Identification of human-selective analogues of the vascular-disrupting agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA)

S M Tijono, K Guo, K Henare, B D Palmer, L-C S Wang, S M Albelda and L-M Ching*

Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, School of Medicine, University of Auckland, Auckland, New Zealand and Thoracic Oncology Research Laboratory, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

Background: Species selectivity of DMXAA (5,6-dimethylxanthenone-4-acetic acid, Vadimezan) for murine cells over human cells could explain in part the recent disappointing phase III trials clinical results when preclinical studies were so promising. To identify analogues with greater human clinical potential, we compared the activity of xanthenone-4-acetic acid (XAA) analogues in murine or human cellular models.

Methods: Analogues with a methyl group systematically substituted at different positions of the XAA backbone were evaluated for cytokine induction in cultured murine or human leukocytes; and for anti-vascular effects on endothelial cells on matrigel. In vivo antitumour activity and cytokine production by stromal or cancer cells was measured in human A375 and HCT116 xenografts.

Results: Mono-methyl XAA analogues with substitutions at the seventh and eighth positions were the most active in stimulating human leukocytes to produce IL-6 and IL-8; and for inhibition of tube formation by ECV304 human endothelial-like cells, while 5- and 6-substituted analogues were the most active in murine cell systems.

Conclusion: Xanthenone-4-acetic acid analogues exhibit extreme species selectivity. Analogues that are the most active in human systems are inactive in murine models, highlighting the need for the use of appropriate in vivo animal models in selecting clinical candidates for this class of compounds.

DMXAA (5,6-dimethylxanthenone-4-acetic acid, Vadimezan) is a small molecule tumour vascular-disrupting agent with immune modulatory activities via its induction of cytokines (Baguley and Ching, 2002). Results from phase II trials of DMXAA in combination with paclitaxel and carboplatin for non-small-cell lung cancer were promising (McKeage et al, 2009), but phase III trials were stopped following interim analysis of results that indicated a lack of utility (Lara et al, 2011). Its poor performance in phase III trials when preclinical studies were so impressive has led us to further investigate potential inter-species differences in the activity of this class of agents that may allow us to develop a compound that has greater activity than DMXAA in the human clinical setting.

5,6-Dimethylxanthenone-4-acetic acid, with a methyl substituent on both the 5- and 6-position, was selected as a clinical candidate from all the xanthenone and flavone derivatives synthesised at the Auckland Cancer Society Research Centre, based on its potency and activity against solid murine cancers that are resistant to conventional chemotherapies (Rewcastle et al, 1991). Preclinical investigations showed that a single administration of DMXAA to tumour-bearing mice, induced selective apoptosis of tumour vascular endothelial cells within 30 min (Ching et al, 2002); caused irreversible vascular shutdown within 4 h and widespread tumour necrosis after 24 h (Zwi et al, 1994; Lash et al, 1998). In contrast to other classes of vascular-disrupting agents, DMXAA has the additional ability of stimulating...
intra-tumoural leukocytes to produce a panel of cytokines in situ (Wang et al., 2009). The cytokines induced in response to DMXAA confer a multitude of secondary host responses that are vital to its overall antitumour effects in mice. TNF-α is induced at higher concentrations within tumour tissue than in the serum (Cao et al., 1999; Joseph et al., 1999; Wang et al., 2009), and its production has been suggested to amplify and prolong the initial blood flow inhibition leading to extensive tumour ischaemia and widespread necrosis (Zhao et al., 2002). Elevated levels of IP-10, RANTES, KC, MIP-1α and MCP-1 observed in the tumour 4–6 h after DMXAA administration have been suggested to promote an influx of macrophages (Jassar et al., 2005) and neutrophils (Wang et al., 2009) into the tumour. DMXAA is also a potent inducer of IFNs (Roberts et al., 2007), and in immunocompetent mice, the IFNs expand the population of tumour-specific cytotoxic T cells (Kanwar et al., 2001; Wallace et al., 2007) that are essential for complete tumour regressions to occur with this treatment (Bibby et al., 1991; Ching et al., 1992).

