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Nitric oxide: its production in host-cell-infiltrated EMT6 spheroids and its role in tumour cell killing by flavone-8-acetic acid and 5,6-dimethylxanthenone-4-acetic acid

Lindy L. Thomsen[1], Bruce C. Baguley[1], W. R. Wilson[2]

Cancer Research Laboratory[1] and Department of Pathology[2], University of Auckland School of Medicine, Auckland, New Zealand

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Summary. Flavone-8-acetic acid (FAA) and its more dose-potent analogue 5,6-dimethylxanthenone-4-acetic acid (5,6-MeXAA), appear to exert their antitumour effects through vascular and other host-mediated mechanisms and are known to induce the synthesis of nitric oxide by murine macrophages. We investigated the role of nitric oxide in the cytotoxic effects of these drugs in host-cell-infiltrated spheroids. EMT6 murine mammary adenocarcinoma cells were grown in culture to produce multicellular spheroids in vitro spheroids), which were then inoculated i. p. into mice. After 6 days the spheroids were removed ex vivo spheroids). Exposure to FAA (890 μM) and 5,6-MeXAA (80 μM) in vitro for 20 h increased nitrite concentrations to 6.7 and 9.7 nmol/spheroid, respectively, as compared with 0.7 nmol/spheroid in the absence of drug. FAA and 5,6-MeXAA did not increase nitrite production in in vitro spheroids in cells obtained by peritoneal lavage. However, mixed cultures of in vitro spheroids and peritoneal cells treated with 5,6-MeXAA produced nitrite (2.4 nmol/spheroid), indicating that interactions between host cells and tumour cells were important for induction. The effects of these drugs on ex vivo spheroids were prevented by co-incubation with N^G-monomethyl-L-arginine, indicating that nitrite originated from the oxidation of L-arginine to nitric oxide. Cell sorting of disaggregated spheroids into EMT6 cells and *Mac-1*-positive macrophage populations indicated that both of these cell populations could be induced to synthesise nitric oxide by subsequent incubation with 5,6-MeXAA. Incubation of ex vivo spheroids with FAA and 5,6-MeXAA decreased the clonogenicity of EMT6 cells, and this effect was wholly (FAA) or partially (5,6-MeXAA) reversed by the presence of N^G-monomethylarginine (250 μM). FAA and 5,6-MeXAA may therefore exert some of their cytotoxic effects on tumour cells through the production of nitric oxide.

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Correspondence to: B. C. Baguley, Cancer Research Laboratory, University of Auckland School of Medicine, Auckland, New Zealand

Introduction

Flavone-8-acetic acid (FAA; Fig. 1) is a synthetic flavonoid that displays marked antitumour activity against experimental solid tumours [17] but exerts no clinical activity as single agent [10]. 5,6-Dimethylxanthenone-4-acetic acid (5,6-MeXAA; Fig. 1) is structurally related to FAA and exhibits similar experimental antitumour activity [19]. The superior potency found for 5,6-MeXAA as compared with FAA and other xanthenone acetic acid (XAA) derivatives synthesised and investigated in this laboratory has led to its selection as a candidate compound for clinical trial.

The mechanism of action of FAA and 5,6-MeXAA in vivo is not fully understood but appears to involve a number of host-mediated killing mechanisms. Tumour necrosis factor-α is produced in response to FAA [14] and XAA analogues [6] and has been implicated in vascular effects [15], which probably involve responses of endothelial cells [16] and lead to cell killing by reducing the tumour blood flow and inducing tumour ischaemia [2, 5, 23]. Although the tumour vasculature is believed to be an important target in the mechanism of action of FAA, perfusion failure following treatment of solid tumours with FAA may not explain all of the cell killing associated with this treatment [23], and other cytotoxic mechanisms may act in concert with vascular effects.

The multicellular spheroid system, as developed and described by Sutherland [20], is a useful in vitro model for the study of the effects of drugs on solid tumours. The technique of i. p. introduction of EMT6 spheroids into mice allows infiltration of host immune effector cells [13]. This model facilitates the investigation of host immune-cell-me-

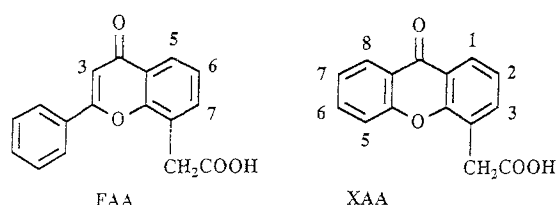

Fig. 1. Structures of FAA and XAA, showing the numbering system

diated cell killing in a tumour-like environment without the problems of systemic host toxicity and of tumour cell death resulting from vascular collapse. Zwi et al. [23, 24] have shown that treatment of mice with FAA (0.8 mmol/kg given either i.p. or i.v.) results in significant killing (approx. 70%) of tumour cells in non-vascularised host-cell-infiltrated EMT6 spheroids (ex vivo spheroids). Ex vivo spheroids removed from mice are sensitive to FAA cytotoxicity in vitro (Zwi et al., manuscript submitted) and therefore provide a useful model for the investigation of the action of these agents.

