ABSTRACT

The antitumor agent flavone-8-acetic acid (FAA) is remarkable because it induces hemorrhagic necrosis, altered tumor blood flow, and cytokine synthesis. We show here that FAA and structurally related analogues increase plasma nitrite plus nitrate (NO$_2^-$/NO$_3^-$) levels in mice. Dose-dependent increases in plasma NO$_2^-$/NO$_3^-$ concentrations, which reached maximum levels at 12 h, were found following administration of FAA. Furthermore, the presence of a palpable s.c. Colon 38 tumor significantly enhanced the response. Tumor-dependent increases were also observed with the active FAA analogues xanthenone-4-acetic acid, 5-methyl XAA, and 5,6-dimethyl XAA, while the inactive analogue 8-methyl XAA failed to increase plasma NO$_2^-$/NO$_3^-$ concentrations substantially above basal levels. Increased plasma NO$_2^-$/NO$_3^-$ levels were also observed in response to endotoxin (100 μg/mouse) and to recombinant human tumor necrosis factor α (4 to 16 μg/mouse). NO$_2^-$/NO$_3^-$ levels may signify nitric oxide production as a result of stimulation of the L-arginine-dependent pathway associated with in vitro cytotoxic and tumoricidal effects of activated macrophages (18, 19). Our in vitro results therefore suggest that FAA and XAA analogues resemble endotoxin in stimulating this pathway in activated macrophages. Since in vivo studies suggest that macrophages may be the major cellular source of increased levels of nitrate in plasma and urine of endotoxin-treated mice (20, 21), plasma nitrate levels may be used to indicate the in vivo response to FAA and XAA analogues.

In this study we have investigated the effect of FAA on the production of NO$_2^-$/NO$_3^-$ in plasma of mice. We have utilized the Colon 38 adenocarcinoma, a dimethylhydrazine-induced tumor (volume doubling time of advanced tumors, approximately 2 days) which has been used extensively for cytotoxic drug development and which is highly sensitive to FAA (3, 22). We have measured plasma NO$_2^-$/NO$_3^-$ levels in normal and tumor-bearing mice and have compared the effects of FAA with those of XAA, 5-MeXAA, 5,6-MeXAA, and 8-MeXAA to determine whether plasma NO$_2^-$/NO$_3^-$ levels correlate with antitumor effects.

INTRODUCTION

FAA $^1$ is a novel antitumor agent with activity against many experimental solid tumors (1–4). Although some activity is observed against human xenografts in mice (4, 5), clinical activity has not been demonstrated (6). The effects of FAA on subcutaneous solid tumors include the induction of hemorrhagic necrosis (3) and changes in tumor blood flow (7–9), the latter appearing at least in part to account for tumor necrosis (9). Discrepancies between in vitro and in vivo antitumor effects (4), together with induction of cytokine production (10) and of tumor cell death by two mechanisms, alteration of blood flow contributing to tumor ischemia and direct tumor cell killing. Plasma NO$_2^-$/NO$_3^-$ concentrations may be a sensitive indication of the antitumor response to this class of compounds.

1 The abbreviations used are: FAA, flavone-8-acetic acid; XAA, xanthenone-4-acetic acid; 5-MeXAA, 5-methyl XAA; 6-MeXAA, 6-methyl XAA; 5,6-MeXAA, 5,6-dimethyl XAA; NO$_2^-$/NO$_3^-$, nitrite plus nitrate; TNF-α, tumor necrosis factor α.

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Materials. FAA was obtained from the National Cancer Institute, USA, through the courtesy of Dr. K. Paull. XAA and its derivatives (Fig. 1) were synthesized in this laboratory by Dr. W. A. Denny, Dr. G. J. Atwell, and Dr. G. W. Rewcastle and were judged pure by thin-layer chromatography. Drug solutions were prepared immediately prior to i.p. injection by dissolving in 5% (w/v) sodium bicarbonate and were protected from light (23). Recombinant human TNF-α, kindly provided by Professor James Watson, Department of Molecular Medicine, University of Auckland Medical School, was dissolved in sterile water. Endotoxin (prepared by phenol extraction from Escherichia coli 055:B5; Sigma) was dissolved in sterile water.