Although cytokine concentrations are higher and more sustained in tumour tissue than in spleen, liver or serum (Cao et al., 1999; Joseph et al., 1999; Wang et al., 2009), the panel of cytokines induced in all the tissues were similar (Wang et al., 2009). Moreover, the panel of cytokines produced in vitro by murine leukocytes cultured with DMXAA is identical to that detected in serum (Wang et al., 2009), indicating that the in vitro response is indicative of the in vivo cytokine response to DMXAA. Subsequent studies with cultured human peripheral blood leukocytes (HPBLS) identified a pattern of human-specific effects that were different to those induced with DMXAA on murine leukocytes (Wang et al., 2009), and the studies were the first to demonstrate experimentally of inter-species differences in the cytokine response to DMXAA. Of the panel of 27 cytokines assayed, highest fold increases in concentrations of IL-8 and IL-6 was induced with DMXAA in the majority of the 12 donors tested, and these cytokines served as the strongest indicators of a positive response from human leukocytes to DMXAA. When phase III trials of DMXAA were stopped, we investigated whether inter-species differences in the response to DMXAA could explain, in part why the clinical response was disappointing when pre-clinical data were so encouraging. In this communication, we examined for differences in the structure–activity relationship (SAR) of a subset of XAA analogues for inducing cytokines in cultures of human or murine leukocytes. Although DMXAA is clearly the most active for murine leukocytes, we show that it was not the best for stimulating human leukocytes, and have identified other analogues that are more selective for human cells.

**MATERIALS AND METHODS**

**Drugs and reagents.** Sodium salts of DMXAA (304.27 Da), xanthone-4-acetic acid (XAA, 276.23 Da) and mono-methyl substituted analogues of XAA (Me-XAA, 290.25 Da) were synthesised at Auckland Cancer Society Research Centre (Rewcastle et al., 1991), and dissolved directly in the culture medium used.

**Mice and A375 xenografts.** Immunodeficient CD-1 nude mice were bred at the Vernon Jansen Unit, Auckland University. All experiments conformed to local institutional guidelines that meet the standards required by the UKCCCR guidelines and in accordance with the declaration of Helsinki. The A375 melanoma line (ATCC # CRL1619) and the HCT116 colon carcinoma line (ATCC # CCL247) were maintained in DMEM media supplemented with 10% FCS, and 100 μg/mL of antibiotics. 5 x 10^6 A375 and 5 x 10^5 HCT116 were maintained in DMEM media supplemented with 10% FCS, and 10^6 cells were inoculated subcutaneously into CD-1 nude mice. Growth inhibition studies were initiated when the tumours were 3–4 mm in diameter. Mice (five per group) with tumours were treated with a single intraperitoneal injection of DMXAA at 25 mg kg⁻¹ or 8-MeXAA and 7,8-MeXAA at 25 and 50 mg kg⁻¹, and another group was left untreated. Tumours were measured thrice weekly thereafter and tumour volumes were calculated as 0.52a²b where a and b are the minor and major axes of the tumour. The arithmetic mean ± s.e.m. were calculated for each time point and expressed as a fraction of the pre-treatment volume. Growth delay was determined as the difference in the number of days between tumours in the treated or untreated groups to quadruple in size.

For haemorrhagic necrosis determinations, mice with tumours were treated with DMXAA, 8-MeXAA or 7,8-MeXAA at 25 mg kg⁻¹, and the tumours excised after 24 h. Tumours were fixed in formalin, paraffin-embedded, sectioned and haematoxylin and eosin stained. Montages of entire tumour sections were acquired (Image Pro PLUS 7.0, Media Cybernetics Inc., Bethesda, MD, USA) at an original 10 x magnification (Nikon TE2000E microscope; Nikon Inc., Tokyo, Japan). Using Image J 1.45s software (National Institutes of Health, Bethesda, MD, USA), a grid with 80 μm intersections was overlaid over each montage and the number of grid intersections over necrotic regions as a percentage of the total number of grid intersections was calculated. One entire section from the widest part of the tumour was scored and the mean ± s.e.m. of n = 3 tumours per group was calculated.

