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Haematological effects in mice of the antitumour agents xanthenone-4-acetic acid, 5,6-methyl-xanthenone-4-acetic acid and flavone acetic acid*

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Summary. Treatment of $C_{57}Bl/6 \times DBA/2$ mice with the maximal tolerated dose of flavone-8-acetic acid (FAA, 1300 μmol/kg), xanthenone-4-acetic acid (XAA, 1090 μmol/kg), or its dose-potent derivative 5,6-dimethyl-xanthenone-4-acetic acid (5,6-MeXAA, 100 μmol/kg) resulted within 24 h in a dramatic reduction in the number of circulating lymphocytes, an elevation in haemoglobin concentrations and a reduction in platelet numbers. Neutrophil counts either remained unchanged or were slightly elevated. All three compounds caused a marked loss of cells in the thymus. Examination of histological sections of thymus at 48 h following treatment with XAA revealed a selective depletion of cortical thymocytes and no effects on the epithelium or other thymic structures. A transient decrease in cell numbers was seen in the spleen and femoral bone marrow, with recovery to normal levels occurring within 3 days. The number of haemopoietic stem cells, colony-forming units in culture (CFU-c), in the femoral bone marrow increased after drug administration despite the occurrence of a decrease in the overall number of cells in the femur. In contrast to the increase in CFU-c numbers seen in vivo, 2 h exposure of bone-marrow cells to FAA, XAA or 5,6-MeXAA in vitro resulted in a decrease in the surviving fraction of CFU-c. The results are consistent with the hypothesis that the in vivo haematological effects of these compounds are indirect, perhaps being mediated through the induction of cytokines, and contrast with the haematological effects of conventional antitumour agents. The biochemical and haematological effects are unlikely to be the cause of the acute toxicity observed for these compounds.

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Introduction

Flavone-8-acetic acid (FAA) is a synthetic flavone derivative that has shown outstanding activity against murine experimental solid tumours [9, 19, 20, 25] but no clinical antitumour activity as a single agent [12, 13]. Possible reasons for this discrepancy might be that the action of FAA is species-specific, acting through a receptor/substrate that is different in mice and humans, or that drug solubility or toxic side effects limit the amount of drug that can be given to humans to an ineffective dose. The low dose potency of FAA, together with its dose-dependent pharmacokinetics [8] and problems of drug precipitation [28], indicates the need for better analogues of this drug.

Towards that end, we have investigated a series of derivatives of xanthenone-4-acetic acid (XAA) [1, 21, 22], which, like FAA, induce haemorrhagic necrosis as well as delays in the growth of tumours [1, 21, 22]. Small changes in the XAA molecule result in large changes in both antitumour [1, 21] and immunomodulatory activities [6, 27], providing a series that includes inactive drugs as well as congeners that are more active and more dose-potent than FAA [1, 21, 22]. Of all the XAA derivatives thus far developed, 5,6-dimethyl-xanthenone-4-acetic acid (5,6-MeXAA; Fig. 1) has been found to be the most active in terms of both tumour regression [22] and immunomodulation [27]. It is also more dose-potent, inducing cures of the colon 38 tumour at doses that are 1 order of magnitude lower than those used for FAA and XAA [22]. However, 5,6-MeXAA is also correspondingly more toxic to mice.

One of the problems of the FAA/XAA class of compounds is that the dose-response curve for antitumour activity is extremely steep, with responses being obtained in mice only at doses approaching the lethal dose. The cause of the lethality in mice induced by this class of compounds is not clear. The toxicity profile of FAA in the clinic differs from those of conventional cytotoxic antitumour agents [12, 13] in that hypotension is the dose-limiting toxicity and the myelosuppression and haematological toxicities that are typically associated with direct cytotoxic agents are not observed [12, 13]. The mechanism underlying the

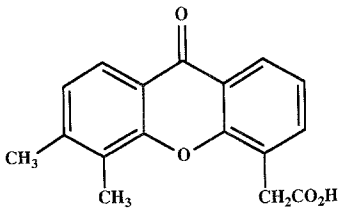

Fig. 1. Structure of 5,6-MeXAA

antitumour activity of FAA is also different from that of direct cytotoxic agents and appears to involve biological response modification in the host [9]. We have shown that the immune-modulating capacities of FAA and XAA derivatives correlate with their ability to induce haemorrhagic necrosis of s.c. tumours, indicating that these activities are associated [6]. In an attempt to establish the basis of the toxicity of this class of compounds, we investigated the biochemical and haematological effects of XAA and its potent derivative 5,6-MeXAA in normal mice.

