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Flavone Acetic Acid and 5,6-Dimethylxanthenone-4-acetic Acid: Relationship between Plasma Nitrate Elevation and the Induction of Tumour Necrosis

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Antitumour agents such as flavone acetic acid, xanthenone acetic acid (XAA), 5,6-dimethylxanthenone-4-acetic acid and tumour necrosis factor-α, following single dose administration to mice with colon 38 adenocarcinomas, induce tumour haemorrhagic necrosis and an elevation in plasma nitrate. The relationship between these two effects has been studied using firstly a series of methyl-substituted XAA derivatives with varying antitumour activity, and secondly the inhibitors N^G-monomethyl-L-arginine (NMMA), N-nitro-L-arginine (NNA) and canavanine, which affect nitric oxide synthesis. Elevation of plasma nitrate resulting from the oxidation of L-arginine by nitric oxide synthase is inhibited by NNA rather than by NMMA or canavanine. The results demonstrate that tumour necrosis can be induced in the absence of a significant elevation of plasma nitrate.
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INTRODUCTION

FLAVONE ACETIC ACID (FAA; NSC 347512), a flavonoid with excellent activity in murine tumours but lacking activity in clinical trials [1], induces haemorrhagic necrosis of subcutaneous murine tumours which is histologically indistinguishable from that induced by tumour necrosis factor-α (TNF-α) [2]. It also induces a rapid reduction in tumour blood flow [3, 4] which is reversed by antibody to TNF-α [5], implying a vascular effect. Since FAA induces the production of TNF-α in mice [6], which itself induces tumour necrosis [7], it is likely that the vascular action of FAA is mediated, at least in part, by TNF-α.

Derivatives of xanthenone-4-acetic acid (XAA; Fig. 1) [8], including the dose-potent clinical candidate 5,6-dimethylxanthenone-4-acetic acid (5,6-dimethyl XAA) [9], have similar antitumour properties to those of FAA. Studies on a series of XAA derivatives, which resemble FAA structurally and vary in antitumour activity, have demonstrated that the induction of tumour necrosis correlates well with reduction of blood flow (Zwi *et al.*, manuscript in preparation) and with the induction of TNF-α [10].

FAA and XAA analogues have been found to induce the production of nitric oxide, both *in vitro* in activated murine macrophages [11] and in mice as indicated by elevation of plasma nitrate concentrations [12]. Tumour excision experiments indicate that the elevation of plasma nitrate is a result of systemic production of nitric oxide rather than local production in the tumour [12]. A comparison of FAA, XAA and three XAA derivatives has demonstrated that antitumour activity, as measured by growth delays of the colon 38 murine adenocarcinoma, is highly correlated with elevation of plasma nitrate, suggesting a relationship between nitric oxide production and antitumour effects. TNF-α also induces an increase in plasma nitrate, raising the question of whether nitric oxide production is required for the vascular effects and consequent antitumour effects of these agents.

In this study, we have tried to answer this question by determining whether tumour necrosis induction can be separated from plasma nitrate elevation, using colon 38 tumour-bearing mice. Firstly, we have examined for correlation between the two variables using a series of antitumour agents of varying activity and chemical structure. Secondly, we have determined whether inhibitors of nitric oxide synthesis can selectively modulate plasma nitrate production by these antitumour agents.

MATERIALS AND METHODS

Materials

FAA was supplied by the National Cancer Institute, USA and dissolved in 5% bicarbonate for injection. XAA and its derivatives (Fig. 1) were synthesised as the sodium salts in this laboratory, were pure as judged by thin layer chromatography, and were dissolved in milli-Q water. N^G-Monomethyl-L-arginine (NMMA), N-nitro-L-arginine (NNA) and canavanine (Sigma, USA) were dissolved, with the appropriate quantity of HCl, in water. Recombinant human TNF-α was kindly provided by Professor J. D. Watson, Department of Molecular Medicine, Auckland Medical School.

Mouse procedures

C_{57}BL/6 × DBA/2 F_1 hybrid mice (Jackson Laboratory, Bar Harbor, Maine, USA) were bred in the laboratory and housed

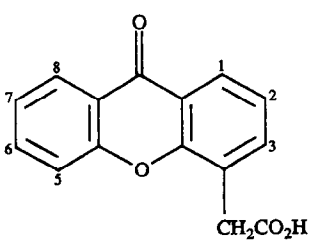

Fig. 1. Structure of XAA, showing the numbering system.

