15. Barnes D, Sato G. Methods for growth of cultured cells in serum-free medium. *Anal Biochem* 1980, **102**, 225–270.
16. Murakami H, Masui B. Hormone control of human colon carcinoma cell growth in serum-free medium. *Proc Natl Acad Sci USA* 1980, **77**, 3464–3468.
17. Huschtscha LI, Lucibello FM, Bodmer WF. A rapid micro method for counting cells *in situ* using a fluorogenic alkaline phosphatase enzyme assay. *In Vitro* 1989, **25**, 105–108.
18. Pignatelli M, Bodmer WF. Genetics and biochemistry of collagen binding triggered glandular differentiation in a human colon carcinoma cell line. *Proc Natl Acad Sci USA* 1988, **85**, 5561–5565.
19. Pinto M, Appay MD, Simon-Assman P *et al.* Enterocytic differentiation of cultured human colon cancer cells by replacement of glucose by galactose in the medium. *Biol Cell* 1982, **44**, 193–196.
20. Bellot F, Luis H, El Battari A, *et al.* Extracellular material secreted by human colonic adenocarcinoma cell lines promotes spreading in serum-free medium and induces neurite outgrowth of PC-12 cells. *Int J Cancer* 1985, **36**, 609–615.
21. Van der Bosch J, Masui H, Sato G. Growth characteristics of primary tissue cultures from heterotransplanted human colorectal carcinomas in serum-free medium. *Cancer Res* 1981, **41**, 611–618.
22. Zirvi KA, Chee DO, Hill GJ. Continuous growth of human tumour cell lines in serum-free media. *In Vitro* 1986, **22**, 369–374.
23. Park J-G, Oie HK, Sugarbaker PH, *et al.* Characteristics of cell lines established from human colorectal carcinoma. *Cancer Res* 1987, **47**, 6710–6718.
24. Boyd DD, Levine AE, Brattain DE, McKnight MK, Brattain MG. A comparison of growth requirements of two human intratumoral colon carcinoma cell lines in monolayer and soft agarose. *Cancer* 1988, **48**, 2469–2474.
25. Sager, R. Tumor suppressor genes: The puzzle and the promise. *Science* 1989, **246**, 406–412.
26. Culouscou J-M, Remaclc-Bonnet M, Garrouste F, Marvaldi J, Pommier G. Simultaneous production of IGF-I and EGF competing growth factors by HT29 human colon cancer line. *Int J Cancer* 1987, **40**, 646–652.
27. Culouscou J-M, Garrouste F, Remacle-Bonnet M, Bettetini D, Marvaldi J, Pommier G. Autocrine secretion of a colorectum-derived growth factor by HT29 colon carcinoma cell line. *Int J Cancer* 1988, **42**, 895–901.
28. Coffey RJ, Goustin AS, Soderquist AM, *et al.* Transforming growth factor α and β expression in human colon cancer lines: implications for an autocrine model. *Cancer Res* 1987, **47**, 4590–4594.
29. Nigro JM, Baker SJ, Preisinger AC *et al.* Mutations in the p53 gene occur in diverse human tumour types. *Nature* 1989, **342**, 705–708.
30. Rodrigues NR, Rowan A, Smith MEF *et al.* p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* 1990, **87**, 7555–7559.

Acknowledgements—We thank Dr Christos Paraskeva for supplying us with the PC/JW line.

Eur J Cancer, Vol. 27, No. 12, pp. 1684–1689, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

In vitro Methods for Screening Agents with an Indirect Mechanism of Antitumour Activity: Xanthenone Analogues of Flavone Acetic Acid

Lai-Ming Ching, Graeme J. Finlay, Wayne R. Joseph and Bruce C. Baguley

Xanthenone-4-acetic acid (XAA) resembles flavone acetic acid (FAA) in its effects on solid tumours in mice. The activity of methyl-substituted XAA derivatives *in vitro* was determined using 18 h ^{51}Cr-release assays, continuous exposure growth inhibition assays and stimulation of tumouricidal activity of cultured murine resident peritoneal macrophages. The macrophage assay identified the high biological activity and dose potency of 5-MeXAA *in vivo*, and was the most accurate *in vitro* predictor of the ability of congeners to induce either haemorrhagic necrosis of subcutaneous Lewis lung and colon 38 tumours or splenic natural killer activity. *In vitro* immune stimulation may be more appropriate than direct cytotoxicity for screening compounds with indirect mechanisms of antitumour activity.
Eur J Cancer, Vol. 27, No. 12, pp. 1684–1689, 1991.