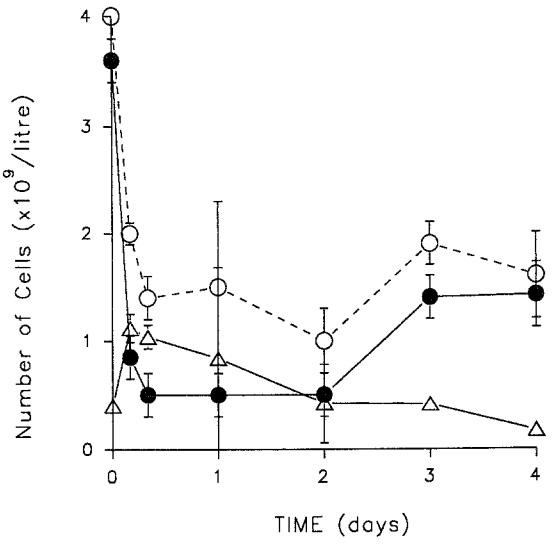

Fig. 2. Peripheral blood total leucocyte (○), lymphocyte (●) and neutrophil (△) counts following i.v. treatment of mice with XAA at the MTD

Materials and methods

Animals and therapeutic agents. For all experiments, we used 6- to 12-week-old $C_{57}Bl/6J \times DBA/2J$ hybrid mice that had been bred in our animal facility and were housed under conditions of constant temperature and humidity using sterile bedding and food according to institutional ethical guidelines. FAA was obtained from the National Cancer Institute (USA) through the courtesy of Dr. K. Paull, and sodium salts of XAA and its analogues were available in our laboratory [1, 21, 22]. Solutions of FAA or XAA and its analogues were prepared by dissolving the drugs in minimal amounts of 5% (w/v) sodium bicarbonate and were then diluted in medium. Because of their photosensitivity [23], the solutions were protected from light during the experiments and were given to mice intravenously (i.v.).

Analysis of peripheral blood. Mice were anaesthetized with ether and blood (approx. 1 ml) was collected from the orbital sinus and placed in microtainer tubes containing lithium heparin. Animals were killed by cervical dislocation immediately after blood collection. Blood from each mouse was centrifuged and the plasma creatinine, bilirubin, alkaline phosphatase, aspartate aminotransferase, γ-glutamyl transpeptidase, albumin, protein, sodium, potassium and calcium concentrations were measured by a Technicon RA 100 random access analyzer. For haematological investigations, blood was collected into microtainer tubes containing ethylenediamine-tetraacetic acid (Becton-Dickinson, Lincoln Park N. J., USA) and diluted in Isoton III (Coulter Electronics, Hialeah, Fla., USA). Haemoglobin, red cell, total white cell and platelet counts were measured by a Coulter S-Plus III cell analyzer (Coulter Electronics). Blood smears stained with Leishman's stain were examined by light microscopy to determine white cell differential counts. Mean values ± standard deviations were calculated for groups of 3–7 mice.

Assessment of the cellularity of lymphoid organs. Mice were killed by cervical dislocation and the thymus, spleen and femora were removed. Cells from the thymus and spleen were gently squeezed out into culture medium (αMEM, Gibco) using forceps. Bone-marrow cells were obtained by removing epiphyses from the femora and rinsing the interior shafts with medium. Single-cell suspensions were obtained by expressing the samples through a 20-gauge needle. Nucleated cells were counted using a haemocytometer.

Histology. Tissues were placed in a phosphate-buffered 10% formaldehyde solution for at least 24 h. The samples were dehydrated and then embedded in paraffin wax. Sections were cut, mounted and stained with haematoxylin and eosin prior to examination by light microscopy.

