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Stimulation of macrophage tumouricidal activity by 5,6-dimethyl-xanthenone-4-acetic acid, a potent analogue of the antitumour agent flavone-8-acetic acid

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Abstract—The new antitumour drug 5,6-dimethylxanthenone-4-acetic acid (5,6-MeXAA; NSC 640488) was 14-fold more potent than the investigational chemotherapeutic drug flavone-8-acetic acid (NSC 347512) in stimulating tumouricidal activity in cultures of resident murine peritoneal macrophages. The tumouricidal activity of thioglycollate-elicited and *Bacillus Calmette-Guérin*-primed macrophages was also significantly enhanced by 5,6-MeXAA. Stimulation of macrophage tumouricidal activity by 5,6-MeXAA was not affected by inhibitors of superoxide and nitric oxide production, but was reduced by cyclosporin A, an inhibitor of protein secretion. Inhibitors of neutral proteases had no effect. Cortisone, dexamethasone, indomethacin, dibutyryl cAMP, prostaglandin E_2 and prostacyclin, but not prostaglandin $F_{2\alpha}$, inhibited stimulation, suggesting the involvement of tumour necrosis factor-α (TNF). However, antibodies to TNF did not inhibit stimulation. The results suggest that 5,6-MeXAA acts on macrophages in a manner similar to that of endotoxin, utilizing a pathway which includes arachidonic acid metabolism and requiring cell–cell contact with target cells for a tumouricidal effect.

The new investigational antitumour drug 5,6-dimethyl-xanthenone-4-acetic acid (5,6-MeXAA*) (NSC 640488) was developed in this laboratory [1] as a potent analogue of FAA (NSC 347512), a drug with impressive activity against murine solid tumours but with no significant activity in clinical trials [2]. A major action of FAA on tumours involves the induction of tumour ischaemia and necrosis through the reduction of tumour blood flow [3, 4], probably as a result of the release of TNF [5]. FAA has the unusual property of being a low molecular mass inducer of the *in vitro* cytotoxicity of adherent resident PC in mice [6]. Although the contribution of this property to the *in vivo* action of FAA is unknown, studies with a series of analogues of FAA have demonstrated it to be highly predictive of *in vivo* antitumour activity [7]. We show here that 5,6-MeXAA is 14-fold more potent than FAA in stimulating tumouricidal activity of resident murine PC, and have investigated the action of a number of potential inhibitors of macrophage cytotoxicity.

Materials and Methods

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, and used between 8–12 weeks of age. Mice were killed by cervical dislocation and PC were collected by washing the peritoneum with 5 mL α-minimum essential medium (Gibco, Grand Island, NY, U.S.A.) supplemented with foetal calf serum (10%, Gibco NZ Ltd), penicillin (100 U/mL) and streptomycin sulphate (100 μg/mL). Resident PC were collected from untreated animals. Elicited PC were collected from mice injected i.p. 4–6 days previously with 0.2 mL/mouse of thioglycollate broth (Becton-Dickinson, Cockeysville, U.S.A.), prepared as a 10% w/v sterile solution. BCG-primed PC were obtained from mice injected i.p. 14 days previously with 10^8 lyophilized BCG (Institute Armand-Frappier, Canada) organisms/mouse, prepared as a suspension (4.2×10^8 organisms/mL) in water. LLTC cells [8] were obtained from Dr R. C. Jackson (Warner-Lambert Co., Ann Arbor, MI, U.S.A.).

The cytotoxicity of resident PC was determined by a standard procedure of measurement of release of chromate from prelabelled target cells [9]. PC were plated at different concentrations in v-bottomed 96-well plates (Linbro, Flow Labs, McLean, VA, U.S.A.) and allowed to adhere for 2 hr at 37°. Non-adherent cells were then discarded and the adherent cells, predominantly macrophages by morphology, were incubated with ^{51}Cr-labelled LLTC carcinoma cells (5×10^3 cells/well in a total volume of 0.2 mL culture medium) at various E:T ratios (based on the number of PC added to the plates) in the presence of FAA (National Cancer Institute, Bethesda, MA, U.S.A.) or 5,6-MeXAA (synthesized in this laboratory by Drs W. A. Denny, G. J. Atwell and G. W. Rewcastle). Drug solutions were prepared fresh for each experiment by dissolving in a minimal amount of 5% w/v sodium bicarbonate and diluting to required concentrations in culture medium and protected from light to prevent decarboxylation [10]. LLTC cells were labelled by incubation for 45 min at 37° with 200 μCi sodium [^{51}Cr]-chromate in saline and washing three times. Lysis of tumour targets was measured after 18 hr incubation at 37° by removing 0.1 mL supernatant and measuring radioactivity in a gamma counter (LKB Wallac 1270 Rackgamma II, Wallac, Finland). Percentage lysis was calculated as [(experimental release − spontaneous release)/total] × 100. All groups were in quadruplicate.