INTRODUCTION

FLAVONE-8-ACETIC ACID (FAA), a synthetic flavonoid [1] has shown impressive preclinical activity against a broad spectrum of murine transplantable solid tumours [2–5], but clinical trials of FAA have been disappointing [6, 7]. Non-linear pharmacokinetics [8], low dose potency and problems of drug precipitation [9] have pointed to the need for better analogues of FAA. Towards this end, work from this laboratory has identified a series of xanthenone-4-acetic acid (XAA) derivatives with a similar antitumour action to that of FAA [10–12] and, in some cases, much greater dose potency.

FAA has been tested *in vitro* and found to have greater toxicity against leukaemic lines than solid tumour lines [2, 13]. On the other hand, solid tumours are more susceptible than leukaemic lines *in vivo* [4]. These discrepancies, as well as other observations, have led to the suggestion that FAA has a mechanism of action different from that of conventional direct cytotoxic agents [2, 13, 14], a suggestion strengthened by the observations

Correspondence to L.-M. Ching.
The authors are at the Cancer Research Laboratory, Auckland University School of Medicine, Auckland, New Zealand.
Revised 24 June 1991; accepted 8 Aug. 1991.

that FAA induces the synthesis of the cytokines tumour necrosis factor-α (TNF-α), interferon-α (IFNα), IFN-γ [15] and nitric oxide [16], as well as promoting macrophage-mediated cytotoxicity [17, 18].

Direct *in vitro* toxicity assays have proved immensely valuable in screening for potential antitumour agents with a direct cytotoxic action, but the above considerations suggest the need to develop alternative *in vitro* screening methods for agents with an indirect mechanism of antitumour activity. We have previously demonstrated that FAA stimulates the ability of mouse resident peritoneal macrophages to lyse a variety of murine target cells, as measured by the release of ^{51}Cr-chromate [17, 18]. However, it is not known whether this activity correlates with *in vivo* activity. The availability of a series of XAA derivatives of closely similar physical properties and widely varying *in vivo* antitumour activity provides a good opportunity for comparing different *in vitro* testing protocols in the assessment of new drugs with a possible indirect mechanism of antitumour action.

In this study we have used a series of monomethyl derivatives of XAA to investigate the relationship of *in vitro* to *in vivo* activity. We have previously shown a good correlation between the ability of these derivatives to enhance natural killer (NK) activity *in vivo* and their ability to induce haemorrhagic necrosis of colon 38 tumours [19]. We have now studied the *in vitro* toxicity of XAA analogues towards tumour lines in both a lytic assay (chromate release) and in an antiproliferative assay. We have also tested the ability of these compounds to stimulate resident mouse peritoneal macrophages in culture to become tumouricidal. The results support the hypothesis that new screening methods are required to detect compounds which have an indirect mechanism of antitumour activity, and that the use of macrophage activation *in vitro* is a useful indicator of the *in vivo* biological activity of XAA derivatives.

MATERIALS AND METHODS

Culture medium and drugs

α-MEM supplemented with 10% fetal bovine serum (Gibco), and antibiotics (penicillin, 100 U/ml; streptomycin sulphate, 100 μg/ml) was used routinely for growing cells. Sodium salts of XAA and analogues were synthesised as described [10–12], dissolved in growth medium, and because of their light sensitivity [20], protected from light.

Mice

C3H/HeN and BDF_1 hybrid [$(C_{57}BL/6 \times DBA/2)F_1$] mice (Jackson Laboratory, Bar Harbor, Maine, USA) were bred in the laboratory and housed 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 animal ethical guidelines.

Cell lines

Murine LLTC and human HCT-8 cell lines were obtained from Dr R. C. Jackson at Warner-Lambert, Ann Arbor, Michigan, USA. The LLTC line was developed from a Lewis lung carcinoma at the Southern Research Institute, Birmingham, Alabama, USA. The murine P815 mastocytoma cell line was obtained from Dr J. Marbrook, Auckland Medical School, Auckland, NZ. The human HT-29 line was obtained from Dr J. Fogh, Sloan-Kettering Institute for Cancer Research, New York, USA. The human SW620, Jurkat and U937 lines were obtained from the American Type Culture Collection, Rockville, Maryland, USA.