Growth of bone-marrow colonies. Colonies were grown as previously described [4] by plating $1-2 \times 10^4$ nucleated bone-marrow cells in culture dishes (35 mm, Nunc) in a 1-ml suspension of methylcellulose (0.8% w/v; Sigma) in culture medium supplemented with 10% foetal calf serum (FCS), 50 μ*M* 2-mercaptoethanol and 40% conditioned medium (CM) that contained colony-stimulating factors. Dishes were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Colonies were counted using a dissection microscope after 5 days. CM was prepared by culturing EL4-B thymoma cells (obtained from Dr. J. J. Farrar, National Institutes of Health, Bethesda, Md., USA) in flasks at 10^6 cells/ml for 48 h with concanavalin A (1 μg/ml; Sigma) and 4β-phorbol-12β-myristate-13α-acetate (20 ng/ml, Sigma) in culture medium supplemented with 10% FCS. Supernatant was collected, filtered and stored at 4°C.

Results

Peripheral blood biochemistry and haematology of XAA-treated mice

Biochemical analyses of peripheral blood in normal mice at 24 h after i.v. treatment with XAA at its maximal tolerated dose (MTD) revealed only one treatment-related abnormality, an elevation in the plasma level of aspartate aminotransferase (183 ± 94 units/l; control value, 63 ± 10 units/l; $P < 0.01$). Sodium, potassium, creatinine, calcium, bilirubin, alkaline phosphatase, γ-glutamyl transpeptidase, albumin and total protein values determined at 24 h after treatment with XAA lay within the normal range.

Groups of mice were injected with XAA at different times prior to the analysis of haematological parameters (Figs. 2, 3). Total white cell counts in the peripheral blood of treated mice were significantly lowered ($P < 0.01$) at 4 h post-treatment and remained low for at least 4 days (Fig. 2). The reduction in white cell counts was attributable to a reduction in the number of circulating lymphocytes, which constitute the predominant circulating white blood cell type in mice [29]. At 4 h the peripheral blood lymphocyte count was approximately 10% of its pre-treatment level, and it remained at these extremely low levels for

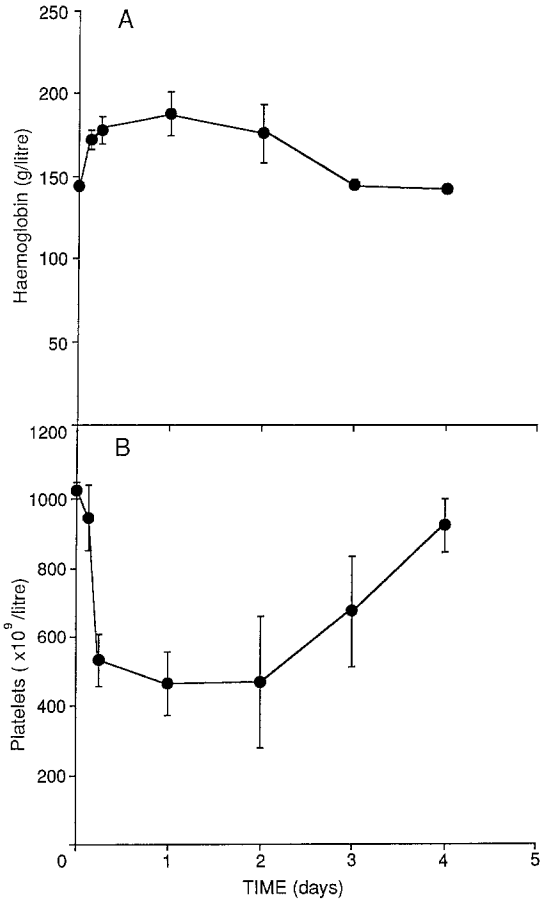

Fig. 3 A, B. Peripheral blood **A** haemoglobin and **B** platelet concentrations following i. v. administration of XAA to mice at the MTD