Results and Discussion

Three differentiation states of macrophages (resident, elicited and activated) are generally recognised [11] and 5,6-MeXAA enhanced the tumouricidal activity in culture of all three types (Fig. 1). 5,6-MeXAA enhanced the tumouricidal activity in culture of all three types (Fig. 1). 5,6-MeXAA-stimulated activity in resident PC (70% lysis at 200:1, E:T) and in elicited PC (100% lysis at 100:1, E:T). Spontaneous tumouricidal activity in the absence of drug was indetectable at these E:T ratios but was detectable at 250:1 in resident PC and at 200:1 in thioglycollate-elicited PC. 5,6-MeXAA stimulated activity in BCG-primed PC at an E:T ratio as low as 25:1, but some spontaneous lysis was also observed at this ratio. The spontaneous cytolytic activity obtained in cultures of BCG-primed PC in the absence of drug (Fig. 1) is consistent with the literature [11]. The spontaneous activity observed at high E:T ratios in resident and thioglycollate-elicited

* Abbreviations: FAA, flavone acetic acid; 5,6-MeXAA, 5,6-dimethyl-xanthenone-4-acetic acid; TNF, tumour necrosis factor-α; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; NDGA, nordihydroguairetic acid; AA861, 2-(12-hydroxy-5,10-dodecadiynyl)-3,5,6-trimethyl-2,5-cyclohexadiene-1,4-dione; BCG, *Bacillus Calmette-Guérin*; PC, peritoneal cells; LLTC, Lewis lung carcinoma tissue culture line; E:T, effector to target cell.

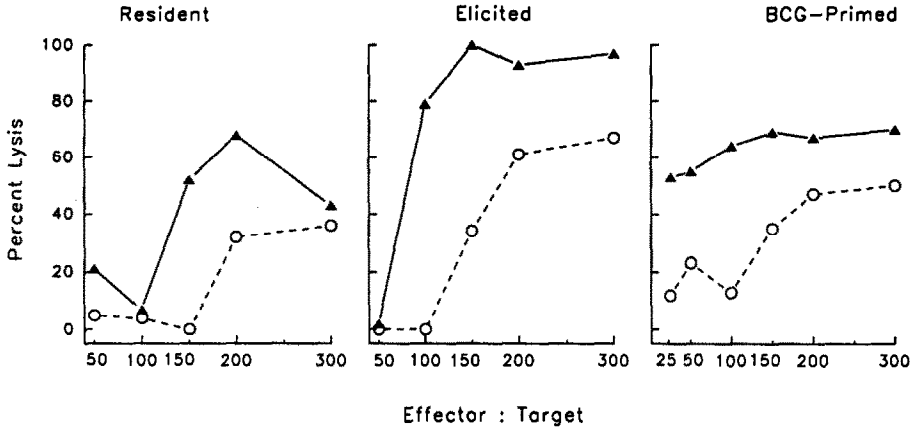

Fig. 1. Enhancement of PC tumouricidal activity by 5,6-MeXAA. PC from untreated (resident), thioglycollate-treated (elicited) or BCG-primed (activated) mice were plated at different E:T cell ratios with (▲) or without (○) 5,6-MeXAA (115 μM). Cytolysis was measured after 18 hr against LLTC targets. Each point represents mean ± SEM of quadruplicate cultures.

PC could be due to a small subpopulation of macrophages which had been activated *in vivo* by environmental stimulants or to stimulation by the tumour targets themselves during the 18-hr incubation. The concentrations of 5,6-MeXAA and FAA required to stimulate 50% lysis of target cells in resident PC cultures (E:T, 200:1) were 50 and 700 μM, respectively, providing a 14-fold increase in potency for the analogue (Fig. 2).

In order to determine which of the variety of known macrophage cytotoxicity mechanisms were stimulated by 5,6-MeXAA (115 μM) appropriate inhibitors were added to cultures of resident (200:1, E:T), elicited (100:1, E:T) or BCG-primed PC (75:1, E:T). Inhibitors of pathways involving activated oxygen (superoxide dismutase, 0.50–150 U/culture; catalase, 12.5–150 U/mL) or nitrogen intermediates (N^G-monomethylarginine, 62.5–500 μM; Calbiochem) were inactive. The lack of effect of inhibitors of the nitric oxide pathway [12] is consistent with the lack of production of nitric oxide by resident or elicited PC [13].