In vitro ^{51}Cr-release assay

Tumour cells ($1–2 \times 10^6$) were incubated with 7.4 mBq sodium ^{51}Cr-chromate in saline for 1 h at 37°C. Excess ^{51}Cr was removed by three washes and the ^{51}Cr-labelled tumour cells placed in 96-well plates (5×10^3 cells/well) with drug in a volume of 0.2 ml culture medium. Quadruplicate wells were plated for each drug dose. After 18 h incubation at 37°C, 0.1 ml of the supernatant was removed from each well and the amount of radioactivity measured in a gamma counter (LKB Wallac 1270 Rackgamma 11, Wallac, Finland). The per cent lysis of tumour cells was calculated as [(^{51}Cr-released with drug-spontaneous ^{51}Cr-released without drug)/(total amount ^{51}Cr incorporated into the cells)] × 100. The LC_{50} was defined as the drug concentration required for 50% lysis of the tumour targets in the ^{51}Cr release assay.

In vitro growth inhibition assay

LLTC, HCT-8, HT-29 and P815 cells were seeded at 10^3 cells per well, and SW620 cells were seeded at 4×10^3 cells per well, and grown in flat-bottomed 96-well plates (Linbro, McLean, Virginia, USA) using 0.2 ml culture medium. Drug was added after 1 day and cultures were harvested after a further 3 days in culture for LLTC and P815 cells and 5 days for SW620, HCT-8 and HT-29 cells. The growth of adherent cells was quantitated by staining with methylene blue as previously described [21]. The number of cells per well was counted using a particle counter (Coulter Electronics, Dunstable, Bedfordshire, UK). The IC_{50} value was defined as the drug concentration required for 50% reduction of cell number in the growth inhibition assay.

Macrophage cytotoxicity assay

C3H/HeN mice (8 and 12 weeks of age) were killed by cervical dislocation and the resident peritoneal cells collected by washing the peritoneum with 5 ml medium. Viable cells which excluded eosin were counted in a haemocytometer and cells were plated (0.2 ml medium per well) in V-bottomed 96-well plates (Linbro, Flow) which were placed in an incubator for 2 h at 37°C. Supernatant and non-adherent cells were then removed by completely inverting the microwells. Fresh medium was added and similarly discarded. Cells adhering to the bottom of the microwells were predominantly macrophages, as determined by morphology after staining with Leishman's stain, and used for experiments. No adjustment to the cell number was made for loss of the non-adherent cells, and the effector to target (E:T) cell ratio was determined using the number of cells initially plated. The adherent cells were incubated 18 h with ^{51}Cr-labelled LLTC tumour targets (5×10^3 per well) in the presence of drug in 0.2 ml medium at 37°C under a humidified atmosphere with 5% CO_2, using quadruplicate wells per drug concentration. E:T ratio was varied by plating different numbers of resident peritoneal cells. Lysis of target cells was assessed from the amount of ^{51}Cr released in 0.1 ml of the supernatant as described by the formula: [(experimental−spontaneous/total) × 100]. The MC_{50} was defined as the drug concentration required for 50% lysis of the tumour targets in the macrophage ^{51}Cr release assay.

Histological assessment of tumour necrosis

Cultured LLTC tumour cells (10^6) were inoculated subcutaneously in BDF_1 mice and allowed to grow until tumours reached a diameter of 5–10 mm for use in experiments. Mice were then injected with a single intraperitoneal dose of drug, killed by

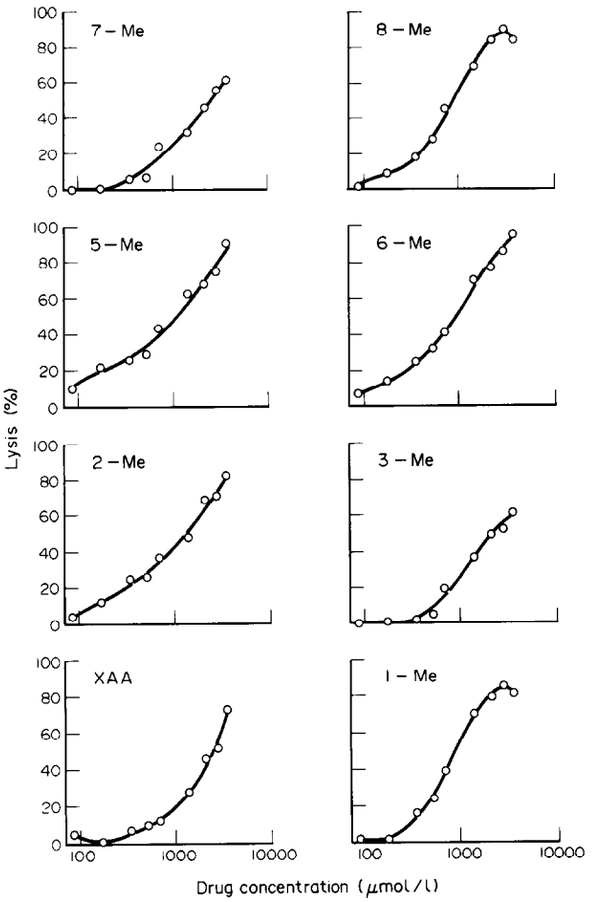

Fig. 1. Lysis of P815 cells by XAA and monomethyl XAA derivatives. P815 cells were labelled with ^{51}Cr and incubated with serial dilutions of each drug. Lysis was measured after 18 h. Each point represents the mean of quadruplicates.