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using sterile cages, bedding, food and water, under conditions of constant temperature and humidity and regular 12-h cycles of light and darkness. All experiments were carried out under institutional ethical guidelines. Colon 38 tumours were implanted subcutaneously and grown to a diameter of 5–10 mm before use. Mice were injected with a single intraperitoneal dose of drug. After 12 h, mice were anaesthetised with ether and blood was obtained by cardiac puncture with heparinised syringes, immediately centrifuged (8000 *g*, 5 min), and the plasma collected and stored at −20°C until analysed. Tumours (if present) were removed and fixed in 10% formalin, and the animals were then killed.

Plasma nitrite/nitrate analysis

Plasma nitrate concentrations were determined after precipitation of plasma proteins with 30% $ZnSO_4$, and reduction of nitrate in the supernatant to nitrite using acid-washed cadmium powder [13]. Nitrite concentrations in the supernatant were analysed using a microplate assay method [11] based on the Griess reaction [14].

Histological assessment of tumour necrosis

Fixed tumours were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. A grid marked at 0.4 mm intervals was placed over the histological section and each intersection was scored as either undamaged or necrotic tumour. The percentage necrosis was determined as the number of grid intersections showing necrotic tumour divided by the total number of intersections counted [2].

Statistics

Results were plotted using Sigmaplot (Jandel Scientific, USA) and variations between groups determined using Student's *t*-test. Significance was defined as $P < 0.05$.

RESULTS

Relationship between increased plasma nitrate and the induction of tumour necrosis

A series of methyl-substituted XAA derivatives was chosen to provide a range of antitumour activity. Plasma nitrate was measured 12 h following administration of drug since this was the maximum found for both FAA and XAA. The results of testing eight compounds over a range of doses up to the maximum tolerated dose in both normal and tumour-bearing mice is shown in Fig. 2. The two drugs which were inactive in promoting tumour necrosis (7-methyl and 8-methyl XAA) were also unable to stimulate increases in plasma nitrate.

In order to determine whether the increased plasma nitrate concentrations and tumour necrosis were correlated, nitrate data from Fig. 2 and from previous studies with FAA and XAA derivatives [12] were plotted against the percentage tumour necrosis (Fig. 3). Increases in plasma nitrate above 200 μmol/l were always associated with induction of tumour necrosis significantly above that of untreated animals. However, necrosis could be induced in some cases with little increase in plasma nitrate concentration. Furthermore, although not consistently associated with any one drug, significantly elevated plasma nitrate concentrations were observed in individual mice in the absence of significantly elevated tumour necrosis (Fig. 3).

Effects of inhibitors of nitric oxide synthesis

The effects of NMMA, NNA and canavanine on the induction of tumour necrosis and on the elevation of plasma nitrate were measured using the optimal dose of the inducing drug and a range of doses of inhibitor. The effects of the inhibitors at their highest tolerated doses are shown in Table 1. NMMA and canavanine caused 40 and 63% reduction, respectively, of the plasma nitrate elevation induced by FAA, but these values were not significant. The effect of NNA was difficult to measure because although NNA was non-toxic when administered alone up to 500 mg/kg, it was lethal when administered at 200 mg/kg in combination with FAA, and induced signs of toxicity (ruffled fur, hunched posture) in combination with TNA-α, 6-methyl XAA and 5,6-dimethyl XAA. Nevertheless, NNA demonstrated significant suppression of plasma nitrate elevation of the latter three compounds (Table 1), and in each case, did not suppress tumour necrosis.

DISCUSSION

Two complementary approaches have been used to demonstrate that the elevation of plasma nitrate can be separated from the induction of tumour haemorrhagic necrosis. The results with the series of XAA analogues, administered at their maximum tolerated doses, indicate that plasma nitrate elevation and tumour necrosis are stimulated in parallel, confirming and extending previous data [12]. Nevertheless, at suboptimal doses these compounds are able to induce necrosis with little or no elevation of plasma nitrate.