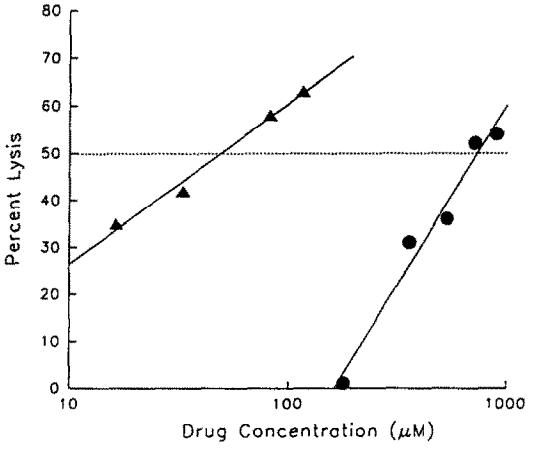

Fig. 2. Relative potencies of 5,6-MeXAA (△) and FAA (●) in stimulating resident PC (200:1, E:T) to kill LLTC targets. Lysis was measured after 18 hr (mean ± SEM of quadruplicate culture). Lysis was not detected in control cultures of LLTC targets incubated without PC in the presence of the drug at all concentrations used.

Cyclosporin A, an inhibitor of secretion [14], inhibited cytotoxicity by 36% at 50 μM and 73% at 200 μM, suggesting that protein secretion was involved in macrophage activity. Since activated macrophages can mediate cytolysis by secreting neutral proteases [15], a number of inhibitors of neutral proteases (Sigma Chemical Co., St Louis, MO, U.S.A.) were tested. Aprotinin (0.0125–0.2 Trypsin Inhibitor units/mL), chymostatin (0.625–10 μg/mL), pepstatin (10^{-8}–10^{-4} M, phenylmethylsulphonyl fluoride (10^{-7}–10^{-3} M), leupeptin (10^{-5}–10^{-3} M), antipain (10^{-5}–10^{-3} M) and soybean trypsin inhibitor (0.24–2 mg/mL) all showed no inhibition. Since TNF has both cytostatic and cytolytic activity against a range of neoplastic cells [16], and because both FAA [17] and 5,6-MeXAA (Dr R. H. Wiltrout, personal communication) induce TNF *in vivo*, polyclonal antibodies to murine TNF (10^2–10^3 neutralizing units/mL; kindly provided by Dr G. R. Adolf, Bender and Co., GmbH, Vienna, Austria, and also purchased from Endogen, Boston, MA, U.S.A.) were tested. No effect on toxicity was observed, consistent with a previous observation that FAA-stimulated PC lyse TNF-insensitive P815 tumour cells [6].

Despite the evidence against the involvement of TNF, studies with a number of inhibitors suggested that regulation of the pathway for release of the cytotoxic species was similar to that for stimulation of TNF secretion. Cortisone (2.5×10^{-3} M) completely inhibited tumouricidal activity of resident or BCG-primed PC induced by 5,6-MeXAA, while dexamethasone (10^{-5}–10^{-8} M) reduced 5,6-MeXAA-induced activity by 80%, consistent with the strong inhibition of TNF synthesis by corticosteroids [16]. Indomethacin, a cyclo-oxygenase inhibitor, inhibited cytotoxicity by 50% at 10^{-6} M (Fig. 3), consistent with its effect on TNF synthesis [18]. Inhibition was also found following addition of either PGE_2 or the stable synthetic prostacyclin, Iloprost (kindly provided by Schering (NZ) Ltd), whereas $PGF_{2\alpha}$ was not inhibitory (Fig. 4). Moreover, dibutyryl-cAMP inhibited 5,6-MeXAA-induced tumouricidal activity by 43% at 100 μM. AA861, a strong lipoxygenase inhibitor [19], had no effect while NDGA, which can inhibit both cyclo-oxygenase and lipoxygenase activity [20], inhibited by 50% at 10^{-5} M and had little effect at lower concentrations (Fig. 3). These results compare with the partial inhibition of TNF activity obtained with both PGE_2 and dibutyryl-cAMP, which act on a