cervical dislocation 24 h later, and the tumours removed and fixed in 10% formalin. Fixed tumours were embedded in paraffin wax, 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 [22].

RESULTS

In vitro toxicity of mono-methyl XAA derivatives by ^{51}Cr release assay

The direct toxicity of XAA and monomethyl XAA derivatives against P815 mastocytoma cells was examined by labelling tumour lines with ^{51}Cr-chromate and then exposing them to XAA derivatives. An exposure time of 18 h was chosen since most of the lysis in the presence of 2.5 mM XAA had taken place by 18 h (data not shown). The per cent lysis at varying drug concentrations was used to determine the LC_{50} value, the amount of drug required for 50% lysis of tumour targets. Titrations carried out using P815 murine mastocytoma cells are shown in Fig. 1. LLTC, a murine tissue culture line derived from the Lewis lung carcinoma [2] as well as three human cell lines, HCT-8 colon, U937 lymphoma and Jurkat leukaemia, were also tested, as summarised in Table 1. LLTC cells were found to be extremely resistant to the compounds and values could be determined only for 1-Me and 8-Me XAA. P815 cells and the human leukaemic lines were the most sensitive and HCT-8 cells showed intermediate sensitivity. The range of LC_{50} values for each cell line was small (2–4 fold). No XAA derivative was consistently more toxic than the others, but on the basis of the logarithmic means, 1-Me and 8-Me XAA were the most toxic.

In vitro growth inhibitory activity of mono-methyl XAA derivatives

IC_{50} values were also determined using a conventional growth inhibition assay for a number of cell lines (Table 1). IC_{50} values for HCT-8 and LLTC cells were considerably lower than the corresponding LC_{50} values, while the IC_{50} and LC_{50} values for P815 cells were comparable in the two assays. The ranking of XAA derivatives in both assays was similar, and the range of IC_{50} values for each cell line was less than 4-fold.

In vitro macrophage-mediated toxicity of mono-methyl XAA derivatives

The ability of each of the monomethyl XAA derivatives to stimulate macrophages in culture to become tumouricidal was assessed. Both P815 and LLTC are susceptible targets for macrophage cytotoxicity [17], but the high degree of resistance of LLTC toward direct toxicity of the drugs in the 18 h ^{51}Cr-release assay (Table 1) rendered them preferable for use as tumour targets for the macrophage cytotoxicity assay. Adherent resident peritoneal cells were incubated with ^{51}Cr-labelled LLTC in the presence of varying concentrations of drug. Lysis of LLTC targets in the presence of drug and resident peritoneal cells was measured after 18 h. Cultures of LLTC in the presence of drug but without resident peritoneal cells were included in all experiments, but the levels of direct drug toxicity were always insignificant. 2-MeXAA and 7-MeXAA did not stimulate resident peritoneal macrophages to become tumouricidal, whereas 1-, 3-, 5-, 6- and 8-MeXAA all stimulated macrophage cytotoxicity to varying extent (Fig. 2). 5-MeXAA was notable for its greater potency, with activity as low as 86 μmol/l.

Relationship of in vivo antitumour and NK responses to in vitro activity

Monomethyl substituted XAA derivatives have been found to vary in both their ability and their dose potency to induce haemorrhagic necrosis in subcutaneous colon 38 tumours [11]. To determine whether they showed similar activity against a tumour which was readily grown in culture, derivatives were tested for their ability to induce haemorrhagic necrosis of subcutaneous LLTC tumours, at doses which were previously used to compare antitumour effects and induction of NK activity in colon 38-bearing mice [19]. The results are shown in Fig. 3. The 6-methyl derivative was the most active, while the 1-, 2-, 3-, and 5-methyl derivatives all induced 50% or greater necrosis of tumour sections.