Results with nitric oxide synthase inhibitors show that plasma nitrate elevation by FAA, TNF-α, 6-methyl XAA and 5,6-dimethyl XAA can be inhibited without affecting the induction of tumour necrosis. These experiments extend previously reported results on the inhibition by NNA of plasma nitrate elevation in non-tumour-bearing animals in response to TNF-α and 5,6-MeXAA [15]. NNA at a dose of 200 mg/kg is lethal in combination with FAA, as reported for non-tumour-bearing mice [15], and produces signs of toxicity in mice when used in combination with the other agents. Toxicity is also present with the highest doses of NMMA and canavanine. All of these drugs are non-toxic when tested alone at corresponding doses. Although the cause of the toxicity is unknown, darkening of the liver and release of liver enzymes into the blood of animals treated with FAA and NNA suggests that liver toxicity may be involved (unpublished data). The results are compatible with the hypothesis that nitric oxide has a protective role under some circumstances, as has been demonstrated in endotoxic shock [16, 17].

The tissue origin of the nitric oxide synthase responsible for systemic elevation of nitrate in mice has not yet been established. Tumour excision experiments indicate that elevation of plasma nitrate is a result of systemic production of nitric oxide rather than local production in the tumour [12]. Nitric oxide synthases can be divided into two broad groups according to their response to NMMA and NNA, and it has been reported that the constitutive enzyme, which is stimulated by calmodulin, is differentially sensitive to NNA whereas the inducible form is sensitive to NMMA [18–20]. The nitric oxide synthase responsible for increased nitrate levels in mice is differentially sensitive to NNA but is clearly inducible. Maximal activity, as judged by rate of nitrate increase following FAA treatment, occurs between 4 and 12 h after drug treatment [12], similar to the time (6 h) for maximal induction of nitric oxide synthase in rat liver [21]. Rat liver nitric oxide synthase, unexpectedly, is both inducible (by endotoxin) and stimulatable by calmodulin [22]. Taken together, these results suggest that the liver is likely to be the major source of nitric oxide in response to these agents. Evidence for the

