extracted twice with dichloromethane (3 mL) to remove FAA. The water phase containing FAA-G was further purified by HPLC using a C_{18} semi-preparative column (Nucleosil, 5 μm, 9 × 235 mm, SFCC, Neuilly-Plaisance, France), and a mobile phase composed of 50% ammonium formate (0.63 g/L, pH 4) and 50% methanol (v/v), at a flow rate of 2 mL/min. The effluent was monitored in UV at 300 nm. The peak at 8.6 min was collected, dried, and further characterized as described below. FAA-G excreted in urine represented 29% of the FAA dose.

High performance liquid chromatography. A Waters system composed of two pumps, a U6K injector, a model 440 absorbance detector, a photodiode array detector (model 990), and a Digital 370 computer was used. For stability and reaction with methanol studies, an analytical octadecylsilane column was used (Nucleosil, 5 μm, 4.6 × 250 mm, SFCC, Neuilly-Plaisance, France), with a mobile phase consisting of 40% ammonium formate (0.63 g/L, pH 4) and 60% methanol (v/v), at a flow rate of 1.3 mL/min.

FAA-G characterization. β-Glucuronidase incubations were accomplished in acetate buffer (0.1 M, pH 3.8; 550 μL) with β-glucuronidase (5000 units) from abalone entrails (Sigma). Control incubations included the denatured enzyme previously heated at 100° for 10 min, the enzyme inhibitor 1,4-saccharolactone (60 mg/mL) and no enzyme. For these controls there was 3% spontaneous degradation to FAA. For base hydrolysis, a NaOH solution (0.1 N) was used at room temperature for 30 min.

FAA-G stability at physiological conditions. FAA-G was incubated in a Tris–HCl buffer (0.5 M, pH 7.4, 37°), and 50 μL were injected at certain times thereafter onto the HPLC system described above.

FAA-G reaction with methanol. The glucuronide was dissolved in methanol and incubated at 37°. Aliquots of 50 μL were injected onto HPLC at certain times thereafter to follow the reaction.

Preparation of FAA methyl ester. Esterification of FAA was accomplished as follows: FAA (400 mg) dissolved in 95% ethanol was added to a solution of potassium hydroxide in water (5 g/8 mL); diazomethane generated from *p*-toluenesulfonylmethylnitrosamide (Diazald®, Aldrich Chemicals) was added as an etheral solution (21.4 g/130 mL diethylether). FAA methyl ester molecular weight was 294 ($MH^+ = 295$) as determined by FAB mass spectrometry (glycerol matrix). The product was analytically pure based on HPLC.

Reaction of FAA with glutathione. Glutathione and FAA were mixed in a 1:1 molar ratio in water (pH 7.4), heated at 50° (to speed up the reaction), and an aliquot was analysed by HPLC at different times. As the reaction proceeded, the FAA peak decreased ($R_t = 4.43$ min) and another peak was detected with a different retention time ($R_t = 3.28$ min). The reaction was complete within 90 min and the resulting major peak was collected and analysed by mass spectrometry.

Mass spectrometry. Mass spectra were recorded in the fast atom bombardment (FAB) mode using a glycerol matrix. The hybrid mass spectrometer (7070-EQ, VG Analytical, U.K.) was operated at 6 kV, and the FAB gun (Ion Tech, U.K.) was used with xenon at 8 kV and 1.2 mA. Cesium iodide was used for calibration. Peak matching was accomplished using glycerol and its sodium clusters as internal standards.

Results

FAA acyl glucuronide (FAA-G) characterization. When purified putative FAA-G ($R_t = 3.75$ min) was subjected to β-glucuronidase it yielded a peak with the same retention time as FAA ($R_t = 5.85$ min). Also, mild alkaline hydrolysis of putative FAA-G, yielded FAA as judged by retention time. UV spectra of FAA-G and FAA were identical with maxima at 255 and 300 nm, and minima at 232 and 270 nm. The identity of FAA-G was further verified by FAB mass spectrometry, and the quasimolecular ion of the purified metabolite was at $MH^+ = 457$ ($C_{23}H_{21}O_{10}$), which corresponded to the acyl glucuronide of FAA (M = 456).

FAA-G stability at physiological conditions. When incubated at pH 7.4 and 37°, FAA-G was unstable and disappeared rapidly with an initial decomposition half-life of 42 min. Two new peaks with longer retention times, and FAA were produced under these conditions (Fig. 1). These two peaks were FAA glucuronide isomers based on their molecular weight of 456.