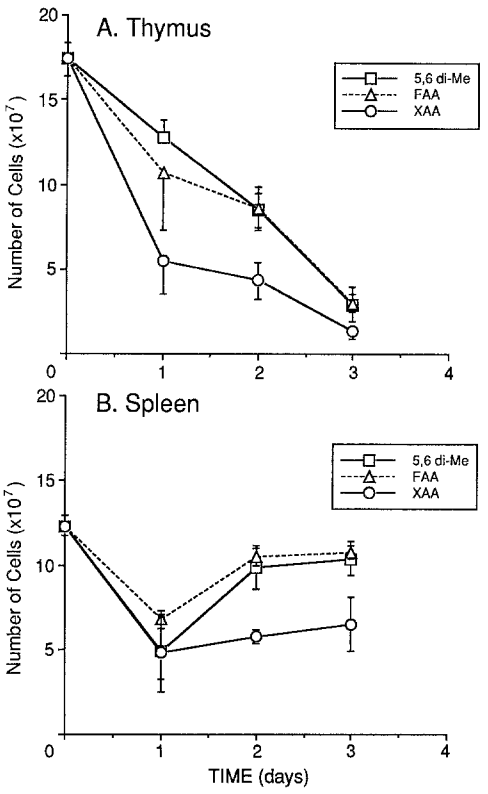

Fig. 4 A, B. Celullarity of the **A** thymus and **B** spleen in mice following i. v. administration of XAA, 5,6-MeXAA (*5,6 di-Me*) and FAA to mice at their respective MTDs. Data represent mean values ± SE for 3–6 mice/group

48 h. In contrast, the neutrophil count rose sharply after treatment before returning to normal levels by 48 h (control vs 4-h neutrophil count, $P < 0.01$; Fig. 2). The haemoglobin level increased to a maximal level at 24 h after treatment and had returned to normal by 72 h (control vs 24-h haemoglobin count, $P < 0.01$, Fig. 3 A). A reduction in platelet count was detectable at 8 h after treatment and had recovered by 96 h (control vs 24-h platelet count, $P < 0.01$; Fig. 3 B).

In another experiment we compared the effects of XAA with those induced by FAA and the dose-potent analogue 5,6-MeXAA, with each being given at its respective MTD (Table 1). After 48 h, all three compounds caused a similar reduction in platelet concentrations, a slight elevation in haemoglobin levels and a marked decrease in lymphocyte numbers. Neutrophil counts either remained unchanged or were elevated.

Cellularity of the thymus and spleen following treatment with XAA, 5,6-MeXAA or FAA

The number of cells in the spleen and thymus of mice in the initial few days following i.v. administration of XAA, 5,6-MeXAA or FAA at their respective MTDs are shown in Fig. 4. In the spleen, there was a decrease in the number of nucleated cells at 24 h after the administration of drug, with some recovery occurring by 48 h (Fig. 4 B). In the

Table 1. Haematological effects of XAA, 5,6-MeXAA and FAA[a]

	Untreated ($n = 7$)	XAA ($n = 4$)	5,6-MeXAA ($n = 3$)	FAA ($n = 3$)
White blood cells ($\times 10^9$)	5.7 ± 0.1	0.8 ± 0.05	0.9 ± 0.1	0.9 ± 0.3
Lymphocytes ($\times 10^9$)	5.1 ± 0.2	0.3 ± 0.03	0.4 ± 0.08	0.2 ± 0.08
Neutrophils ($\times 10^8$)	5.7 ± 0.6	6 ± 0.1	6.5 ± 0.2	5 ± 0.2
Platelets ($\times 10^9$)	1025 ± 25	646 ± 6	322 ± 8	185[b]
Haemoglobin (g)	128 ± 2.5	154 ± 1	149 ± 1	142 ± 1

[a] Mice were treated i. v. with either XAA (1090 µmol/kg), 5,6-MeXAA (100 µmol/kg) or FAA (1300 µmol/kg) and were bled after 48 h for haematological analyses

