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Modulation of superoxide production from murine macrophages by the antitumour agent flavone acetic acid and xanthenone acetic acid analogues

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Flavone-8-acetic acid (FAA*) is a synthetic flavonoid compound whose antitumour activity against a broad range of experimental solid tumours [1] contrasts sharply with a lack of activity in clinical trials [2]. The mechanism of action of FAA *in vivo* is not fully understood but involves the production of cytokines [3] and nitric oxide [4]. FAA also increases the direct *in vitro* cytotoxicity of murine macrophages against tumour targets [5] and stimulates the formation of active nitrogen intermediates in activated macrophages [6]. Cultures of elicited macrophages release substantial amounts of superoxide when stimulated with PMA [7]. Antiinflammatory agents can inhibit the production of superoxide from appropriately stimulated macrophages or polymorphonuclear leukocytes [8–10] and there is evidence that some flavonoids are antiinflammatory, and modulate the release of reactive oxygen intermediates [11, 12]. XAA, which has been used as the basis for the synthesis in this laboratory of novel compounds with antitumour properties similar to those of FAA [13, 14], is also reported to have antiinflammatory properties [15]. These observations raise the question of whether the antitumour action of FAA and XAA analogues involves modulation of the production of active oxygen intermediates.

In this study, the effect of FAA on superoxide production from murine macrophages in the presence or absence of PMA is described and the effects of FAA with those of XAA derivatives compared. These derivatives include 5,6-MeXAA, the most dose potent of antitumour agents [14], and 8-MeXAA, an inactive analogue [13]. The use of this series with diverse dose potency and activity provides an excellent basis for determining whether superoxide production correlates with antitumour effects. Thus, comparisons are also made here between the effects of these agents on superoxide production and their antitumour effects against the experimental s.c. Colon 38 murine tumour.

* Abbreviations: FAA, flavone-8-acetic acid; PBS, phosphate buffered saline; SPBS, supplemented phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; XAA, xanthenone-4-acetic acid; 5-MeXAA, 5-methyl XAA; 5,6-MeXAA, 5,6-dimethyl XAA; 8-MeXAA, 8-methyl XAA; DMSO, dimethyl sulphoxide.

Materials and Methods

Materials. α-Minimal essential culture medium (Gibco, Grand Island, NY, U.S.A.) was supplemented with foetal calf serum, 2-mercaptoethanol (50 μM), penicillin (100 units/mL) and streptomycin sulphate (100 μg/mL). SPBS consisted of PBS supplemented with calcium chloride (2.7 mM), magnesium chloride (3.4 mM) and glucose (5.6 mM). FAA (National Cancer Institute, U.S.A.) and XAA derivatives (synthesized as described [13, 14] by Drs W. A. Denny, G. J. Atwell and G. W. Rewcastle) were dissolved immediately prior to use in a minimal amount of 5% (w/v) sodium bicarbonate and diluted in SPBS. Indomethacin (Merck Sharpe and Dohme (NZ) Ltd) was dissolved in a minimal amount of DMSO and diluted in SPBS. Sodium bicarbonate and DMSO at the highest concentrations used (0.001% and 0.2%, respectively) were shown not to alter cell viability or superoxide production in the *in vitro* macrophage cultures. PMA (Sigma Chemical Co., St. Louis, MO, U.S.A.) was prepared as a 1.62 mM stock solution in DMSO and stored as 2.5 μL aliquots in glass vials at −70°. Ferricytochrome *c* (Sigma) was prepared as a 1.5 mM stock solution in SPBS, stored in 1 mL aliquots at −20° and diluted in SPBS immediately prior to use. Superoxide dismutase (Sigma) was prepared in SPBS (7.5 units/μL) and stored at −20°. Thioglycollate (Beckton Dickinson and Co., Cockeysville, U.S.A.) (10% w/v) broth was in sterile water.

Macrophage preparation and culture. C_3H/HeN mice were bred in the laboratory animal facility under constant temperature and humidity with sterile bedding, water and food according to institutional ethical guidelines. Mice between 6 and 12 weeks of age were injected i.p. with thioglycollate broth (0.2 mL/mouse). Mice were killed by cervical dislocation 4–6 days later and peritoneal exudate cells were collected in PBS. Adherent macrophages were collected by plating 1.25×10^5 cells in 0.5 mL culture medium in 24-well plates (or alternatively, 3×10^5 cells in 100 μL culture medium in 96-well flat-bottom microwell trays) and incubating for 2 hr at 37° in 95% air/5% CO_2.