When the LC_{50} and IC_{50} values of the monomethyl XAA derivatives against cultured cells (Table 1) were compared either with the induction of haemorrhagic necrosis of subcutaneous LLTC tumours, or with the induction of NK activity in mice, no significant linear correlation was seen. However, when the maximum percentage lysis values obtained for each of the compounds in the macrophage cytotoxicity assay (data from Fig. 2) were plotted against the degree of haemorrhagic necrosis induced *in vivo* against LLTC and colon 38 tumours, a weak but significant linear correlation ($r = 0.65$; $P<0.01$) was observed (Fig. 3). When the macrophage cytotoxicity data were

Table 1. *In vitro toxicity of monomethyl substituted XAA derivatives*

	XAA	1-Me	2-Me	3-Me	5-Me	6-Me	7-Me	8-Me	FAA
^{51}Cr release LC_{50} (mmol/l)									
LLTC murine lung carcinoma	>32	23	>32	>32	>32	>32	>32	18	>32
P815 murine mastocytoma	2.5	1.0	1.3	2.5	1.0	1.0	2.5	1.0	ND
HCT-8 human colon carcinoma	11	8.3	8.6	9.3	11	16	17	9.0	ND
U937 human histiocytic lymphoma	3.2	1.4	3.9	2.4	5.0	1.7	5.4	1.4	ND
Jurkatl human T-cell leukaemia	8.2	3.6	8.2	2.1	8.2	5.7	5.7	3.6	7.1
Growth inhibition IC_{50} (mmol/l)									
LLTC murine lung carcinoma	1.3	0.5	1.3	0.8	0.5	0.8	1.1	0.5	0.6
P815 murine mastocytoma	0.9	0.4	0.6	0.7	0.4	0.8	0.6	0.4	ND
HCT-8 human colon carcinoma	0.96	0.60	0.69	0.28	0.43	0.25	0.82	0.36	<0.25
HT-29 human colon carcinoma	1.4	0.59	1.0	0.60	1.2	0.95	1.2	0.55	0.62
SW620 human colon carcinoma	1.6	0.7	1.4	1.1	0.8	0.9	1.5	0.8	1.2
Macrophage-mediated cell lysis MC_{50} (mmol/l)									
LLTC murine lung carcinoma	>1.4	0.34	>1.4	>1.4	0.07	0.51	>1.4	>1.4	0.70
In vivo dose used for assessment of tumour necrosis (mmol/kg)									
Lewis lung/colon 38	0.80	0.52	1.72	0.76	0.15	0.52	1.14	1.14	1.18

ND = not determined.

plotted against NK activity an even higher degree of correlation ($r = 0.83$; $P<0.01$) was obtained (Fig. 4).

DISCUSSION

The results show that small changes in the chemical structure of the XAA series result in large changes in the potency and biological activity (Fig. 2). Bearing in mind that the induction of tumour necrosis is steeply dependent on drug dose and sometimes quite variable between individual experiments, the pattern of activity of XAA and XAA analogues against subcutaneous LLTC tumours (Fig. 3) is similar to that previously observed for colon 38 tumours [11]. The parent compound XAA was inactive against LLTC tumours at the dose used in this study, but XAA sometimes also shows poor activity against colon 38 tumours at this dose [19]. Substitution at postion 2 gave higher activity than that expected, but again this is variable in the case of colon 38 data [19]. Methyl substitution at positions 3 or 6 resulted in highly active compounds, while substitution at position 5 produced an active compound with increased dose potency (Table 1).

Ideally, an *in vitro* drug screening system should be able to predict whether or not a compound has high biological activity as well as its dose potency. Two direct cytotoxicity assays, ^{51}Cr-release and growth inhibition, were compared (Table 1). The ^{51}Cr-release assay demonstrated that XAA and its derivatives were capable of inducing direct cell lysis at high concentrations, although LLTC cells were much more resistant than the other cell lines tested. An 18 h exposure was chosen since most of the lysis had taken place by this time, and so that results could be directly compared with the macrophage-mediated cytotoxicity assay. The ^{51}Cr-release assay differentiated only slightly between the analogues and the LC_{50} values neither correlated with nor predicted *in vivo* dose potency or antitumour activity. LLTC and HCT-8 cells were more sensitive to the drugs in continuous exposure growth inhibition assays, although again the concentrations required for inhibition, at least for XAA, were higher than those encountered *in vivo* [23]. However, none of the sets of IC_{50} values for FAA, XAA and XAA derivatives correlated with *in vivo* dose potency or antitumour activity.