[b] Only 1 sample was available for testing because of clotting of samples

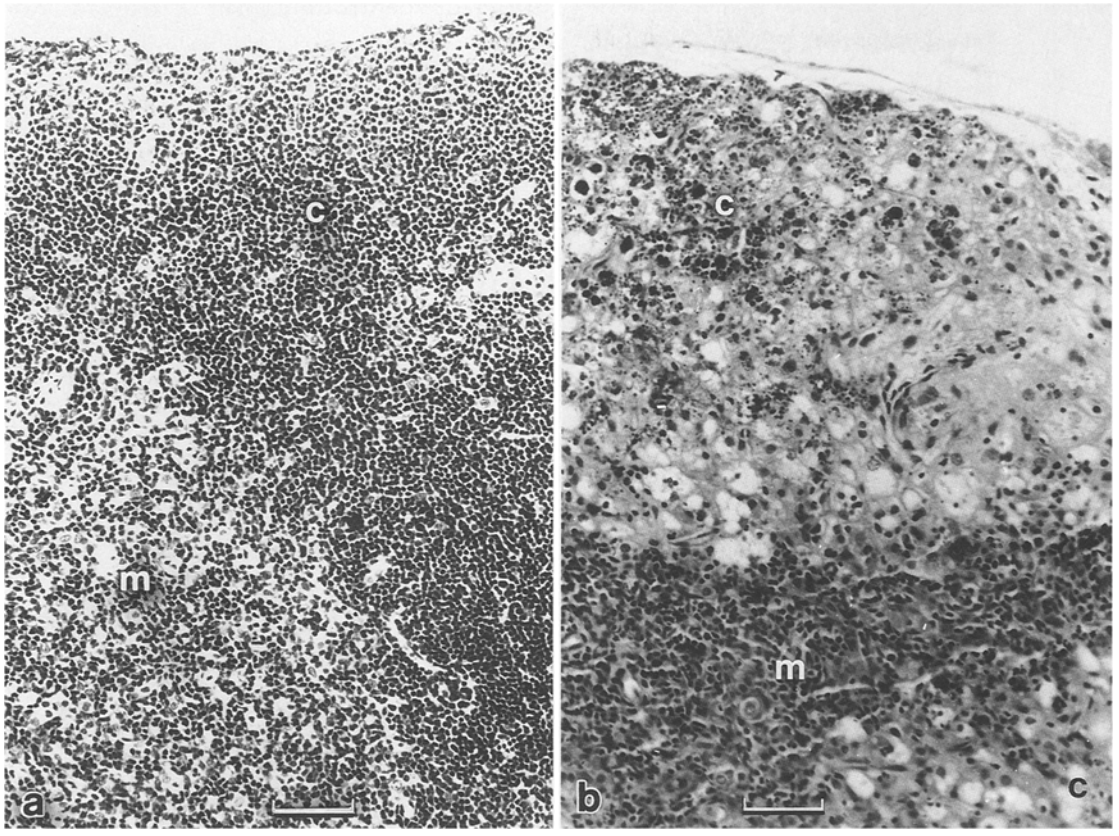

Fig. 5 a, b. Histological sections of thymus from **a** untreated mice, showing the cortex (*c*) and medulla (*m*), and **b** mice treated 48 h previously with XAA (1090 μmol/kg), showing the unchanged medulla (*m*), depletion of thymocytes in the cortex (*c*) and nuclear fragmentation of residual thymocytes (*upper left*). Bar = 50 μm

thymus, a progressive decrease in cell numbers occurred following XAA treatment, with a 90% reduction being noted after day 3 (Fig. 4A). The effects of the other two compounds were similar (Figs. 4A, B).

Histological sections of spleen did not show any distinct changes following treatment. However, sections of thymus from XAA-, 5,6-MeXAA- or FAA-treated animals demonstrated a marked loss of cortical thymocytes exposing large pale-staining thymic epithelial cells that appeared undamaged. An example of a thymic section obtained from animals at 2 days after XAA treatment is shown in Fig. 5. The remaining cortical thymocytes showed nuclear fragmentation, shrinkage and increased staining (nuclear pyknosis). The medulla and thymic blood vessels remained unchanged. Treatment with all three compounds at the MTD caused selective necrosis of cortical thymic lymphocytes, leaving the remaining thymic structures unaffected.

