Cancer Chemother Pharmacol (1989) 24: 273–276

Cancer Chemotherapy and Pharmacology

© Springer-Verlag 1989

Flavone acetic acid (LM-975; NSC-347512) activation to cytotoxic species in vivo and in vitro*

Guy G. Chabot, Marie-Christine Bissery, and Alain Gouyette**

Unité de Biochimie-Enzymologie, URA158 CNRS and U140 INSERM, Laboratoire de Pharmacologie Clinique, Institut Gustave-Roussy, F-94805 Villejuif Cedex, France

Summary. Flavone acetic acid (FAA; LM 975; NSC 347512) is a new anticancer agent with unprecedented, broad antitumor activity in murine models. Although FAA is very effective in vivo against solid tumors, including colon 38 adenocarcinoma, it was not cytotoxic in vitro against colon 38 cells and human colon adenocarcinoma cells HCT116 at pharmacologically achievable concentrations and exposure times. For example, a concentration of 300 μg/ml for a 10-day exposure time was required to obtain <1 log cell kill. After the administration of an effective FAA dose (180 mg/kg, i.v.) to mice, plasma cytotoxicity against HCT116 cells attained a 2 log cell kill between 0.5 and 2 h, which decreased to 1 log cell kill at 4 h. No cytotoxicity was observed 6, 12 or 21 h after drug administration. The controls used comprised mouse plasma containing FAA concentrations similar to those assayed in the above plasma samples from in-vivo-dosed mice. These spiked plasma were not cytotoxic, indicating that other cytotoxic species, formed in vivo, were responsible for the increased cytotoxicity. Mouse hepatocytes co-cultured with HCT116 cells increased FAA cytotoxicity to 1 log cell kill at 30–100 μg/ml. The addition of phenobarbital-induced mouse liver supernatant S-9000xg also markedly increased FAA cytotoxicity to a 2 log cell kill at 300 μg/ml. We conclude that FAA can be activated both in vivo and in vitro to cytotoxic species that are more active than the parent compound.

Introduction

Flavone-8-acetic acid (FAA; 2-phenyl-8-(carboxymethyl)-benzopyran-4-one; LM 975; NSC 347512) is a new anticancer agent with unprecedented, broad antitumor activity in murine models [1, 10, 20]. Unlike most of the clinically used anticancer agents, only modest activity is observed against either P388 or L1210 leukemia [1, 10, 20]. Although very active in preclinical trials, this drug has shown a steep dose-response behavior that was correlated to its nonlinear pharmacokinetics in mice [7]. Nonlinear pharmacokinetics has also been reported in phase I clinical studies [16, 19, 25].

However, a marked discrepancy between FAA's good antitumor activity in vivo and its poor cytotoxicity in vitro has been observed by many authors [2, 5, 6, 12, 14, 23]. A possible reason for the latter could be the need for an in vivo activation of the drug, which, like cyclophosphamide and dacarbazine, could act as a prodrug, requiring bioactivation to exert its activity. The present experiments were designed primarily to examine the possible activation of FAA in vivo (in mice) and in vitro. In this study, we present evidence that FAA can be activated both in vivo and in vitro to species that are more cytotoxic than the parent compound.

Materials and methods

Drug. FAA sodium salt was kindly provided by Dr. P. Briet, Lyonnaise Industrielle Pharmaceutique (LIPHA; Lyon, France). It was dissolved in distilled water and sterilized by filtration (0.22-μm filter).

Cells and cytotoxicity assay. Colon adenocarcinoma 38 tumors [9] were passaged in C57Bl/6 mice and fresh cell suspension for cytotoxicity assay was prepared by mechanical disruption using a Stomacher [3]. HCT116 human carcinoma cells [4] were obtained from the American Type Culture Collection (Rockville, Md) and passaged twice a week. The culture medium used for in vitro soft-agar assay was McCoy's 5A medium with heat-inactivated fetal bovine serum (20%), gentamycin (50 μg/ml), and amphotericin B (Fungizone, 1 μg/ml). Cells were incubated with FAA at various concentrations and exposure times. After the different treatments in vitro, cells were seeded in soft agar (0.3%) at a density of 10^4 cells/ml (3 ml) in 35 mm petri dishes (triplicate). Cells were incubated (37° C, 100% humidity, 5% CO_2) for 10 days, and colonies were counted under the microscope. Results are expressed as the number of colonies in the treatment plates divided by the number of colonies in the control plates (survival fraction).