For cytokine determinations in A375 xenografts, mice with tumours were treated with XAA analogues at 25 mg kg⁻¹, and after 4 h, mice were killed and tumours were excised, weighed and homogenised in 200 μl of PBS containing 1:100 v/v Sigma (St Louis, MO, USA) Protease Inhibitor Cocktail. Tumour homogenates were stored at −80 °C until assayed for both murine (stromal cell derived) or human (melanoma cell derived) cytokines using non-cross-reacting murine and human multiplex cytokine kits (murine 6-plex, and human 7-plex, Milliplex MAP, Millipore Corporation, Billerica, MA, USA). Concentration of each cytokine present was read using the Luminex 100 instrument (Luminex Corporation, Austin, TX, USA). The cytokine concentration (pg g⁻¹ of tumour) from three tumours per group were expressed as mean ± s.e.m.

**Cytokine production in leukocyte cultures.** Blood from healthy human donors who had consented for their blood to be used for research, was obtained from NZ Blood Services for this study, which has ethical approval from the Auckland Regional Health and Disabilities Ethics Committee. Leukocytes were isolated using ficoll–paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density centrifugation. Murine leukocytes were obtained from spleens from C57Bl/6 mice. Spleen cells were squeezed out into culture medium, aspirated to form a single-cell suspension, and red blood cells were removed by osmotic lysis as previously described (Wang et al., 2009). Leukocytes (10⁶ per well) were cultured in flat-bottomed 96-well plates with xanthene derivatives in a final volume of 200 μl of culture medium (α-MEM; Gibco BRL, Grand Island, NY, USA), supplemented with FCS (10%) and antibiotics (100 U μl⁻¹ penicillin and 100 μg ml⁻¹ streptomycin). Tumour-associated macrophages (TAM) were obtained by isolating CD11b⁺ cells from pleural effusions from mesothelioma patients being treated at the University of Pennsylvania Medical Centre. MidiMACS separator cell isolation kit (catalogue #130-049-601, Miltenyi Biotec, Auburn, CA, USA) was used following the manufacturer’s instructions to enrich for macrophages. Briefly, cells in pleural effusions were collected by centrifugation and the cell pellet was resuspended with phosphate-buffered saline containing 2% bovine serum albumin and magnetically labelled antibody to CD11b. The positively selected cells (2 x 10⁵ cells ml⁻¹ per well) were then cultured in 24-well plates with XAA analogues (300 μg ml⁻¹).
Supernatants from murine or human leukocyte cultures were harvested 4 and 18 h, respectively, after incubation at 37°C in an atmosphere of 5% CO₂. Supernatants were stored at −20°C until assay for cytokine concentrations using enzyme-linked immunosorbent assay kits (OptEIA, BD Biosciences, San Diego, CA, USA), according to the manufacturer’s instructions. Triplicate cultures were assayed per treatment group and the results were expressed as mean ± s.e.m.

Inhibition of tube formation on matrigel. Inhibition of tube formation by endothelial-like cells on matrigel layers was used as an in vitro assay of anti-vascular activity. ECV304 (CRL-1998) from ATCC (Manassas, VA, USA) were cultured in M199 culture medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FCS and antibiotics (100 U ml⁻¹ penicillin plus 100 µg ml⁻¹ streptomycin) at 37°C under humidified atmosphere of 5% CO₂. Cells were used for experiments after two or three passages from frozen master stocks of the line. Matrigel Basement Matrix (100 µl undiluted from BD Biosciences) at 4°C was added to each well of pre-chilled 24-well plates and allowed to polymerise at 37°C for 1 h. ECV304 cells (10⁵ cells per well) were added to the matrigel layer together with the required concentration of XAA analogues in a final volume of 1 ml supplemented culture medium. After 18-h incubation at 37°C, tubular structures on the matrigel layer in each well were photographed using a Camedia C-5050 camera attached to a CKX41 microscope (Olympus, Tokyo, Japan) with a 4× objective. The number of tubes per set area of each well was counted, and duplicate wells were assessed for each treatment.

Cytotoxicity assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colourimetric assay was used to assess viability of ECV304 cells after exposure to XAA analogues at the same concentrations used for the inhibition of tube formation assay on that day. ECV304 cells (10⁵ per well) with different concentrations of XAA analogues were plated into the wells of a 96-well flat-bottom plates in a final volume of 200 µl per well, and cultured 18 h. MTT (20 µl at 5 mg ml⁻¹) was added per well and the cultures were further incubated until formation of purple formazan crystals was observed. Culture supernatants were removed and DMSO (100 µl per well) was added to dissolve the crystals, and the absorbance at 550 nm of each well was measured immediately with an automated microplate reader ELx808 (Bio-Tek Instruments Inc., Winooski, VT, USA). Triplicate cultures were used for each XAA concentration, and the mean viability was calculated and expressed as percentage of untreated controls.