Animal Procedures. C57BL/6 × DBA/2 F1, mice, bred in the laboratory animal facility, were housed under constant temperature and humidity with sterile bedding, water, and food according to institutional ethical guidelines. Mice without tumors (non-tumor bearing) or with s.c. Colon 38 tumors (tumor bearing) 4 to 10 mm in diameter (10 to 11 days after implantation) were given injections i.p. of FAA at doses up to the maximum tolerated dose (1180 μmol/kg) or of XAA, 5-MeXAA, 5,6-MeXAA, or 8-MeXAA at maximum tolerated doses (800, 160, 100, and 640 μmol/kg, respectively). Endotoxin and recombinant
human TNF-α were injected i.p. Control mice that received no drug treatment were included in each experiment.

In one experiment, tumor-bearing mice either were anesthetized with pentobarbital (90 mg/kg) and had their tumors excised or were sham operated, or they received no anesthetic and operation, 2 h prior to FAA administration. Similarly, non-tumor-bearing mice either were sham operated or received no anesthetic and operation, 2 h prior to FAA administration.

Blood Collection. Blood was obtained by heart puncture under ether anesthesia with heparinized syringes. The blood samples were immediately centrifuged at 8000 x g for 5 min, and the plasma was removed and stored at −70°C.

Plasma NO₂⁻/NO₃⁻ Analysis. Plasma samples were diluted with milli-Q (distilled) water, and the proteins were precipitated with 30% ZnSO₄ (0.05 ml/ml of plasma) and centrifuged (8000 x g, 5 min). After reducing NO₃⁻ in the supernatants to NO₂⁻ using acid-washed cadmium powder (24), NO₂⁻ concentrations were measured using a microplate assay method (17) based on the Griess reaction (25). The NO₂⁻/NO₃⁻ value for each plasma sample was obtained from duplicate assays. Data from experiments using at least three mice per data point were expressed as the mean ± SEM. In selected samples, NO₂⁻ concentrations were assayed without prior reduction of NO₃⁻ by cadmium and were found to be below the level of detection.

Assay of Tumor-hemorrhagic Necrosis. Immediately after each blood sample was collected, tumors were removed, sliced in half, fixed in 10% formalin, and embedded in paraffin wax. Sections were stained with hematoxylin and eosin and assessed on a grid marked at 0.4-mm intervals by scoring the percentage of intersections that were necrotic (22).

RESULTS

Plasma NO₂⁻/NO₃⁻ Concentrations following Administration of FAA. Plasma NO₂⁻/NO₃⁻ concentrations increased in mice after administration of FAA at the maximum tolerated dose (1180 μmol/kg). However, the presence of a s.c. Colon 38 tumor dramatically enhanced this increase (Fig. 2). Peak plasma NO₂⁻/NO₃⁻ levels were reached after 12 h, and at 24 h they remained elevated in tumor-bearing animals but had returned to near basal levels in non-tumor-bearing mice (Fig. 2).

Plasma NO₂⁻/NO₃⁻ levels showed a steep response to FAA dose. For tumor-bearing mice, a low but significant increase in plasma NO₂⁻/NO₃⁻ levels was found 12 h after administration of 790 μmol/kg, and no increase was found following doses of <540 μmol/kg (Fig. 3). For non-tumor-bearing mice NO₂⁻/NO₃⁻ levels were not significantly increased above basal levels at doses of <790 μmol/kg (Fig. 3).

Excision of the tumor prior to drug administration did not significantly affect the FAA-induced rise in plasma concentrations of NO₂⁻/NO₃⁻ (Table 1). The slightly lower plasma NO₂⁻/NO₃⁻ concentrations observed at 12 h in the tumor-bearing groups which received operations (excised and sham operated) were not significantly different from the levels found in the tumor-bearing group which received no operation. In non-tumor-bearing mice the sham operation had no significant effect on FAA-induced plasma NO₂⁻/NO₃⁻ concentrations at 12 h (Table 1).

Comparison of Plasma NO₂⁻/NO₃⁻ Concentrations following Administration of FAA and XAA Analogues. Plasma NO₂⁻/NO₃⁻ levels increased substantially following administration of the active FAA analogues, XAA, 5-MeXAA, and 5,6-MeXAA,
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at maximum tolerated doses. Again the increase was enhanced by the presence of a s.c. Colon 38 tumor (Fig. 4). After 12 h, plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels in tumor-bearing mice were significantly higher than those in non-tumor-bearing mice for FAA and 5,6-MeXAA (P < 0.05). 5,6-MeXAA induced a significantly greater response (820 ± 140 μM) than did FAA (540 ± 50 μM, P < 0.05), 5-MeXAA (230 ± 80 μM, P < 0.01), or XAA (120 ± 20 μM, P < 0.001). Only a slight increase above basal levels was found following administration of the inactive analogue, 8-MeXAA (Fig. 4). Endotoxin (100 μg/mouse) and recombinant human TNF-α (4 to 16 μg/mouse) also stimulated an increase in plasma NO\textsuperscript{2-/NO\textsuperscript{-}} in tumor-bearing mice (450 ± 100 μM and 590 to 700 μM, respectively).