Mouse plasma sampling for in vitro cytotoxicity. $B_6D_2F_1$ mice (28 g) were injected i.v. with FAA at 180 mg/kg in a volume of 0.5 ml. Blood was aseptically collected by cardiac puncture at 0.5, 1, 2, 4, 6, 12, and 21 h after FAA ad-

* Part of this work has been published in abstract form in: Proc Am Assoc Cancer Res 29: 484, 1988

** *Present address:* Rhône-Poulenc Santé, Centre de Recherche de Vitry, F-94403 Vitry Sur Seine, France

Offprint requests to: Guy G. Chabot, Institut Gustave-Roussy, Pavillon de Recherche, 39, rue Camille-Desmoulins, F-94805 Villejuif Cedex, France

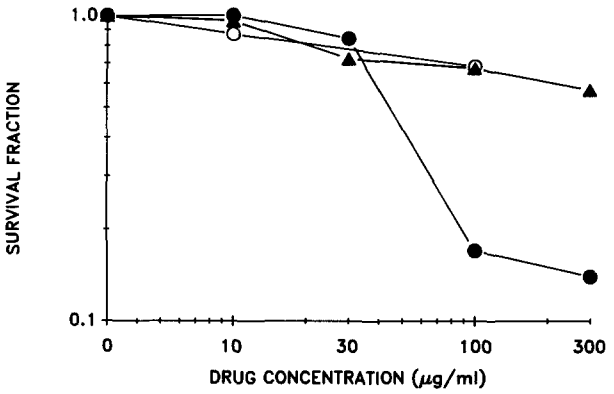

Fig. 1. FAA cytotoxicity in vitro against mouse adenocarcinoma 38 cells freshly isolated from a tumor. Cells were exposed to FAA at the indicated concentrations for 4 h (*empty circles*), 24 h (*triangles*), or 240 h (*closed circles*). Colonies were counted 10 days after plating. Each point represents the mean of a triplicate determination; SE, < 18% on each point

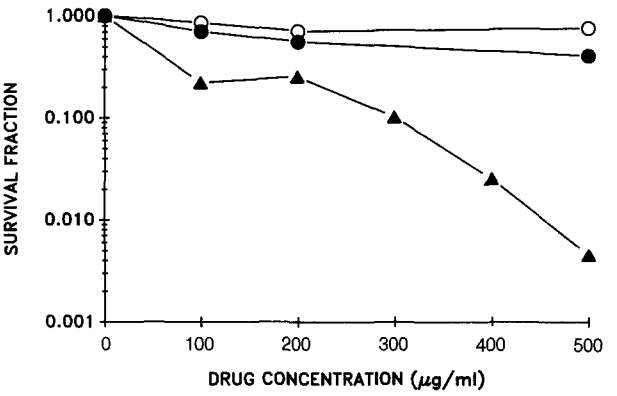

Fig. 2. FAA cytotoxicity in vitro against human colorectal carcinoma cells (HCT116). Cells were exposed to FAA at the indicated concentrations for 4 h (*empty circles*), 24 h (*closed circles*), or 240 h (*triangles*). Colonies were counted 10 days after plating. Each point represents the mean of a triplicate determination; SE, < 10% on each point

ministration (5 mice/time point). Blood was immediately centrifuged (4° C) and 1 ml plasma was then added to 9 ml HCT116 cell suspension (10^4 cells/ml) and plated in soft agar for 10 days. FAA plasma concentrations were determined by HPLC [7] in an aliquot of the above plasma. For controls, FAA was added ex vivo to fresh mouse plasma.

Preparation of mouse hepatocytes. Mouse hepatocytes were prepared using the retrograde vena cava cannulation method [21]. Following pentobarbital anesthesia (100 mg/kg i.p.), the liver was first perfused with a calcium-free HEPES buffer (pH 7.4) at 5 ml/min for 2 min, followed by perfusion with a collagenase solution (type I, Sigma, St. Louis, Mo) for 8 min. Hepatocytes were washed twice and 5×10^5 viable cells were plated in triplicate with HCT116 cells as described above. Controls with HCT116 cells and hepatocytes without drug were used.