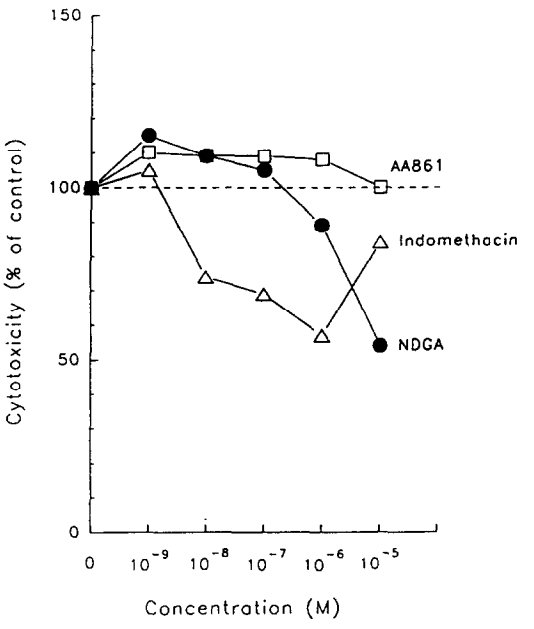

Fig. 3. Effect of inhibitors of arachidonate metabolism on tumouricidal activity. Indomethacin (△), AA861 (□) or NDGA (●) was added to cultures of thioglycollate-elicited PC (125:1 E:T) in the presence of 5,6-MeXAA (115 μM) and lysis of LLTC targets measured after 18 hr. The means of triplicate cultures were expressed as per cent of cytotoxicity measured in control cultures.

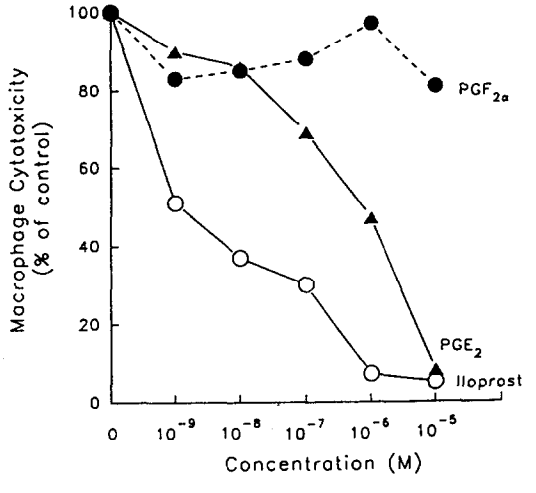

Fig. 4. Effect of prostaglandins on tumouricidal activity. PGE_2 (▲), iloprost (○) or $PGF_{2\alpha}$ (●) was added to cultures of resident PC (200:1, E:T) with 5,6-MeXAA (115 μM) and lysis of LLTC targets measured after 18 hr. The means of triplicate cultures were expressed as per cent of cytotoxicity measured in control cultures.

negative feed-back loop, but not with $PGF_{2\alpha}$, which is inactive in this loop [16, 18].

A possible explanation for the results is that 5,6-MeXAA induces the expression of an endosome-localized cytotoxin which is released on to the tumour cell surface only during cell–cell contact [21]. Alternatively, 5,6-MeXAA may induce release of TNF only during cell–cell contact [22, 23]. This model is consistent with the failure of previous experiments to isolate an active cytolytic agent in culture supernatants of resident PC incubated with FAA and tumour cells [6], or to demonstrate killing in double chamber experiments where PC are separated from the tumour targets by a porous membrane (unpublished). The stimulation of the cytocidal action of mouse macrophages by 5,6-MeXAA is consistent with the production of a non-secretable factor which is active during cell–cell contact.