FAA, XAA and some XAA derivatives were found to stimulate resident peritoneal cells to become tumouricidal in culture (Table 1, Fig. 2). As discussed previously for FAA [17], this activity is likely to result from macrophages. Although NK cells are present in the peritoneum, both the P815 [17] and LLTC (unpublished results) target cells are resistant to NK-mediated lysis. In contrast to the two direct cytotoxicity assays, the maximum lysis values obtained using the resident peritoneal macrophage assay correlated with the ability of the 3-, 5-, and

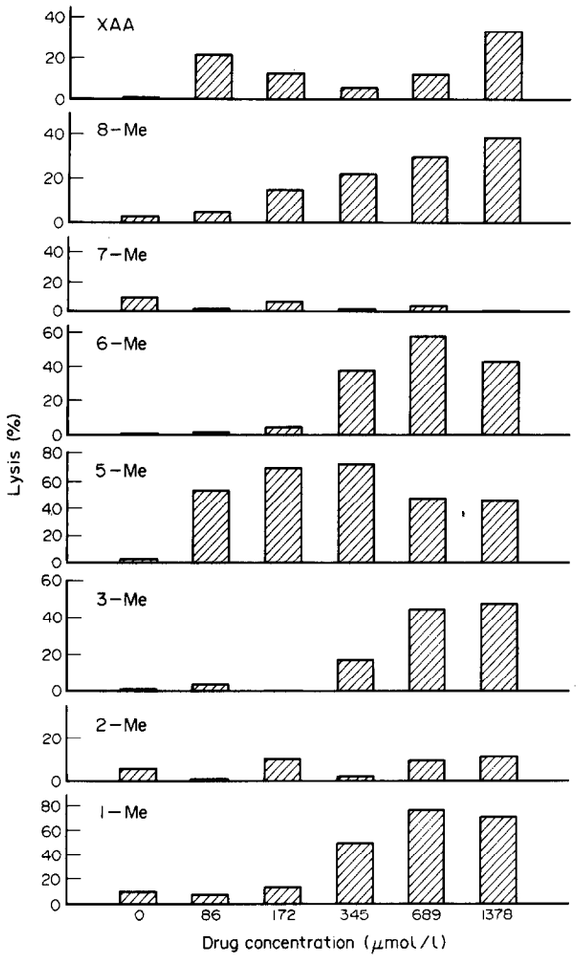

Fig. 2. Stimulation of macrophage tumouricidal activity by monomethyl XAA derivatives. Adherent resident peritoneal cells were incubated with ^{51}Cr-labelled LLTC targets in the presence of varying concentrations of drug. Per cent lysis was measured after 18 h. Mean (S.E.) of quadruplicate cultures measured at 200:1 E:T.

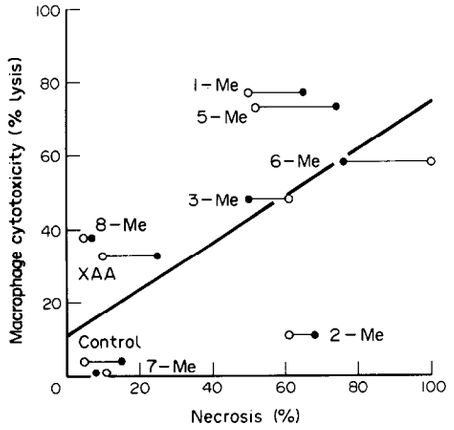

Fig. 3. Relationship between the induction of necrosis of subcutaneous LLTC tumours (○) or colon 38 tumours (●) and the highest level of macrophage cytotoxicity against LLTC cells for each of the XAA derivatives (data from Fig. 2). *In vivo* doses used are shown in Table 1 and were optimal for natural killer induction [19]. The regression line ($r = 0.65$) is drawn through all of the data points.

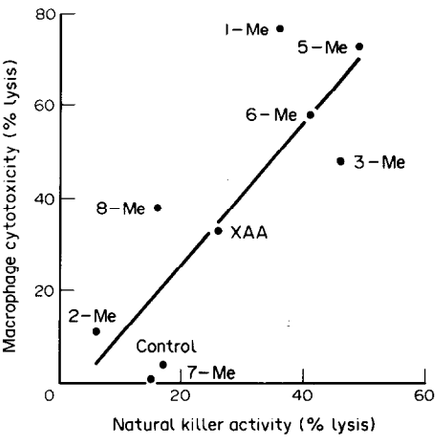

Fig. 4. Relationship between induction of splenic NK activity (YAC-1 targets; data from Fig. 6 of [19]) and the highest level of macrophage cytotoxicity against LLTC cells for each of the XAA derivatives (data from Fig. 2). *In vivo* doses used are the same as in Fig. 3.