Relationship between Plasma NO\textsuperscript{2-/NO\textsuperscript{-}} Levels and Antitumor Effects. A striking relationship was found between plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels and growth delays of s.c. Colon 38 tumors in mice following administration of FAA or the XAA analogues (Fig. 5). 5,6-MeXAA (100 μmol/kg), the analogue which induced the greatest increase in plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels, also induced the greatest tumor growth delay (20 days). The tumor growth delays of 17, 13, and 11 days for FAA (1180 μmol/kg), 5-MeXAA (160 μmol/kg), and XAA (1200 μmol/kg), respectively, reflected the ranking of plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels observed following administration of these compounds. At a lower dose of FAA (790 μmol/kg), which produced a much lower increase in plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels, an insignificant growth delay of 1.2 days was observed. No tumor growth delay was observed following administration of an inactive dose of FAA (540 μmol/kg) or the inactive analogue 8-MeXAA (640 μmol/kg) (Fig. 5).

A relationship was also found between the degree of tumor necrosis and the corresponding plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels when the two were compared in individual mice following administration of FAA or XAA analogues to mice bearing s.c. Colon 38 tumors (Fig. 6). FAA at the maximum tolerated dose (1180 μmol/kg) substantially elevated plasma levels of NO\textsuperscript{2-/NO\textsuperscript{-}} and induced extensive hemorrhagic necrosis in all tumors at 12 h (100 ± 0%). For a lower dose of FAA (790 μmol/kg) which induced a smaller increase in plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels, tumor hemorrhagic necrosis was incomplete (87 ± 9%). Tumor-hemorrhagic necrosis was only slightly above the control range after administration of FAA at doses (≤540 μmol/kg) which failed to increase plasma NO\textsuperscript{2-/NO\textsuperscript{-}} concentrations.

5,6-MeXAA, the analogue which produced the greatest increase in plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels, also induced complete tumor-hemorrhagic necrosis (100 ± 0%) when administered at the maximum tolerated dose (100 μmol/kg). 5-MeXAA (160 μmol/kg) and XAA (800 μmol/kg), which induced intermediate plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels, induced incomplete tumor-hemorrhagic necrosis (69 ± 11% and 10 ± 6%, respectively). The inactive analogue 8-MeXAA failed to induce tumor-hemorrhagic necrosis or to increase plasma NO\textsuperscript{2-/NO\textsuperscript{-}} levels substantially above control levels.

DISCUSSION

The results show that the antitumor agents FAA, XAA, 5-MeXAA, and 5,6-MeXAA stimulate in vivo production of...
NO$_2^-$/NO$_3^-$ in mice. Plasma NO$_2^-$/NO$_3^-$ levels are higher in mice bearing a s.c. Colon 38 tumor and are positively correlated with tumor growth delay (Fig. 5). The highest plasma concentration of NO$_2^-$/NO$_3^-$ was found following administration of the most active compound (5,6-MeXAA), while no substantial increase was found following administration of the inactive compound (8-MeXAA).

Recent evidence indicates that nitrite and nitrate are derived from the oxidation of L-arginine and that nitric oxide is an intermediate molecule in this biochemical pathway (19). Many cell types are known to generate nitric oxide including macrophages, Kupffer cells, neutrophils, endothelial cells, platelets, and cells in the nervous system (26–28). However, in vitro studies show that the capacity of the activated macrophage to generate nitric oxide is relatively much larger than that of other cell types (27). Nitric oxide production by activated macrophages is stimulated in vitro by endotoxin (20), TNF-α (29), and FAA and XAA analogues (17). The evidence presented here, as well as that for endotoxin from other laboratories (20, 21), suggests that these agents also stimulate nitric oxide production in vivo. Since it has previously been shown that FAA induces the synthesis of TNF-α (10), nitric oxide production induced by FAA and analogues may occur either by direct stimulation of L-arginine oxidation or via the induction of TNF-α.