Effect on bone-marrow and haemopoietic progenitor cells

Femoral bone-marrow cell counts decreased to approximately half those found in untreated controls at 24 h after treatment but had returned to control levels by day 4 (Fig. 6A). The number of CFU-c haemopoietic progenitor cells in the femur increased after treatment (Fig. 6B); by day 3, the quantity of CFU-c had risen to approximately 3-fold that noted in untreated controls.

We also examined the in vitro effects of these drugs on CFU-c and compared the findings with observations in vivo. Bone-marrow cells were removed from healthy mice, incubated with varying concentrations of drug for 2 h, washed and then plated for determination of the growth of CFU-c colonies. The number of colonies detected after 5 days were expressed as a percentage of the number grown from untreated bone marrow. The results (Fig. 7) show that a 2-h exposure to high concentrations of these compounds resulted in a decrease in the surviving fraction of CFU-c, in contrast with the elevation in CFU-c numbers observed after in vivo treatment.

Discussion

The immunotherapeutic and immunomodulating effects of FAA have generally been attributed to its ability to induce cytokine synthesis, and levels of tumour necrosis factor (TNF) and interferons are elevated after FAA treatment [11, 14]. Increased natural killer cell (NK) activity in FAA-treated mice [3, 10] is a consequence of interferon induction [11], whereas TNF has been shown to be responsible for FAA-induced inhibition of tumour blood flow [15], an important component in the antitumour activity of the drug [30]. Using a series of XAA derivatives, we have found correlations between the ability of individual analogues to elevate NK activity [6], to induce tumouricidal macro-

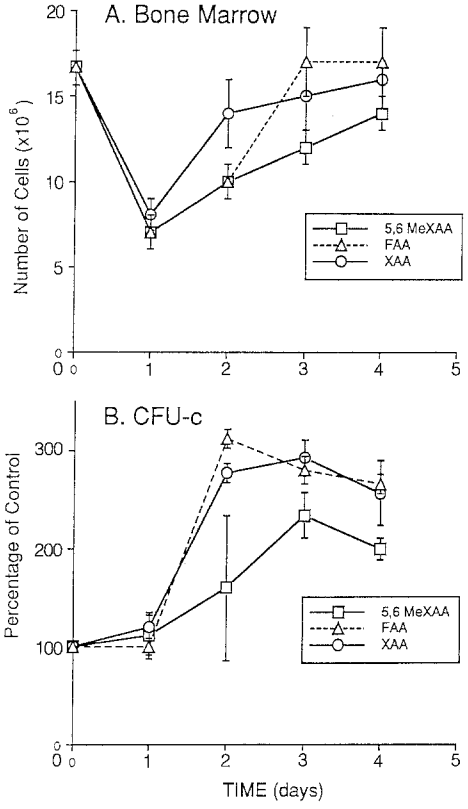

Fig. 6. Numbers of cells and CFU-c in both femurs in mice following i. v. administration of XAA, 5,6-MeXAA and FAA at the MTD. Data represent mean values ± SE for 5 mice/group

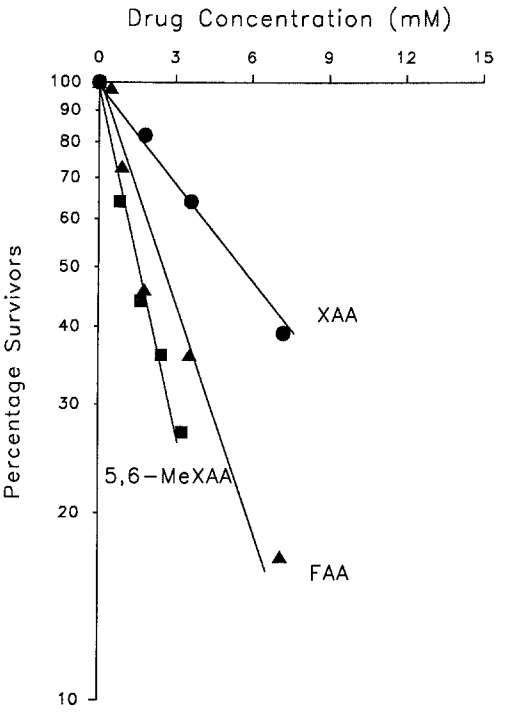

Fig. 7. Survival of CFU-c after in vitro exposure to FAA, XAA and 5,6-MeXAA. Bone-marrow cells were incubated for 2 h with drug at varying concentrations and were then plated for determination of the growth of CFU-c colonies. Points represent means of quadruplicate cultures

phage activity in vitro [5] and to induce tumour necrosis, indicating that these activities are linked.