Preparation of phenobarbital-induced mouse liver supernatant S-9000xg. $B_6D_2F_1$ mice received phenobarbital for 4 days at 60 mg/kg i.p. and were sacrificed by decapitation; livers were aseptically excised, trimmed of debris, washed, and homogenized in 4 times their weight in cold 1.15% KCl using a motor-driven Teflon-glass homogenizer. The liver homogenate was centrifuged for 10 min at 4° C and the supernatant was harvested (S-9000xg). Aliquots of 10 or 50 μl S-9000xg (containing 0.04 and 0.2 mg protein/ml, respectively) were added to petri dishes containing HCT116 cells and plated in soft agar for 10 days. $MgCl_2$ (5 m*M*) and NADPH (2.4 m*M*) were added to the cell medium. Controls with co-factors and S-9000xg were used.

Results

FAA cytotoxicity in vitro

FAA cytotoxicity was first evaluated in vitro against colon 38 mouse adenocarcinoma cells, because of the known sensitivity of this cell line to the drug in vivo [1, 10, 20]. Figure 1 shows that FAA was not cytotoxic at concentrations and exposure times achievable in vivo in mice. A 4- or 24-h exposure time at drug concentrations up to 300 μg/ml did not achieve a 0.5 log cell kill. For comparison to the in vivo situation, an effective dose of FAA (180 mg/kg) gives plasma concentrations above 100 μg/ml for only about 2 h and tumor concentrations between 10 and 100 μg/g for about 8 h [7]. To achieve almost 1 log cell kill in vitro, a 10-day exposure time was needed at concentrations of 100–300 μg/ml. These long exposure times and high concentrations are not achievable in vivo in mice.

FAA cytotoxicity was also evaluated against HCT116 human colon adenocarcinoma cells (Fig. 2), and we also observed that FAA was not cytotoxic at concentrations and exposure times achievable in vivo in man: high concentrations (500 μg/ml) for 4 or 24 h were not cytotoxic. Only the 10-day exposure time at high concentrations (300–500 μg/ml) could kill more than 1 log of tumor cells. This 10-day exposure time (at high concentrations) is not achievable in mice or humans. In view of the poor cytotoxicity of FAA observed in vitro against either a murine or a human cell line, we then tested the hypothesis of FAA activation in vivo and in vitro.

Mouse plasma cytotoxicity after in vivo FAA administration

Plasma cytotoxicity of mice injected with an effective dose of FAA (180 mg/kg, i.v.) was evaluated. The cytotoxicity of mouse plasma harvested at different times after drug administration is presented in Fig. 3. We observed a marked cytotoxicity of about a 2 log cell kill (99% cell kill) with plasma samples harvested as early as 0.5, 1, and 2 h after FAA administration. At 4 h, plasma cytotoxicity decreased to about 1 log (90% cell kill), and no cytotoxicity was observed 6, 12, and 21 h after drug administration. To verify whether cytotoxicity was due to FAA alone, FAA concentrations were determined by high-performance liquid chromatography (HPLC) in an aliquot of the plasma samples, and the known concentrations of FAA were added to blank mouse plasma as controls. We observed that FAA added to mouse plasma was not cytotoxic (Fig. 3), even at the highest drug concentration tested (217 μg/ml).

FAA cytotoxicity in vitro with mouse hepatocytes

Mouse hepatocytes co-cultured with HCT116 cells increased FAA cytotoxicity to about 1 log cell kill at drug concentrations of 30–100 μg/ml (Fig. 4).