6-methyl derivatives both to induce tumour necrosis and to induce NK activity (Figs 3 and 4). Furthermore, the *in vitro* dose response curves (Fig. 2) predicted both the moderately increased dose potency of 6-MeXAA and the highly increased dose potency of 5-MeXAA (Table 1).

In conclusion, we have shown using the monomethyl XAA series that measurement of direct *in vitro* drug cytotoxicity does not accurately reflect *in vivo* selectivity and potency of antitumour activity. The ability of this series to stimulate the tumouricidal activity of macrophages did, however, correlate with *in vivo* biological responses. Antitumour activity is not simply related to the induction of tumour necrosis but is rather a consequence of a number of mechanisms, possibly including generation of T-cell immunity [24, 25], NK induction [26] and nitric oxide synthesis [16]. FAA-induced tumour haemorrhagic necrosis is not caused by NK induction [27, 28], but may result from decreased tumour blood flow [29] induced by release of TNF-α [15] since antibody to TNF-α inhibits FAA-induced reduction of tumour blood flow [30]. It is not yet clear whether macrophages are involved in the antitumour response to this class of agents, but irrespective of their role, the relationships demonstrated in this study indicate that activation of macrophage cytotoxicity may be useful as an *in vitro* test of *in vivo* antitumour activity. Such *in vitro* assays of immune stimulation could augment direct drug cytotoxicity assays in the screening of new compounds for antitumour activity.

1. Atassi G, Briet P, Berthelon J-J. Collonges F. Synthesis and antitumour activity of some 8-substituted-4-oxo-4H-1-benzopyrans. *Eur J Med Chem* 1985, 5, 393–402.
2. Finlay GJ, Smith GP, Fray LM, Baguley BC. Effect of flavone acetic acid (NSC 347512) on Lewis carcinoma: evidence for an indirect effect. *J Natl Cancer Inst* 1988, **80**, 241–245.
3. Smith GP, Calveley SB, Smith MJ, Baguley BC. Flavone acetic acid (NSC 347512) induces haemorrhagic necrosis of mouse colon 26 and 38 tumours. *Eur J Cancer Clin Oncol* 1987, **23**, 1209–1212.
4. Plowman J, Narayanan VA, Dykes D, *et al.* Flavone acetic acid: a novel agent with preclinical antitumor activity against colon adenocarcinoma 38 in mice. *Cancer Treat Rep 1986*, **70**, 631–638.
5. Pratesi G, Manzotti, Damia G, D'Incalci M. Response of chemically induced primary colon tumours of the mouse to flavone acetic acid (NSC 347512). *Br J Cancer* 1988, **58**, 144–146.
6. Kerr DJ, Kaye SB. Flavone acetic acid—preclinical and clinical activity. *Eur J Cancer Clin Oncol* 1989, **25**, 1271–1272.