Reaction of FAA-G with methanol. When purified FAA-G was incubated at 37° with methanol (as a model nucleophilic reagent), FAA-G ($R_t = 3.55$ min) disappeared and a new peak ($R_t = 9.88$ min), with a longer retention time than FAA ($R_t = 5.35$ min), appeared on the HPLC chromatogram. This reaction was rapid with an apparent half-time of 18 min, and was almost complete after a 2-hr incubation. The new peak had a UV spectrum identical to FAA and FAA-G indicating that transesterification probably occurred, since alteration elsewhere on the molecule should have led to alteration of the UV spectrum. To identify the putative FAA methyl ester, the reaction product was collected and submitted to FAB mass spectrometry. A quasimolecular ion MH^+ at $m/z = 295$

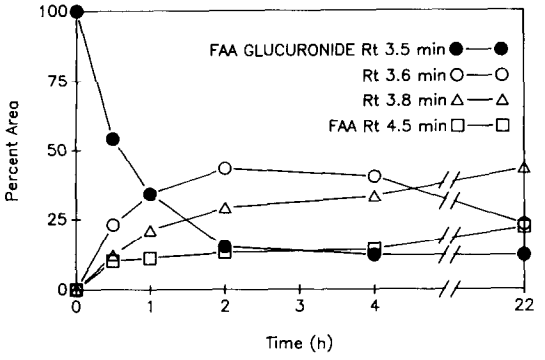

Fig. 1. FAA acyl glucuronide stability at pH 7.4 and 37°. Purified FAA acyl glucuronide was incubated at 37° in Tris–HCl buffer (0.5 M, pH 7.4), and 50 μL were injected at the indicated times onto the HPLC system described in Materials and Methods.

was observed, indicating a molecular weight of 294. Corrected peak matching confirmed the quasimolecular ion formula $C_{18}H_{15}O_4$ (experimental: m/z = 295.0948; theoretical: m/z = 295.0970) (Fig. 2A). FAA did not react with methanol in the same experimental conditions. This putative methyl ester of FAA had the same HPLC retention time as an authentic chemically synthesized methyl ester of FAA. FAB mass spectrometry of the synthesized FAA methyl ester yielded a spectrum identical to the transesterification reaction product (Fig. 2B). Evidence was thus obtained that a transesterification reaction occurred between the glucuronic acid moiety and methanol.

Reaction of FAA with glutathione. FAA could react with glutathione *in vitro* in a water solution (pH 7.4, 50°). The resulting reaction product had a R_t shorter (3.28 min) than FAA (4.43 min) in reversed-phase HPLC. A UV spectrum of the reaction product showed only one absorption maximum at 267 nm, compared to two maxima of absorption for FAA at 255 and 300 nm. On the mass spectrum, two peaks were observed at m/z = 588 and 610, corresponding to the quasi-molecular ion (MH^+ = 588) of the glutathione–FAA adduct and the addition of sodium ($M + Na^+$ = 610) confirming the molecular weight of 587 for the adduct. Peak matching confirmed the proposed structure $C_{27}H_{30}N_3O_{10}S$ (found: m/z = 588.1639; theoretical: m/z = 588.1652) for the quasi-molecular ion MH^+.

Discussion

Because metabolism plays an important role in FAA cytotoxic activity *in vivo* and *in vitro* [4], we are presently studying its biotransformation products. In the course of these studies, it was noted that a metabolite was unstable and reactive *in vitro*. In this paper, we presented evidence that FAA-G, a major metabolite of FAA in both mouse and man [5, 6], is unstable and can spontaneously react with a model nucleophilic agent. In addition to FAA-G reactivity, it was also observed that FAA could react directly with glutathione.

The instability and rearrangement reactions of acyl glucuronides of carboxylic drugs and endogenous molecules have been documented in recent years [7–9]. These glucuronides can undergo an intramolecular migration from the initial 1-β-glucuronide position to other positions on the glucuronic acid explaining the formation of β-glucuronidase-resistant FAA-G species.

The reactivity of FAA-G was verified by its reaction with the model nucleophile methanol, which yielded FAA methyl ester by a transesterification reaction. Methanol [10], as well as other model nucleophilic species and endogenous molecules [11, 12] were shown to react with acyl glucuronides. For example, van Breeman and Fenselau [11] reported covalent binding of carboxylic drugs to albumin *in vitro* through their acyl glucuronides. Such glucuronides are prone to react with other hydroxyl or sulfhydryl derivatives.