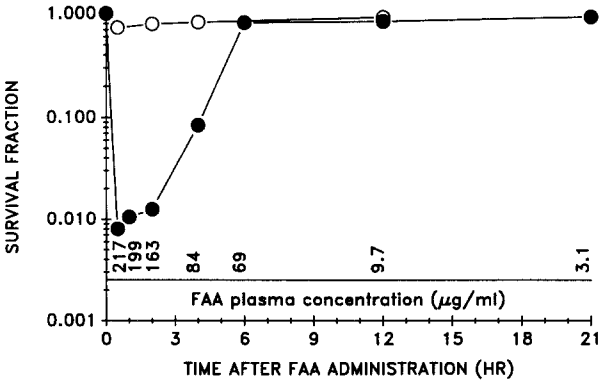

Fig. 3. In vitro cytotoxicity of mouse plasma after in vivo FAA administration against HCT116 cells. *Solid circles*, mouse plasma from mice that received FAA in vivo (180 mg/kg, i.v. bolus), harvested at different times after drug administration; *empty circles*, control mouse plasma with added FAA, at the same concentrations that were determined (HPLC) in an aliquot of the above plasmas from mice that received FAA. Plasma concentrations are indicated at their corresponding times after in vivo drug administration. Colonies were counted 10 days after plating. Each point represents the mean of a triplicate determination; SE, <10% on each point

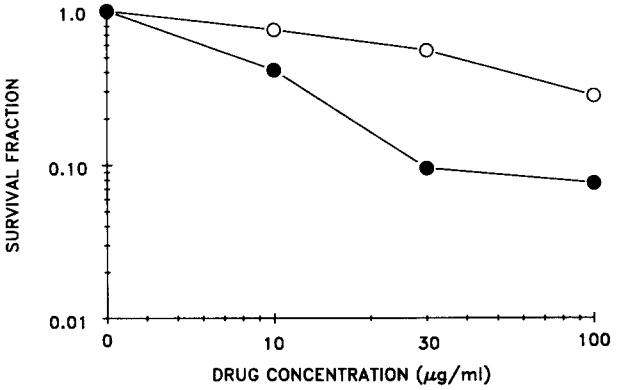

Fig. 4. FAA cytotoxicity in vitro against human HCT116 cells cocultured with mouse hepatocytes. Cells were continuously exposed to FAA at the indicated concentrations without (*empty circles*) or with hepatocytes (*closed circles*). Colonies were counted 10 days after plating. Each point represents the mean of a triplicate determination; SE, <7% on each point

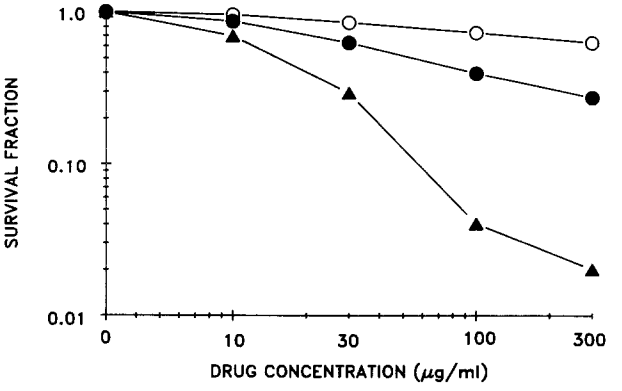

Fig. 5. FAA cytotoxicity in vitro against human HCT116 cells in the presence or absence of phenobarbital-induced mouse liver supernatant S-9000xg: controls (*empty circles*); 10 μl S-9000xg/ml (0.04 mg protein/ml) (*closed circles*); and 50 μl S-9000xg/ml (0.2 mg protein/ml) (*triangles*). Colonies were counted 10 days after plating. Each point represents the mean of a triplicate determination; SE, <7% on each point

FAA cytotoxicity in vitro with phenobarbital-induced mouse liver supernatant S-9000xg

HCT116 cells were also exposed to FAA in the presence of phenobarbital-induced mouse liver supernatant S-9000xg. Cytotoxicity was markedly increased to about a 2 log cell kill, as shown in Fig. 5. The increase in FAA cytotoxicity was dependent on both FAA and S-9000xg concentration.

Discussion

Although FAA is markedly active in vivo in murine models [1, 10, 20], its poor activity in vitro at pharmacologically achievable concentrations and exposure times [7] was soon observed by us [6] and others [2, 5, 12, 14, 23]. The discrepancy between in vivo and in vitro cytotoxicity could be due to in vivo activation of FAA to species that are more cytotoxic than the parent compound. In this paper, we present evidence that FAA can be activated both in vivo and in vitro to species that are more cytotoxic than the parent drug.