A feature of our results is that NO$_2^-$/NO$_3^-$ production is stimulated by FAA to a greater extent in tumor-bearing mice than in non-tumor bearing animals (Figs. 2 to 4). The development of a population of activated macrophages in tumor-bearing animals could explain this finding. In vitro evidence that only activated macrophages produce nitrite and nitrate in response to FAA and active XAA analogues (17) is consistent with previous studies in which macrophage activation procedures, such as exposure to strong immunostimulants (e.g., Bacillus Calmette-Guérin) in vivo or γ-interferon treatment in vitro, are prerequisites for NO$_2^-$/NO$_3^-$ production from macrophages treated with endotoxin (30). It is thus possible that the immunological stimulus induced by s.c. implantation of tumor cells into the animals results in macrophage activation and, hence, enhanced NO$_2^-$/NO$_3^-$ production. The failure of tumor excision to significantly reduce NO$_2^-$/NO$_3^-$ production suggests that, if activated macrophages are indeed present 10 to 11 days after tumor implantation, they are not located exclusively within tumor tissue. In preliminary experiments we have not yet been able to stimulate nitrite formation in vitro by adherent cells extracted from Colon 38 or from peritoneal macrophages prepared from tumor-bearing animals (results not shown). It is possible that the in vivo production of nitric oxide involves the cooperation of macrophages with other host components which are not present in our in vitro system.

The correlation between plasma NO$_2^-$/NO$_3^-$ levels and growth delays of s.c. Colon 38 tumors in mice (Fig. 5) suggests that the L-arginine cytotoxicity pathway (18) may be involved in the antitumor effects of these compounds. This finding is supported by in vitro evidence that L-arginine-dependent reactive nitrogen intermediates play a major role in effector mechanisms of tumor cell killing by activated macrophages (31). Inhibition of essential iron-containing enzymes, including respiratory enzymes, by nitric oxide (32) may be one cytotoxic mechanism. Others have suggested that nitric oxide may react with peroxide (33), another product of activated macrophages (34), to form the highly toxic peroxynitrite species (35). Synergism between nitric oxide and TNF-α (35), which is also produced in response to FAA (10), constitutes a third possible cytotoxic mechanism.

Analysis of hemorrhagic necrosis within tumors taken from mice at the time when plasma samples were obtained has enabled the assessment of the relationship between plasma NO$_2^-$/NO$_3^-$ levels and antitumor effects for individual mice. It is clear from Fig. 6 that considerable hemorrhagic necrosis can develop in the absence of a large increase in plasma NO$_2^-$/NO$_3^-$ concentrations. Since NO$_2^-$/NO$_3^-$ concentrations are directly correlated with tumor growth delay (Fig. 5), it may be concluded that hemorrhagic necrosis is necessary but not sufficient for a pronounced tumor growth delay. The results may be explained if it is postulated that at least two effects are induced by FAA and XAA analogues: (a) the development of hemorrhagic tumor necrosis resulting from vascular changes and (b) direct tumor cell killing requiring the production of a cytotoxic species. This supports the findings of Zwi et al. (9) that tumor ischemia resulting from reduction of blood flow may not alone account for all FAA-induced cytotoxicity.

Vascular changes in tumors are induced by tumor necrosis factor α, and at least some of the effects observed may be attributable to its induction by FAA (10). It is possible that nitric oxide is also involved, since nitric oxide mediates vasorelaxation and plays an important role in controlling blood pressure (27). Hypotension, observed following clinical administration of FAA (6), may be an important factor influencing tumor blood flow. Moreover, the inhibitory effects of nitric oxide on platelet aggregation and adhesion (26, 28) may result in increased leakiness and extravasation of red blood cells from tumor blood vessels and contribute to the hemorrhagic appearance of FAA-treated tumors (3). Only low concentrations of nitric oxide are required for vascular effects (27), while direct tumor cell killing requires larger amounts of nitric oxide (19). Substantial growth delays or cures may occur only when both vascular and cytotoxic events act in concert.

The results of this study, which show increased plasma NO$_2^-$/NO$_3^-$ levels in mice in response to the antitumor agents FAA, XAA, 5-MeXAA, and 5,6-MeXAA, provide important indirect evidence for the in vivo nitric oxide in the action of this class of compounds. The increased response in mice bearing tumors suggests that tumor cell-host cell relationships are important for their action. In view of our previous results (17) the responsiveness of macrophages may be an important factor to investigate in the future application of these drugs as clinical agents.

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Tumor-dependent Increased Plasma Nitrate Concentrations as an Indication of the Antitumor Effect of Flavone-8-acetic Acid and Analogues in Mice

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