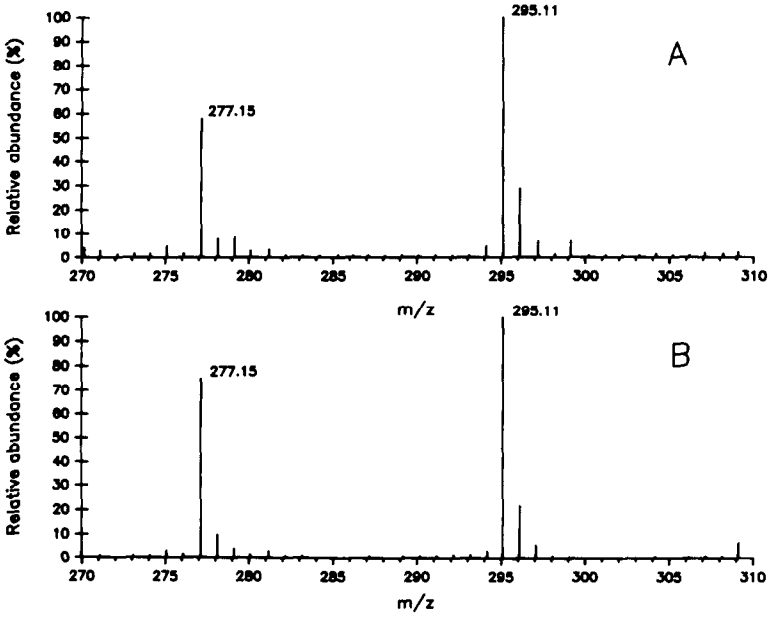

Fig. 2. FAB mass spectra of the purified reaction product of methanol with FAA acyl glucuronide (A), and of the chemically synthesized FAA methyl ester (B). The peak at 277.1 is due to the glycerol matrix (uncorrected m/z are indicated).

Although FAA-G is not cytotoxic *per se* when applied externally to neoplastic cells, because it does not enter cells [6], it could nevertheless play an indirect role in the anticancer activity of FAA *in vivo* through binding to biological molecules needed for cancer cell division. It is also possible that a reactive metabolite, like FAA-G, when formed intracellularly in neoplastic cells, could be cytotoxic to these cells. Binding of FAA to macromolecules like albumin or DNA is therefore possible through this mechanism. Cytotoxicity and immunogenicity have also been proposed as some possible consequences of chronic exposure to acyl glucuronides through their electrophilic reactions [12].

FAA-G production is dose-dependent in both mouse and man, although in opposite directions: in mice FAA-G production increases up to the maximum tolerated dose (180 mg/kg) [5], whereas in man, the production of FAA-G decreases with dose (maximum tolerated dose, 8.6 g/m^2, or about 210 mg/kg) [6]. These observations indicate a major difference between the two species, with respect to FAA biotransformation. It is also possible that other metabolic routes are present in mice, because of the lower formation of FAA-G in this species compared to man. These other metabolic routes could be important for anticancer activity.

The reaction between FAA and glutathione showed that the α,β-conjugated ketone of FAA is reactive and can give the corresponding adduct. The reactivity of this double bond is similar to the conjugated double bond of prostaglandin A_1, which is metabolized to glutathione-, glutamylcysteine- and cysteine-adducts [13, 14]. This reaction could possibly occur *in vivo* with other bionucleophilic molecules.

The reactivity of FAA and its acyl glucuronide demonstrated in this paper could also indicate that these species can react *in vivo* with numerous other nucleophilic compounds. Such compounds could be, for example, the superoxide anion $O_2^{\cdot-}$, and nitric oxide $NO^{\cdot}$, since the former can be formed *in vivo*, and the latter accounts for the biological activities of endothelium-derived relaxing factor (EDRF) [15]. Recently, Moncada *et al.* [16] provided evidence that nitric oxide plays a major role in cell function and communication. This, coupled with the fact that FAA can act as an antitumor drug through various mechanisms, e.g. modification of blood flow [17, 18], and immune modulation [19, 20], could be reconciled by the ability of FAA or FAA-G to react with nitric oxide.

In conclusion, FAA-G was shown to be an unstable and reactive metabolite, and FAA 2-3 double bond was also shown to be reactive. These data suggest that FAA and FAA-G could react *in vivo* with nucleophilic species, and this chemical reactivity could play a direct or indirect role in the anticancer activity of this compound.