Activation to cytotoxic species is probably not the only mechanism of action implicated in FAA antitumor activity in vivo because FAA seems to act on many biological systems, like other flavonoids [17]. The precise mechanism of action is presently unknown, but the following list of reported effects could explain in part its antitumor activity in vivo: (a) extensive irreparable DNA single-strand breaks in tumor cells after FAA treatment in mice [3]; (b) rapid decrease in tumor ATP levels [13]; (c) decrease in tumor blood flow [13]; (d) increase in natural killer-cell activity and interferon production [8, 18, 26, 27]; and (e) interference with the coagulation process [11, 22].

Our data on the poor cytotoxicity of FAA in vitro against murine colon adenocarcinoma 38 cells were unexpected because this cell line is sensitive to FAA in vivo [1, 10, 20]. The insensitivity of the human colon adenocarcinoma cell line HCT116 corroborates the poor sensitivity of other human colon cancer cell lines in vitro [12].

The marked cytotoxicity of mouse plasma against human HCT116 cells after in vivo administration of FAA indicates that metabolites and/or other factors are responsible for the enhanced cytotoxicity compared with that obtained with FAA alone. Human colorectal carcinoma cells were also reported to be sensitive to FAA after in vivo administration in nude mice, showing that bioactivation also occurs in nude mice [15]. Taken together, these data show that human cell lines can also be sensitive to FAA after appropriate bioactivation.

Although mouse plasma from FAA-dosed mice was markedly more cytotoxic than that with FAA added, this experiment did not rule out previously cited factors that could be involved in cytotoxic FAA action in vivo. However, because plasma cytotoxicity was assessed in vitro in this experiment, we can rule out factors such as natural killer-cell activity [8, 26, 27], hemorrhagic necrosis [3, 24], tumor blood flow [13], and anticoagulation effect [22] that are implicated in FAA activity in vivo.

Interferon production cannot be ruled out as a contributor to mouse plasma cytotoxicity, but our data on the kinetics of cytotoxicity are markedly different from the interferon production reported by Hornung et al. [18]. For example, our data showed maximal cytotoxicity (2 log cell kill) between 0.5 and 2 h, decreasing to 1 log cell kill at 4 h, and no cytotoxicity was observed at 6 h and beyond,

whereas interferon peak production was shown to occur between 3 and 5 h [18]. Therefore, it seems unlikely that cytokine production can explain the cytotoxicity observed in plasma as early as 0.5–2 h after FAA administration.

However, our in vitro data, showing enhanced cytotoxicity with mouse hepatocytes and phenobarbital-induced mouse liver supernatant S-9000xg, rule out any contribution of the immune system to the cytotoxicity. These data strongly suggest the participation of FAA metabolites in cytotoxicity both in vitro and in vivo. Our data indicate that FAA metabolism plays an important role in its cytotoxicity both in vivo and in vitro; therefore, the identification of FAA metabolites is of primary importance in the understanding of FAA's mechanism of action. The identification of FAA metabolites formed in vivo and in vitro is presently under study in our laboratory and will be published separately.

Acknowledgements. This work was supported in part by the Association pour la Recherche contre le Cancer, Villejuif, France. Part of this work was initiated under a grant from the Harper Medical Staff Trust Fund, Wayne State University School of Medicine, Detroit, Michigan. We are very grateful to Kathleen Rutkowski for excellent technical assistance.