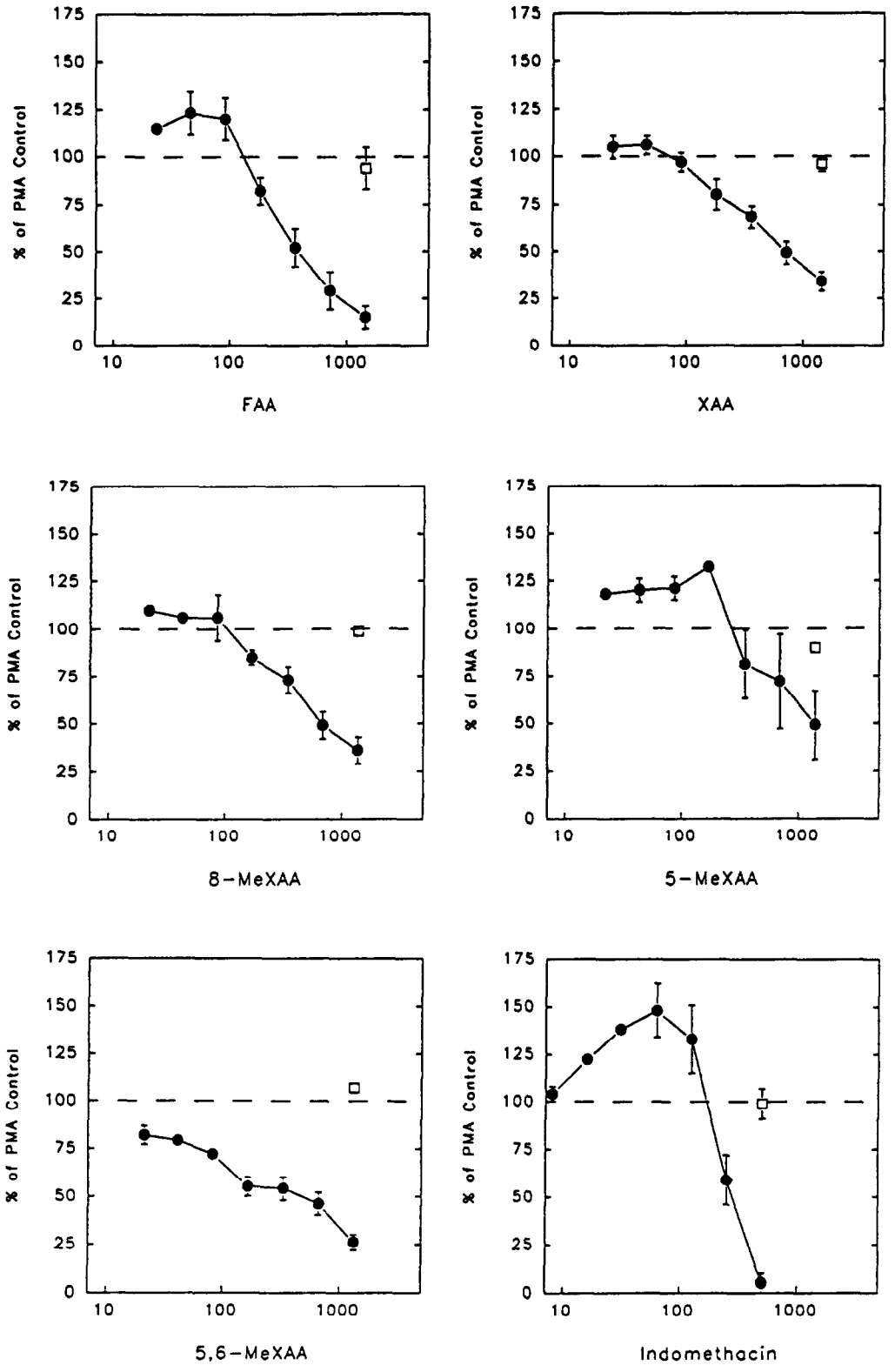

Fig. 1. The effect of FAA, XAA, 8-MeXAA, 5-MeXAA, 5,6-MeXAA, and indomethacin (μM) on PMA-stimulated superoxide release from elicited murine peritoneal macrophages. Superoxide production with PMA alone (PMA control) ranged from 21 to 49 nmol/10^6 cells/90 min for all experiments. (□) Indicates cell viability after 90 min culture in the presence of PMA and the highest concentration of drug relative to PMA control. Points (means ± SEM) represent the results of 2–5 experiments. Error bars are not shown when bars fall within the area of a point.

Non-adherent cells were removed with the supernatant followed by three further vigorous washings with PBS. The adherent population was judged to be >82% macrophages by differential counts of Leishman stained cells and <2% T-lymphocytes by fluorescence microscopy after fluorescent antibody labelling with anti-Thy 1 antibody. Macrophage monolayers were tested for viability in 24-well plates containing one glass cover slip per well. Duplicate cultures exposed to PMA (200 nM) alone (PMA control) or PMA and the highest concentrations of drugs tested in the superoxide assay, were assessed for their ability to exclude eosin as described previously [6] after culture for 90 min.