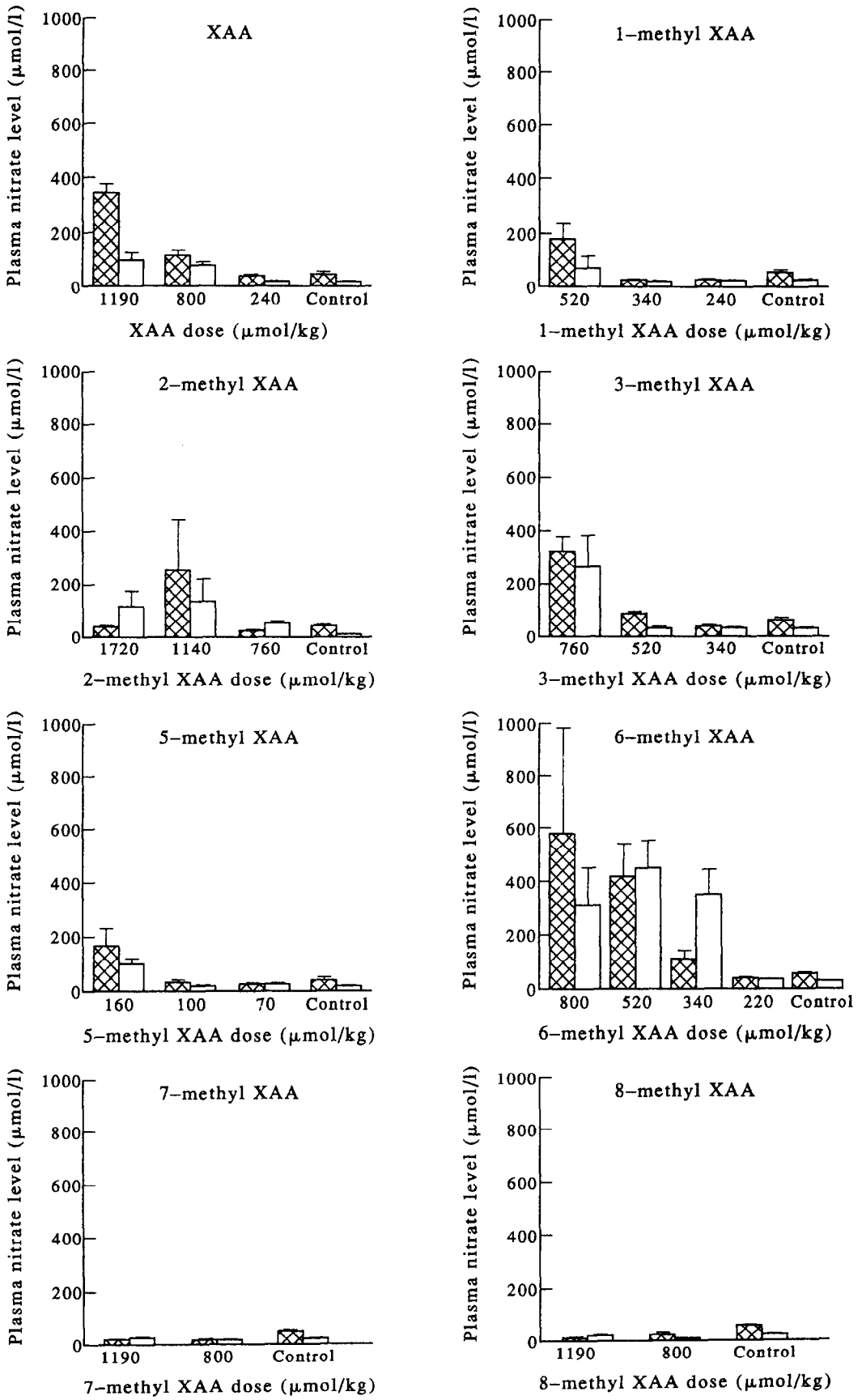

Fig. 2. Plasma nitrate concentrations 12 h after intraperitoneal injection of XAA and methyl XAA derivatives. Hatched bars, tumour-bearing mice; open bars, non-tumour-bearing mice. Error bars indicate standard errors of the mean of at least three animals per experiment.

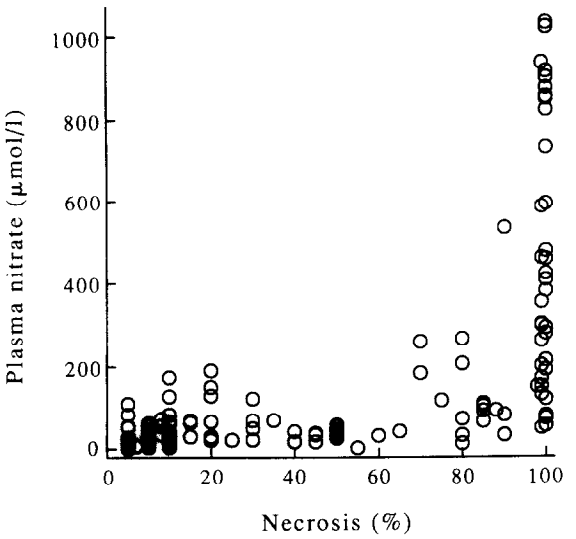

Fig. 3. Relationship between plasma nitrate concentrations and induction of tumour necrosis. Each point represents one mouse.

Table 1. *Effect of inhibitors, at their highest tolerated doses, on plasma nitrate elevation and induction of haemorrhagic necrosis in colon* 38 *tumours*

Treatment	Dose (mg/kg)	Plasma nitrate* (μmol/l)	Tumour necrosis (%)
FAA	330	650 ± 130	100 ± 0
+ NMMA	200	360 ± 160	100 ± 0
+ NNA	200	Toxic	
+ canavanine	200	240 ± 8	100 ± 0
TNF-α	0.8	710†	90
+ NNA	200	57†	99
6-Methyl XAA	150	410 ± 62	99 ± 0
+ NNA	300	1 ± 1	95 ± 5
5,6-Dimethyl XAA	30	820 ± 140	95 ± 5
+ NNA	200	0 ± 0	99 ± 1

*The results, except where indicated are averaged from at least three mice, with tumour tissue and plasma removed 12 h after treatment. Plasma nitrate of untreated (control) animals has been subtracted.
†One mouse per experiment. A similar reduction in plasma nitrate was observed for non-tumour-bearing animals [15].

elevation of plasma nitrate has also been obtained following clinical administration of FAA [23], suggesting that this pathway also exists in humans.