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Effect of ethanol feeding on fatty acid ethyl ester synthase activity in the liver and pancreas of rats fed a nutritionally adequate diet or a low protein diet

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Alcohol dehydrogenase (ADH) and the microsomal ethanol-oxidizing system (MEOS) are enzymes which catalyse the biological oxidation of ethanol in the liver. Chronic ethanol consumption results in a 2–3-fold enhancement of the MEOS activity [1, 2]. ADH activity has been reported to be increased, decreased and unchanged in the livers of chronic ethanol-fed rats [1–6].

Recently, the presence of non-oxidative ethanol metabolism has been demonstrated in alcohol target organs. In the pancreas, myocardium and brain, where there is little or no ADH activity, the metabolites of this non-oxidative pathway, fatty acid ethyl esters (FAEE), are reported to play an important role in the development of alcoholic organ damage [7–12]. We also reported that ethanol feeding had no effect on pancreatic FAEE synthase activity, but pancreatic FAEE content, increased 5-fold in chronic ethanol-fed rats, correlated inversely with amylase activity [11]. Although FAEE synthase activity is low in the rat liver [11], there is no report about the effect of chronic ethanol feeding on this enzyme activity in liver. In general, alcoholics have low protein intake, so hepatic ADH activity may be decreased [13, 14]. Therefore, the non-oxidative ethanol pathway may work in the liver after ethanol feeding with dietary protein deficiency.

In the present study, we examined the effects of ethanol feeding with a standard or low protein diet on FAEE synthase activity in the liver and pancreas of rats, as well as on hepatic ADH activity.

Materials and Methods

Animals. Twenty-four male Wistar strain rats, weighing about 200 g, were divided into four groups and fed the following diets in individual cages for 7 weeks as described previously [15]. Six rats received a diet in which 18% of the total calories was protein, 35% was fat and 47% was carbohydrate (a standard diet). Six rats received a low protein diet, in which 8% of the calories was protein, 35% was fat and 57% was carbohydrate (a low protein diet). In the ethanol formula, carbohydrate was partially replaced by ethanol. The ethanol amounted to 36% of the total calories in the standard diet (a standard ethanol diet) and also in the low protein diet (a low protein ethanol diet), while the total calories was held constant. All diets contained adequate essential trace elements and vitamins. These experimental diets were purchased from CLEA Japan Inc. (Osaka, Japan). After fasting for 12 hr, the rats were killed by exsanguination from the aorta. The liver and pancreas were removed and subjected to biochemical analysis.

Assay for FAEE synthase activity. FAEE synthase activity was determined by the method of Mogelson and Lange [16] as described previously [11].

Assay for ADH activity. ADH activity was determined by the method of Estival *et al.* [17].

Protein determination. Protein contents were determined by the method of Lowry *et al.* [18].

Statistical analysis. All values were expressed as the mean ± SEM, and the significance of the differences was assessed by one-way analysis of variance.

Results

Body weight, weight gain, organ weights and enzyme activities of the liver and pancreas in the four groups are shown in Table 1. In spite of pair feeding, ethanol feeding resulted in smaller mean weight gain and body weight than the corresponding control groups on both the standard and the low protein diet. However, liver weight per 100 g body weight was greater than the control in the standard ethanol diet group, and did not differ from the control in the low protein ethanol diet group. Rats fed the low protein diet had lower body weight than those fed the standard diet, but liver weight was similar.

The liver ADH activity of rats in the standard ethanol diet did not differ from control. However, these animals did show decreased liver FAEE synthase activity, which was statistically significant when expressed as μmol ethyl oleate/hr/g liver and as μmol ethyl oleate/hr/liver. Rats given the low protein diet had lower liver ADH and FAEE synthase activity than rats given the standard control diet. Liver ADH and FAEE synthase activities in rats given the low protein ethanol diet was very low. It was even lower than the low protein control group.

FAEE synthase activity in the pancreas was remarkably higher than in the liver. There were no significant differences in pancreatic FAEE synthase activity between any of the groups.

Discussion

ADH and MEOS are responsible for the oxidation of ingested ethanol and are abundant in the liver. Ethanol feeding is known to enhance MEOS activity by about 3-fold [19]. After chronic alcohol consumption, liver MEOS activity increases in experimental animals and in humans [6, 20, 21], and the increased rate of ethanol elimination can largely be accounted for by the increase in total liver MEOS activity. However, there is a controversy about the effect of chronic ethanol consumption with nutritionally