Nitric oxide, an important mediator of L-arginine-dependent tumour cell killing by activated murine macrophages [8, 9], is produced in response to FAA and XAA derivatives both in vitro by activated macrophages [21] and in vivo in normal and tumour-bearing mice [22]. Moreover, the correlation between antitumour activity and elevation of plasma nitrate concentrations in mice found for FAA and XAA analogues [22] provides circumstantial evidence that nitric oxide contributes to the action of these compounds. However, the question of whether nitric oxide might be produced in cytotoxic concentrations in tumours has not been resolved. In the experiments reported herein we used the ex vivo spheroid system to investigate whether cytotoxic concentrations of nitric oxide might be produced in response to FAA and XAA derivatives.

Materials and methods

Materials. FAA (NSC 347512) was obtained from the National Cancer Institute (Bethesda, Md., USA). 5,6-MeXAA (NSC 649488) and 8-MeXAA (Fig. 1) were synthesised in this laboratory by Drs. W. A. Denny, G. J. Atwell and G. W. Rewcastle and were judged to be pure by thin-layer chromatography. Immediately prior to their use, drug solutions were dissolved in a minimal amount of 5% (w/v) sodium bicarbonate and were diluted to the final concentration ($\leq 0.001\%$) in supplemented culture medium. N^G-Monomethylarginine (N^GMMA; Sigma was dissolved in supplemented culture medium. α-Modified minimal essential culture medium (Gibco, Grand Island, N. Y.) was supplemented with 10% foetal calf serum, penicillin (100 units/ml) and streptomycin sulphate (100 μg/ml). An additional supplement of 2-mercaptoethanol (50 μM) was included for assays of nitric oxide production [21].

Preparation of in vitro spheroids. The EMT6/Ak murine mammary adenocarcinoma cell line [23] was used to initiate EMT6 spheroid growth by culturing 5×10^5 cells in 100 mm non-tissue-culture petri dishes in supplemented culture medium at 37° C in an atmosphere of 95% air/5% CO_2. After 4–5 days the resultant small spheroids were transferred to spinner flasks containing 250 ml supplemented culture medium, which was replenished as required to maintain the pH above 7.0. The density of spheroids was progressively reduced to give approximately 1 spheroid/ml after 2 weeks. These spheroids, each of which measured 500–800 μm in diameter, were used for all experiments.

Preparation of ex vivo spheroids. BALB/c mice were bred under constant temperature and humidity and were provided with sterile bedding, water and food according to institutional ethical guidelines. In vitro spheroids (20/mouse) were implanted into the peritoneum of anaesthetised mice through a 16.5-gauge needle [23]. After 6 days, mice were killed by cervical dislocation and spheroids were recovered under sterile conditions and washed twice with culture medium.

Disaggregation of spheroids. Spheroids were disaggregated by incubation at 37° C for 15–20 min in an enzyme cocktail of 0.5 mg Pronase/ml (Calbiochem) and 0.2 mg DNAase/ml (Sigma) in supplemented culture medium, and the cells were then washed twice in culture medium by centrifugation.

Separation of host cells and tumour cells from ex vivo spheroids. After disaggregation of ex vivo spheroids, host cells and tumour cells were separated in a FACS 440 cell sorter (Becton Dickinson, Mountain View, Calif.) according to forward and side scatter (related to size and granularity, respectively). Collection parameters were chosen to ensure that discrete populations of tumour and host cells were obtained. Cell separation was verified by fluorescent microscopy after labeling with anti-*Mac 1* antibody (M1/70.15.1; Serotec, Oxford, England) or anti-*Thy 1* antibody (T24 ascites, kindly donated by Prof. J. D. Watson, University of Auckland, New Zealand) followed by staining with fluorescein isothiocyanate (FITC)-labeled anti-IgG second antibody.