References

1. Atassi G, Briet P, Berthelon JJ, Collonges F (1985) Synthesis and antitumor activity of some 8-substituted-4-oxo-4H-1-benzopyrans. Eur J Med Chem-Chim Ther 20: 393
2. Bibby MC, Double JA, Phillips RM, Loadman PM (1987) Factors involved in the anti-cancer activity of the investigational agents LM985 (flavone acetic acid ester) and LM975 (flavone acetic acid). Br J Cancer 55: 159
3. 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
4. Brattain MG, Fine WD, Khaled FM, Thompson J, Brattain DE (1981) Heterogeneity of malignant cells from a human colonic carcinoma. Cancer Res 41: 1751
5. Capolongo LS, Balconi G, Ubezio P, Giavazzi R, Taraboletti G, Regonesi A, Yoder OC, D'Incalci M (1987) Antiproliferative properties of flavone acetic acid (NSC 347512; LM 975), a new anticancer agent. Eur J Cancer Clin Oncol 23: 1529
6. Chabot GG, Rutkowski K (1988) Activation of flavone-8-acetic acid to cytotoxic metabolites in vitro. Proc Am Assoc Cancer Res 29: 484
7. Chabot GG, Bissery MC, Corbett TH, Rutkowski K, Baker L (1989) Pharmacodynamics and causes of dose-dependent pharmacokinetics of flavone-8-acetic acid (LM-975; NSC-347512) in mice. Cancer Chemother Pharmacol 24: 15–22
8. Ching LM, Baguley BC (1987) Induction of natural killer cell activity by the antitumour compound flavone acetic acid (NSC 347512). Eur J Cancer Clin Oncol 23: 1047
9. 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
10. 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
11. Davis HP, Newlands ES, Allain T, Hegde U (1988) Immune thrombocytopenia caused by flavone-8-acetic acid. Lancet I: 412
12. Drewinko B, Yang LY (1986) The activity of flavone acetic acid (NSC-347512) on human colon cancer cells in vitro. Invest New Drugs 4: 289
13. Evelhoch JL, Bissery MC, Chabot GG, Simpson NE, McCoy CL, Heilbrun LK, Corbett TH (1988) Flavone acetic acid (NSC 347512)-induced modulation of tumor physiology monitored by in vivo nuclear magnetic resonance spectroscopy. Cancer Res 48: 4749
14. Finlay GJ, Smith GP, Fray LM, Baguley BC (1988) Effect of flavone acetic acid on Lewis lung carcinoma: evidence for an indirect effect. J Natl Cancer Inst 80: 241
15. Giavazzi R, Garofalo A, Damia G, Garattini S, D'Incalci M (1988) Response to flavone acetic acid (NSC 347512) of primary and metastatic human colorectal carcinoma xenografts. Br J Cancer 57: 277
16. Gouyette A, Kerr DJ, Kaye SB, Setanoians A, Cassidy J, Bradley C, Forrest G, Soukop M (1988) Flavone acetic acid: a nonlinear pharmacokinetic model. Cancer Chemother Pharmacol 22: 114
17. Havsteen B (1983) Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol 32: 1141
18. Hornung RL, Young HA, Urba WJ, Wiltrout RH (1988) Immunomodulation of natural killer cell activity by flavone acetic acid: occurrence via induction of interferon alpha/beta. J Natl Cancer Inst 80: 1226
19. Kerr DJ, Kaye SB, Cassidy J, Bradley C, Rankin EM, Adams L, Setanoians A, Young T, Forrest G, Soukop M, Clavel M (1987) Phase I and pharmacokinetic study of flavone acetic acid. Cancer Res 47: 6776
20. 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
21. Renton KW (1987) Cytochrome P-450-dependent monooxygenase systems in mouse hepatocytes. In: Rauckman EJ, Padilla GM (eds) The isolated hepatocyte, use in toxicology and xenobiotic biotransformations. Academic, Orlando, Florida, p 69
22. Rubin J, Ames MM, Schutt AJ, Nichols WL, Bowie EJW, Kovacs JS (1987) Flavone-8-acetic acid inhibits ristocetin-induced platelet agglutination and prolongs bleeding time. Lancet II: 1081
23. Schroyens WA, Dodion PF, Sanders C, Loos M, Dethier NE, Delforge AR, Stryckmans PA, Kenis Y (1987) In vitro chemosensitivity testing of flavone acetic acid (LM 975; NSC 347512) and its diethylaminoethyl ester derivative (LM 985; NSC 293015). Eur J Cancer Clin Oncol 23: 1135
24. Smith GP, Calveley SB, Smith MJ, Baguley BC (1987) Flavone acetic acid (NSC 347512) induces haemorrhagic necrosis of mouse colon 26 and 38 tumors. Eur J Cancer Clin Oncol 23: 1209
25. 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
26. Urba WJ, Longo DL, Lombardo FA, Weiss RB (1988) Enhancement of natural killer activity in human peripheral blood by flavone acetic acid. J Natl Cancer Inst 80: 521
27. Wiltrout RH, Boyd MR, Back TC, Salup PR, Arthur JA, Hornung RL (1988) Flavone-8-acetic acid augments systemic natural killer cell activity and synergizes with IL-2 for treatment of murine renal cancer. J Immunol 9: 3261

Received 30 November 1988/Accepted 10 March 1989