All of the in vivo haematological effects described in this report can be explained by the production of cytokines. The profound lymphocytopaenia, neutrophil cytosis and slight reduction in platelet counts that were noted within 24 h of XAA treatment in mice are similar to the disturbances observed in clinical trials of TNF [2, 24]. The main effect was a selective reduction in the number of circulating (Fig. 2B) and thymic lymphocytes (Fig. 4A). Histological examination of thymus glands from treated mice revealed a severe depletion of cortical thymocytes but no discernible effects on the thymic epithelium (Fig. 5), results that are consistent with the findings of Zwi et al. [31], who described necrosis in the thymus and in follicles of peripheral lymphoid tissues, indicating that both the T- and B-lymphocytes were susceptible. Interleukin-1 (IL-1) has been shown to cause lymphocytopaenia by inducing increases in the levels of corticosteroids, which in turn induce thymic hypoplasia and lympholysis [18]. The histological appearance of thymic lobes from XAA-treated mice (Fig. 5) was very similar to that previously observed in mice that had undergone treatment with IL-1 [18]. IL-1 production could explain the thymic hypoplasia and dramatic reduction in peripheral blood lymphocytes observed after drug treatment, although IL-1 production has not yet been detected in FAA-treated mice [14]. Thus, it is possible that all of the in vivo effects described for these compounds are indirectly mediated through the release of cytokines or other products by host cells in response to the drugs.

When bone marrow cells were exposed to the drugs in vitro at drug concentrations in the millimolar range, the number of surviving CFU-c decreased (Fig. 7), indicating that the drugs themselves do not directly stimulate CFU-c production. The maximal plasma concentrations of XAA and FAA range from 1- to 2 mM, whereas that of 5,6-MeXAA is 0.6 mM [17]. These might be expected to cause modest reductions in the number of surviving CFU-c, but CFU-c numbers actually increased 2–3 times following in vivo drug administration (Fig. 6B), although there was an overall decrease in the number of cells in the femur (Fig. 6A). The increase in CFU-c numbers in vivo cannot be entirely explained by the host's normal response to hypoplasia, since increases in the numbers of CFU-c have not been observed following hypoplasia induced by radiation or other types of cytotoxic drugs (unpublished observations). A possible explanation of this phenomenon might be that haemopoietic growth factors are amongst the cytokines induced by FAA, XAA or 5,6-MeXAA.

Despite a 10-fold difference in potency for 5,6-MeXAA over FAA or XAA, the haematological effects of all three drugs at their respective MTDs are quite similar and are unlikely to be the cause of the acute lethality induced by these drugs, particularly since an analogue that exhibits no antitumour activity, 8-methyl XAA, can induce lethality but does not cause haematological changes in the peripheral blood at its MTD (520 μmol/kg i.v.; unpublished observations). The only abnormality observed in the blood bio-

chemistry was an elevation in levels of serum aspartate aminotransferase. This finding is often regarded as an indicator of liver damage, although this enzyme can be released from other tissues in the course of many pathological diseases [7].

The results of the present study are consistent with the reports that myelosuppression was not observed in clinical trials of FAA [12, 13] and contrast with the haematological toxicities observed using conventional anticancer agents that cause reductions in white blood cell counts that are not detectable until 1–2 weeks after treatment. Recovery from directly cytotoxic agents is delayed until 3–8 weeks after treatment [26]. Hypotension is the major toxicity that has been reported in clinical trials of FAA [12, 13], and we have shown that FAA, XAA and 5,6-MeXAA stimulate the production of nitric oxide [27], a vasodilator [16]. Experiments investigating the effects of nitric oxide and hypotension on the toxicity of this class of compounds are in progress.

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