Assay for nitric oxide production. Intact in vitro or ex vivo spheroids (one/well) were placed into 96-well plates with 250 μl supplemented culture medium containing FAA, 5,6-MeXAA or 8-MeXAA in the presence or absence of N^GMMA and were incubated for 20 h at 37° C in an atmosphere of 95% air/5% CO_2. Controls were cultured in medium without drugs. Nitric oxide production by macrophages in the absence of other oxidation or reduction results in the formation of equal quantities of nitrite and nitrate [21]. Nitrite concentrations were measured in culture medium supernatants using a microplate assay method [21] based on the Griess reaction [7].

Peritoneal cells (10^5/well) obtained from unprimed BALB/C mice as previously described [21] were also added to wells containing an intact in vitro spheroid or to wells containing no spheroid and were incubated in 96-well plates with 5,6-MeXAA. Nitrite in the medium was measured after culturing for 20 h. Cells from disaggregated ex vivo spheroids (10^5 cells/well) and host cells (4×10^4 cells/well) or tumour cells (8×10^4 cells/well) separated from disaggregated ex vivo spheroids were incubated with 200 μl culture medium in the presence or absence of 5,6-MeXAA (80 μM) and nitric oxide production was assayed as described above.

Clonogenicity assays. After 20 h culture in 96-well plates in the presence or absence of drugs, spheroids were disaggregated and washed as described above. Cells were seeded in 60-mm dishes (10^2 cells/5 ml culture medium) and cultured for 7 days. Colonies were stained with 0.5% methylene blue in 50% ethanol, and those comprising more than 50 cells were counted.

Statistical analysis. The results are expressed as mean values ± SEM. Differences between groups were analysed using Student's *t*-test.

Results

Nitric oxide production by EMT6 spheroids

EMT6 spheroids that had been grown for 6 days in the peritoneal cavity of BALB/c mice (ex vivo spheroids), removed and cultured for 20 h produced nitrite concentrations ranging for individual spheroids from 0.1 to 0.8 nmol/spheroid. Nitrite production was significantly enhanced ($P < 0.05$) when ex vivo spheroids were treated with FAA or 5,6-MeXAA (Fig. 2; Table 1). Optimal concentra-

Table 1. A representative experiment comparing cell killing with nitrite production by ex vivo EMT6 spheroids treated with 890 μM FAA or 80 μM 5,6-MeXAA in the presence or absence of 250 μM N^GMMA

Treatment	Plating efficiency[a] (colonies/100 cells)	Nitrite[b] (nmol spheroid)
FAA	15±0*	6.7±0.5
FAA + N^GMMA	42±1**	<0.1
5,6-MeXAA	12±0*,**	9.7±0.4
5,6-MeXAA + N^GMMA	24±2*,***	0.3±0
N^GMMA	40±2	<0.1
Control	40±4	0.7±0.2

[a] Colonies (mean ± SEM of duplicates) formed by cells obtained from disaggregated ex vivo spheroids (9/group) after they had been cultured for 20 h with the indicated treatments
[b] In vitro nitrite production by the ex vivo spheroids (mean ± SEM of 9 spheroids/group) as indicated above
[c] Ex vivo spheroids cultured without drugs
*,**,*** Significantly different (P <0.05) as compared with control, FAA, and 5,6-MeXAA values, respectively

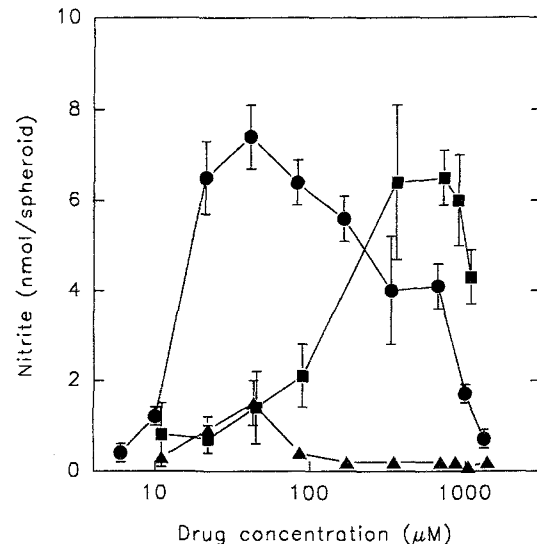

Fig. 2. Representative experiment showing the stimulation of nitrite production by ex vivo spheroids cultured in the presence of FAA (■), 5,6-MeXAA (●) or 8-MeXAA (▲). *Points*, mean values ± SEM for triplicates. The control value (no drug) was 0.5±0.2 nmol/spheroid

tions for stimulation of nitrite production were 20–80 μM for 5,6-MeXAA and 360–890 μM for FAA. 8-Methyl XAA, an inactive analogue (8-MeXAA; Fig. 1) [18] had no significant effect (Fig. 2).