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REFERENCES

1. Rewcastle GW, Atwell GJ, Zhuang L, Baguley BC and Denny WA, Potential antitumor agents. 61. Structure–activity relationships for *in vivo* colon-38 activity among disubstituted 9-oxo-9*H*-xanthene-4-acetic acids. *J Med Chem* **34**: 217–222, 1991.
2. Kerr DJ and Kaye SB, Flavone acetic acid—preclinical and clinical activity. *Eur J Cancer Clin Oncol* **25**: 1271–1272, 1989.
3. Evelhoch JL, Bissery M-C, Chabot GG, Simpson NE, McCoy CL, Heilbrun LK and Corbett TH, Flavone acetic acid (NSC 347512)-induced modulation of murine tumor physiology monitored by *in vivo* nuclear magnetic resonance spectroscopy. *Cancer Res* **48**: 4749–4755, 1988.
4. Zwi LJ, Baguley BC, Gavin JB and Wilson WR, Blood flow failure as a major determinant in the antitumor action of flavone acetic acid (NSC 347512). *J Natl Cancer Inst* **81**: 1005–1013, 1989.
5. Mahadevan V, Malik STA, Meager A, Fiers W, Lewis GP and Hart IR, Role of tumor necrosis factor in flavone acetic acid-induced tumor vasculature shutdown. *Cancer Res* **50**: 5537–5542, 1990.
6. Ching L-M and Baguley BC, Enhancement of *in vitro* toxicity of mouse peritoneal exudate cells by flavone acetic acid (NSC 347512). *Eur J Cancer Clin Oncol* **24**: 1521–1525, 1988.
7. Ching L-M, Finlay GJ, Joseph WR and Baguley BC, *In vitro* methods for screening agents with an indirect mechanism of antitumour activity: xanthenone analogues of flavone acetic acid. *Eur J Cancer* **27**: 1684–1689, 1991.
8. Wilkoff LJ, Dulmadge E and Chopra DP, Viability of cultured Lewis lung cell populations exposed to β-retinoic acid (40753). *Proc Soc Exp Biol Med* **163**: 233–236, 1980.
9. Adams DO and Marino PA, Evidence for a multistep mechanism of cytolysis by BCG-activated macrophages: the interrelationship between the capacity for cytolysis,

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target binding, and secretion of cytolytic factor. *J Immunol* **126**: 981–987, 1981.
10. Rewcastle GW, Kestell P, Baguley BC and Denny WA, Light-induced breakdown of flavone acetic acid and xanthenone analogues in solution. *J Natl Cancer Inst* **82**: 528–529, 1990.
11. Stewart CC, Stevenson AP and Hibbs J, Effector mechanisms for macrophage-induced cytostasis and cytolysis of tumour cells. In: *Macrophages and Cancer* (Eds. Heppner GH and Fulton AM), pp. 39–59. CRC Press, Boca Raton, Florida.
12. Marletta MA, Nitric oxide—biosynthesis and biological significance. *Trends Biochem Sci* **14**: 488–492, 1989.
13. Thomsen LL, Ching LM and Baguley BC, Evidence for the production of nitric oxide by activated macrophages treated with the antitumor agents flavone-8-acetic acid and xanthenone-4-acetic acid. *Cancer Res* **50**: 6966–6970, 1990.
14. Nguyen DT, Eskandari MK, Deforge LE, Raiford CL, Strieter RM, Kunkel SL and Remick DG, Cyclosporin A modulation of tumor necrosis factor gene expression and effects *in vitro* and *in vivo*. *J Immunol* **144**: 3822–3828, 1990.
15. Adams DO, Effector mechanisms of cytolytically activated macrophages. Secretion of neutral proteases and effect of protease inhibitor. *J Immunol* **124**: 286–292, 1980.
16. Taffet SM and Russell SW, Macrophage-mediated tumour cell killing: regulation of expression of cytolytic activity by prostaglandin E. *J Immunol* **126**: 424–427, 1980.
17. Mace KF, Hornung RL, Wiltrout RH and Young HA, Correlation between *in vivo* induction of cytokine gene expression by flavone acetic acid and strict dose dependency and therapeutic efficacy against murine renal cancer. *Cancer Res* **50**: 1742–1747, 1990.
18. Schultz RM, Pavlidis NA, Styles WA and Chirigos MA, Regulation of macrophage tumouricidal function: a role for prostaglandins of the E series. *Science* **202**: 320–321, 1978.
19. Riendeau D, Fagneyret J-P, Nathaniel DJ, Rokach J, Ueda N and Yamamoto S, Sensitivity of immuneaffinity-purified porcine 5-lipoxygenase to inhibitors and activating lipid hydroperoxides. *Biochem Pharmacol* **38**: 2313–2321, 1989.
20. Larrick JW and Wright SC, Cytotoxic mechanism of tumor necrosis factor-alpha. *FASEB J* **4**: 3215–3223, 1990.
21. Bucana C, Hoyer LC, Hobbs B, Breesman S, McDaniel M and Hanna MC, Morphological evidence for the translation of lysosomal organelles from cytotoxic macrophages into the cytoplasm of tumour target cells. *Cancer Res* **36**: 4444–4458, 1976.
22. Perez C, Albert I, Defay K, Zachariades N, Gooding L and Kriegler M, A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. *Cell* **63**: 251–258, 1990.
23. Decker T, Lohman-Matthes M-L and Gifford GE, Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. *J Immunol* **138**: 957–962, 1987.