Superoxide assay. Superoxide was measured as the reduction of ferricytochrome *c* in 90-min culture by methods described previously for 24-well or 96-well assays [7, 16]. Superoxide production was investigated in the presence of FAA, XAA, 8-MeXAA, 5-MeXAA or 5,6-MeXAA (concentration range 21–1450 μM), or indomethacin (concentration range 8–500 μM), with or without PMA (200 nM). Each drug was tested in duplicate (24-well assay) or quadruplicate (96-well assay). Superoxide released was calculated from the reduced ferricytochrome *c* concentration using the extinction coefficient $\varepsilon_{550} = 2.1 \times 10^{-3}\,M^{-1}\,cm^{-1}$. To assess the specificity of ferricytochrome *c* reduction, superoxide dismutase (75 or 150 units/well) was added to selected ferricytochrome *c*-containing wells before drugs and PMA were added. Drug effects on PMA-stimulated superoxide release were expressed as mean percentages ± SEM of the absorbance values obtained for the cultures stimulated with drug and PMA divided by absorbance values for cultures stimulated with PMA control.

Assay of tumour necrosis and tumour growth delay. Groups of 3–5 B6D2F$_1$ mice with s.c. Colon 38 tumours 5–12 mm in diameter (randomized with respect to tumour size) were given i.p. injections of drug 10–11 days after implantation and tumours were assayed for haemorrhagic necrosis, and growth delays as described previously [17].

Results and Discussion

Superoxide production, measured as superoxide dismutase-inhibitable reduction of ferricytochrome *c*, was not observed when elicited murine macrophages were incubated with FAA, XAA, 8-MeXAA, 5-MeXAA or 5,6-MeXAA alone. However, PMA-stimulated superoxide production was significantly enhanced (Student's *t* test; $P < 0.05$) in the presence of low concentrations of FAA, 8-MeXAA and 5-MeXAA (Fig. 1) with maximal enhancement at 170 μM 5-MeXAA (32 ± 1%), 90 μM FAA (20 ± 11%) and 22 μM 8-MeXAA (10 ± 1%). On the other hand, PMA-stimulated superoxide production was progressively inhibited in the presence of increasing concentrations of these agents, with maximal inhibition ranging from 51 ± 18% for 5-MeXAA (1380 μM) to 85 ± 6% for FAA (1440 μM). 5,6-MeXAA progressively inhibited superoxide production at all concentrations tested, ranging from 18 ± 5% at 21 μM to 74 ± 4% at 1320 μM (Fig. 1). Thus, these agents alone do not act as "triggering agents", stimulating the production of superoxide from elicited murine macrophages, but modulate the release of superoxide from elicited murine macrophages cultured in the presence of PMA. Similar results were found for the antiinflammatory agent indomethacin which showed maximal enhancement of PMA-stimulated superoxide production (48 ± 14%) at low concentrations (63 μM) and inhibition (95 ± 5%) at higher concentrations (500 μM) (Fig. 1). Since no significant difference in cell viability was observed between cultures exposed to PMA alone (82 ± 7% viable) and to PMA and the highest concentrations tested for each of these agents (Fig. 1), inhibition of superoxide production is unlikely to be due to drug toxicity. Modulation of superoxide production is believed to be an important property of antiinflammatory agents [9, 10, 16]. The resemblance between the effects of FAA, XAA analogues and indomethacin, as well as a number of other non-steroidal antiinflammatory agents [9, 10], on superoxide production suggests that FAA and XAA analogues may possess *in vivo* antiinflammatory properties.

Comparison of *in vitro* and *in vivo* effects (Table 1) shows that there is no significant correlation between the effects of FAA, XAA analogues or indomethacin on superoxide production, and their induction of haemorrhagic necrosis or growth delays in s.c. Colon 38 murine tumours. Thus the antiinflammatory activity of these compounds is unrelated to their action against experimental solid tumour models.

Table 1. Comparison between the *in vitro* and *in vivo* effects of FAA, XAA derivatives and indomethacin

	In vitro			*In vivo*			
	Superoxide stimulation		Superoxide inhibition	Tumour necrosis		Tumour growth	
Treatment	Concn* (μM)	% of PMA control†	IC_{50}‡ (μM)	Dose (μmol/kg)	Necrosis§ (%)	Dose (μmol/kg)	Delay‖ (days)
FAA	45	+23 ± 11	380	1200	94 ± 4	1200	17
XAA	45	+6 ± 5	700	800	77 ± 19	800	11
8-MeXAA	22	+10 ± 1	650	1450	27 ± 7	640	0
5-MeXAA	170	+32 ± 1	1350	160	74 ± 11	160	13
5,6-MeXAA	NS	—	380	100	85 ± 14	100	20
Indomethacin	63	+48 ± 14	280	870	13 ± 8	ND	—

* Concentration producing the greatest enhancement of PMA-stimulated superoxide production from thioglycollate-elicited murine macrophage cultures.

† PMA-stimulated superoxide production in the presence of drug, expressed as a percentage of superoxide production with PMA control.

‡ Concentration required to inhibit PMA-stimulated superoxide production by 50%.

§ Haemorrhagic necrosis (24 hr) at the maximal tolerated dose in s.c. Colon 38 tumours. Values of <50% are considered not to represent significant activity [17].

‖ Growth delay of s.c. Colon 38 tumours treated with the maximal tolerated dose. Some of these data have been published previously [13, 14].

NS, no stimulation of PMA-stimulated superoxide production at all concentrations tested; ND, not done.

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