Nitrite production was below the level of detection (<0.1 nmol/spheroid) in EMT6 spheroids grown only in vitro (in vitro spheroids) and cultured for 20 h in medium alone or in the presence of 5,6-MeXAA (Fig. 3), FAA or 8-MeXAA (concentration range, 10–1400 μM). In contrast, nitrite production was observed in in vitro EMT6 spheroids cultured for 20 h with 10^5 peritoneal cells and treated with 5,6-MeXAA (Fig. 3). The optimal concentration of 5,6-MeXAA (40 μM) was within the range obtained for ex vivo spheroids (Fig. 2), although the maximal nitrite production was lower (2.4±0.2 nmol/spheroid). Peritoneal cells alone did not produce nitrite when treated with 5,6-MeXAA.

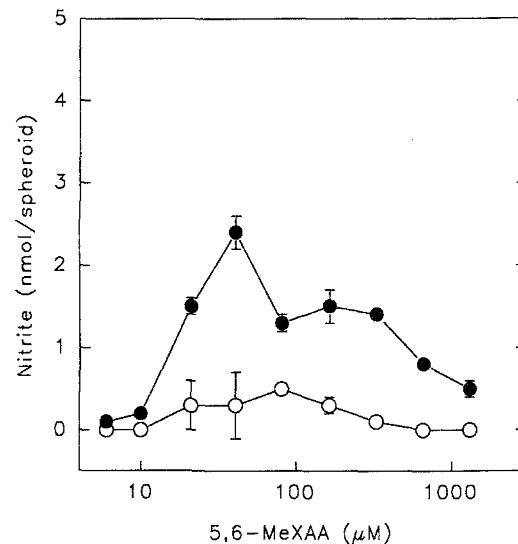

Fig. 3. Representative experiment showing nitrite production by in vitro spheroids cultured in the presence (●) or absence (○) of peritoneal cells and treated with 5,6-MeXAA. *Points*, mean values ± SEM for duplicates

Nitric oxide production by host cells and tumour cells separated from ex vivo spheroids

Separation of cells from enzymatically disaggregated ex vivo spheroids by flow cytometric cell sorting provided host cell (approx. 50% of total cells) and tumour cell populations. In the host fraction collected, 80% of the cells were found to measure >15 μm in diameter and >99% of these were labeled with anti-*Mac 1* antibody. Of the remaining 20% that measured <15 μm in diameter, 75% were labeled with anti-*Thy 1* antibodies. All cells in the tumour cell population had a diameter of >20 μM, and no *Mac 1*- or *Thy 1*-positive cell was detected.

The host cell and tumour cell populations as well as the mixed cell population from unsorted disaggregated spheroids produced detectable nitrite when cultured for 20 h in the absence of drugs (Table 2). Nitrite production by each population was significantly increased (P <0.05) in the presence of 5,6-MeXAA (80 μM). Diminished production of nitric oxide was noted in cells that had been recovered by cell sorting of the disaggregated spheroids as compared with freshly disaggregated spheroids (P <0.05; Table 2). This may have resulted from the time (4 h) required for the separation and collection of tumour cells and host cells in numbers sifficient for the performance of these experiments.

Killing of EMT6 tumour cells by FAA and 5,6-MeXAA

For assessment of the cytotoxic activity of the antitumour agents, clonogenic tumour cells were assayed after culture of ex vivo and in vitro spheroids with FAA (890 μM) or 5,6-MeXAA (80 μM) for 20 h. For ex vivo spheroids, a

Table 2. In vitro nitrite production by unfractionated cells from disaggregated ex vivo EMT6 spheroids and by fractionated host cells and tumour cells from disaggregated ex vivo EMT6 spheroids

Treatment	Nitrite (nmol/10^6 cells)[a]		
	Unfractionated cell population	Host cells[c]	Tumour cells[c]
Control[d]	3.9 ± 0.3	0.3 ± 0.2	3.2 ± 0.1
5,6-MeXAA[e]	14.5 ± 0.5*	3.3 ± 0*,**	7.7 ± 0*,**