7. Kerr DJ, Maughan T, Newlands E, *et al.* Phase II trials of flavone acetic acid in advanced malignant melanoma and colorectal cancer. *Br J Cancer* 1989, **60**, 104–106.
8. Gouyette A, Kerr DJ, Kaye SB, *et al.* Flavone acetic acid: a nonlinear pharmacokinetic model. *Cancer Chemother Pharmacol* 1988, **22**, 114–119.
9. O'Dwyer PJ, Shoemaker D, Zaharko DS, *et al.* Flavone acetic acid (LM 975, NSC 347512): a novel antitumour agent. *Cancer Chemother Pharmacol* 1987, **19**, 6–10.
10. Atwell GJ, Rewcastle GW, Baguley BC, Denny WA. Synthesis and antitumour activity of topologically-related analogues of flavoneacetic acid. *Anti-Cancer Drug Design* 1989, **4**, 161–169.
11. Rewcastle GW, Atwell GJ, Baguley BC, Calveley SB, Denny WA. Potential antitumor agents. 58. Synthesis and structure-activity relationships of substituted xanthenone-4-acetic acids active against the colon 38 tumor *in vivo*. *J Med Chem* 1989, **32**, 793–799.
12. Atwell GJ, Rewcastle GW, Baguley BC, Denny WA. Potential antitumour agents. 60. Relationships between structure and *in vivo* Colon 38 activity for 5-substituted 9-oxoxanthene-4-acetic acids. *J Med Chem* 1990, **33**, 1375–1379.
13. Capolongo LS, Balconi G, Ubezio P, *et al.* Antiproliferative properties of flavone acetic acid (NSC 347512) (LM 975) a new anticancer agent. *Eur J Cancer Clin Oncol* 1987, **23**, 1592–1535.
14. Schroyens, WA, Dodion PP; Sanders C, *et al*. *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* 1987, **23**, 1135–1139.
15. Mace KF, Hornung RL, Wiltrout RH, Young HA. Induction of cytokine expression *in vivo* by flavone acetic acid: strict dose dependency and correlation with therapeutic efficacy against murine renal cancer. *Cancer Res* 1990, **50**, 1742–1747.
16. Thomsen, LL, Ching L-M, Zhuang L, Gavin JB, Baguley BC. Tumor-dependent increased plasma nitrate concentrations as an indication of the antitumour effect of flavone-8-acetic acid and analogues in mice. *Cancer Res* 1991, **51**, 77–81.
17. Ching L-M, Baguley BC. Enhancement of *in vitro* toxicity of mouse peritoneal exudate cells by flavone acetic acid (NSC 347512). *Eur J Cancer Clin Oncol* 1988, **24**, 1521–1525.
18. Ching L-M, Baguley BC. Hyporesponsiveness of macrophages from C3H/HeJ (endotoxin-resistant) mice to the antitumour drug flavone acetic acid (NCS 347–512). *Eur J Cancer Clin Oncol* 1989, **25**, 1313–1315.
19. Ching L-M, Joseph WR, Zhuang L, *et al.* Induction of natural killer activity by xanthenone-4-acetic acids: relation with antitumor activity. *Eur J Cancer* 1991, **27**, 79–83.
20. Rewcastle GW, Kestel P, Baguley BC, Denny WA. Light-induced breakdown of flavone acetic acid and xanthenone analogues in solution. *J Natl Cancer Inst* 1990, **82**, 528–829.
21. Finlay GJ, Baguley BC. The use of human cancer cell lines as a primary screening system for antineoplastic compounds. *Eur J Cancer Clin Oncol* 1984, **20**, 947–954.
22. Baguley BC, Calveley SB, Crowe KK, Fray LM, O'Rourke SA, Smith GP. Comparison of the effects of flavone acetic acid, fostriecin and homoharringtonine and tumour necrosis factor-α on colon 38 tumors in mice. *Eur J Cancer Clin Oncol* 1989, **25**, 263–269.
23. Kestell P, McKeage MJ, Baguley BC. Determination of xanthenone-4-acetic acid in mouse plasma by high performance liquid chromatography. *J Chromatogr* 1991, **564**, 315–319.
24. Pratesi G, Rodolfo M, Rovetta G, Parmiani G. Role of T cells and tumour necrosis factor in antitumour activity and toxicity of flavone acetic acid. *Eur J Cancer* 1990, **26**, 1079–1083.
25. Bibby MC, Phillips RM, Double JA, Pratesi G. Anti-tumour activity of flavone acetic acid (NSC-347512) in mice—influence of immune status. *Br J Cancer* 1991, **63**, 57–62.
26. Hornung RA, Back TC, Zaharto DS, Urba WJ, Longo DL, Wiltrout RH. Augmentation of natural killer (NK) activity, induction of interferon and development of tumor immunity during the successful treatment of established murine renal cancer using flavone acetic acid (FAA) and interleukin 2. *J Immunol* 1988, **141**, 3671–3679.
27. Ching L-M, Baguley BC. Effect of flavone acetic acid (NSC 347512) on splenic cytotoxic effector cells and their role in tumour necrosis. *Eur J Cancer Clin Oncol* 1989, **25**, 821–828.
28. Ching L-M, Baguley BC. Reduction of cytotoxic effector cell activity in Colon 38 tumours following treatment with flavone acetic acid. *Eur J Cancer Clin Oncol* 1989, **25**, 1061–1065.
29. Zwi LJ, Baguley BC, Gavin JB, Wilson WR. Blood flow failure as a major determinant in the antitumor action of flavone acetic acid (NSC 347512). *J Natl Cancer Inst* 1989, **81**, 1005–1013.
30. Mahadevan V, Malik STA, Meager A, Fiers W, Lewis GP, Hart IR. Role of tumor necrosis factor in flavone acetic acid-induced tumor vasculature shutdown. *Cancer Res* 1990, **50**, 5537–5542.

Acknowledgements—This study was supported by the Auckland Division of the Cancer Society of New Zealand, the Medical Research Council of New Zealand and a Warner-Lambert Laboratory Fellowship. The authors thank Li Zhuang for histological assessment of tumours and Lynden Wallis and Wendy Hodgson for secretarial assistance.