The dissociation of nitrate production and tumour necrosis, as demonstrated by these studies, appears to argue against the involvement of nitric oxide in the antitumour action of these compounds. However, the inhibition of production of nitric oxide in the liver is not necessarily related to local production in the tumour, which if controlled by tumour-associated macrophages would be inhibited more by NMMA than by NNA [19]. Evidence for a role of nitric oxide in the *in vitro* killing of tumour cells has been obtained in spheroids formed from EMT-6 cells and infiltrated with host macrophages. Treatment of these spheroids with FAA or 5,6-MeXAA results in the production of nitric oxide and in killing of tumour cells. Both of these processes are inhibited by NMMA [24]. Further work is required to determine the contribution of nitric oxide to the cytotoxic effects of FAA and 5,6-MeXAA in tumours.

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Chemosensitisation and Drug Accumulation Effects of Cyclosporin A, PSC-833 and Verapamil in Human MDR Large Cell Lung Cancer Cells Expressing a 190k Membrane Protein Distinct from P-glycoprotein

M.A. Barrand, T. Rhodes, M.S. Center and P.R. Twentyman

The doxorubicin-selected multidrug resistant (MDR) human large cell lung cancer line COR-L23/R, lacks P-glycoprotein but shows a drug accumulation deficit. It does however overexpress a 190k membrane protein which shares an epitope with, but is otherwise distinct from, P-glycoprotein. The resistant cells show only a small sensitisation to vincristine and daunorubicin on treatment with cyclosporin A and its more potent analogue, PSC-833 despite an increase in drug accumulation. Verapamil, another effective resistance modifier in P-glycoprotein MDR cells, is slightly more effective. Fluorescent daunorubicin distributes in the cytoplasm and nucleus of sensitive parent COR-L23 cells but is confined to cytoplasmic perinuclear vesicles in resistant cells. Addition of cyclosporin A or PSC-833 slightly increases cytoplasmic fluorescence whereas verapamil also increases nuclear fluorescence. Resistance in this non-P-glycoprotein MDR line, COR-L23/R where these resistance modifiers have little effect may be associated with expression of the 190k protein.
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INTRODUCTION

DECREASED DRUG accumulation is one of a variety of mechanisms which have been shown to account for the multidrug resistance observed in tumour cells in culture. Hyperexpression of the drug efflux pump, P-glycoprotein, has been associated in many cases with this decrease in drug accumulation [1]. However there are now an increasing number of reports of multidrug resistant (MDR) cells that show decreased accumulation of drugs of the type normally expelled by the efflux pump but that do not express *mdr* mRNA or contain P-glycoprotein [2–5]. One such resistant cell line has been developed from a human large cell lung cancer cell line, COR-L23. The MDR variant, COR-L23/R was derived by continuous *in vitro* exposure to doxorubicin [6] and shows cross resistance to vincristine and to colchicine [7]. It does not hyperexpress the *mdr* 1 gene as determined by northern blot analysis [8] and shows no evidence of the *mdr* gene product, P-glycoprotein, either from western blot analysis using a single anti-P-glycoprotein monoclonal antibody, C219 [8] or from immunocytochemical staining using a panel of anti-P-glycoprotein antibodies, JSB-1, C219 and MRK16 [9]. The mechanisms underlying the chemoresistance and decreased drug accumulation observed in these non-P-glycoprotein MDR cells are unknown, but we provide evidence here that there is overexpression in our resistant cells of a membrane protein slightly larger in size than P-glycoprotein (approximately 190k), but containing a short amino acid sequence antigenically similar to a region of P-glycoprotein close to one of its nucleotide binding sites.

It has been shown that at least partial reversal of resistance in MDR cells containing P-glycoprotein may be brought about by verapamil and by cyclosporins [10], this reversal being associated usually but not invariably with reduction in the drug accumulation deficit. There is evidence to suggest that these resistance modifiers may exert their actions at least in part by binding directly to P-glycoprotein so reducing its drug efflux capability [10]. The actual sites for binding of each modifier to the protein

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