[a] In vitro nitrite production (mean of duplicates ± SEM) by cells treated with the indicated drugs
[b] Mixed (unsorted) population of cells (i. e. host and tumour cells) obtained following enzymatic disaggregation of ex vivo spheroids
[c] Host cells and tumour cell collected from enzymatically disaggregated ex vivo spheroids by flow cytometric cell sorting
[d] Cultured without drug
[e] The concentration of 5,6-MeXAA was 80 µM
* Significantly different (P <0.05) as compared with the control
** Significantly different (P <0.05) as compared with the unfractionated cell population

significant decrease in plating efficiency (clonogenic cells/100 cells plated) was observed after culture with FAA or 5,6-MeXAA as compared with controls (P <0.05). Furthermore, the effect of 5,6-MeXAA was significantly greater than that of FAA (P <0.05). The number of clonogenic cells (37 ± 2/100 cells) in in vitro spheroids did not decrease after treatment with 890 µM FAA (40 ± 2/100 cells) or 80 µM 5,6-MeXAA (41 ± 2/100 cells).

Relationship between nitric oxide production and tumour cell killing by FAA and 5,6-MeXAA

The addition of N^G^MMA (250 µM) to cultures inhibited nitrite production and significantly reversed (P <0.05) the decrease in plating efficiency observed for ex vivo spheroids treated with 5,6-MeXAA or FAA (Table 1). The plating efficiency found for 5,6-MeXAA plus N^G^MMA remained substantially lower than control values (P <0.05), whereas that observed for FAA plus N^G^MAA and for N^G^MMA alone did not significantly differ from the control values (Table 1).

Discussion

The present study shows that the antitumour agents FAA and 5,6-MeXAA are capable of stimulating nitric oxide production by ex vivo EMT6 spheroids that have been infiltrated with host cells during growth in the peritoneal cavities of mice. Mixed cultures of in vitro spheroids and peritoneal cells are also stimulated to produce nitric oxide, albeit at a lower level than ex vivo spheroids. Neither peritoneal cells alone nor in vitro spheroids alone produce nitric oxide in response to these agents. The induction of nitric oxide production in host-cell-infiltrated spheroids has not previously been demonstrated, although both EMT6 tumour cells and peritoneal macrophages are known to produce nitric oxide following incubation with interferon-γ in combination with endotoxin or tumour necrosis factor-α [1, 4].

Experiments performed to ascertain the cellular source of nitric oxide in ex vivo spheroids indicate that both EMT6 tumour cell and host cell populations are responsible (Table 2). One possible explanation for this result is that both EMT6 cells and the infiltrating macrophages are activated by exposure to interferon-γ and/or other products during the 6 days of growth of the spheroids in vivo and that such activated cells can then be stimulated to produce nitric oxide in response to FAA and 5,6-MeXAA. Furthermore, nitric oxide production by mixed cultures of in vitro spheroids and peritoneal cells indicates that factors required for the induction of detectable nitric oxide production are also produced in response to interactions between host cells and tumour cells in culture. The production of nitric oxide by EMT6 cells in response to low molecular-weight compounds has not previously been demonstrated.

Clonogenic assays indicate that at concentrations providing maximal stimulation of nitric oxide production, both FAA and 5,6-MeXAA are cytotoxic to ex vivo EMT6 spheroids but are not cytotoxic to in vitro spheroids. This suggests that either a direct interaction between peritoneal cells and tumour cells or an indirect interaction via the production of diffusible factors produced by these cells induces cytotoxicity. N^G^MMA, a specific inhibitor of nitric oxide production, fully reverses the cytotoxicity induced by FAA and partially reverses that induced by 5,6-MeXAA, implicating nitric oxide as a principal cytotoxic factor in this system. The mechanism of the cytotoxic action of nitric oxide was not investigated in these studies, but it probably involves complexation to non-haem iron-containing proteins and consequent inactivation of electron-transport proteins essential for cellular respiration [3].

The induction of a cytotoxic pathway involving nitric oxide in ex vivo spheroids suggests that this pathway may also be induced in experimental solid tumours. Endothelial cells, absent from avascular ex vivo spheroids but abundant in vascularised tumour tissue, can also be induced to produced nitric oxide [11]. It is not yet known whether nitric oxide production by endothelial cells can be stimulated by FAA and its analogues, but such a process could also contribute to the antitumour effect of these agents [12]. Our results suggest that stimulation of nitric oxide production in tumour-infiltrating host cells (probably macrophages) provides an additional cytotoxic mechanism operating in concert with the vascular effects induced by FAA and 5,6-MeXAA.

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