Statistical analyses. Data between untreated and treated groups were compared using Student’s t-tests or ANOVA if multiple comparisons were made and were considered significant when P ≤ 0.05. Differences between growth of treated and untreated tumours were also compared using unpaired Student’s t-tests according to Braun and Vistisen (2012) and were considered significant when P ≤ 0.05.

RESULTS

SAR of xanthenone analogues for inducing cytokines in murine or human leukocytes. Our laboratory holds a set of XAA analogues, where the hydrogen at each position of the parent XAA molecule, has been systematically replaced with a methyl group (Figure 1A). This set of mono-methyl substituted XAA analogues is a useful tool for SAR studies of this class of compounds. Previous studies indicated that substitution at positions 3, 5 and 6 resulted in compounds with increased antitumour activity in mice (Ching et al, 1991; Woon et al, 2005). This led to the subsequent synthesis of 3,5- and 5,6 di-substituted XAA analogues, of which 5,6-dimethylxanthenone-4-acetic acid, later abbreviated to DMXAA, was shown to be the most potent in murine tumour models (Rewcastle et al, 1991). With the observation that murine and human leukocytes respond differently to DMXAA (Wang et al, 2009), we investigated whether or not the SAR for the mono-methyl XAA analogues in murine and human leukocytes may also be different.

Murine leukocytes extracted from mouse spleens, or human leukocytes extracted from blood of healthy donors were cultured with each of the mono-methyl XAA analogues, XAA, DMXAA and flavone acetic acid. Culture supernatants were assayed for cytokines of interest after an appropriate period of incubation. IL-6 produced by murine leukocytes cultured with each analogue at 300 µg ml⁻¹ is shown in Figure 1B. DMXAA induced the highest levels of IL-6. Of the mono-methyl XAAs, 5-MeXAA was the best, followed by 6-MeXAA, and then 3-MeXAA, whereas 2-MeXAA, 7-MeXAA and 8-MeXAA showed no induction of IL-6. In contrast, 8-MeXAA, inactive in murine leukocytes, induced the highest amounts of IL-8 (Figure 1C), IL-6 (Figure 1D) and TNF-α (Figure 1F) in cultures of HPBL. We also examined the response in culture of human macrophages isolated from pleural effusions of patients with mesothelioma as a model of TAM for production of IL-6 (Figure 1E) and TNF-α (Figure 1G). Again, 8-MeXAA induced the highest amount of those cytokines compared with the other analogues.

IL-8 is one of the most abundant cytokines produced and we chose that as the read-out in a study to determine the inter-individual variability between nine different donors in the responsiveness of their HPBLs to XAA analogues. The constitutive production of IL-8 in untreated HPBL cultures was highly variable between donors (Supplementary Table 1) and we have presented the data in Figure 2A as fold increase in IL-8 over that of the corresponding untreated control cultures for each donor. 8-MeXAA induced highest fold increases in IL-8 production followed by 7-MeXAA, although a considerable spread in the response between individuals is observed (Figure 2A). IL-8 production by HPBLs from two donors at different drug concentrations was determined and 8-MeXAA induced higher amounts of IL-8 than 7-MeXAA or DMXAA at all concentrations between 100 and 500 µg ml⁻¹ (Figure 2B).

As 8-MeXAA and 7-MeXAA were the most active of the mono-methyl XAAs in HPBLs, the 7,8-disubstituted analogue was custom synthesised and evaluated. The fold increase in IL-8 concentrations induced with 7,8-MeXAA in HPBLs from six different donors at 300 µg ml⁻¹ is compared with those induced with 8-MeXAA, 7-MeXAA, DMXAA in Figure 2C. The fold increase in IL-8 induced with 7,8-MeXAA for HPBLs from that cohort of donors was 55.4 ± 18.6, higher than that obtained with 8-MeXAA (18.5 ± 7.1), 7-MeXAA (3.4 ± 1.2) and DMXAA (1.7 ± 0.2). In HPBLs from two individuals tested, 7,8-MeXAA induced higher concentrations of IL-8 than 8-MeXAA over the concentration range of 200–500 µg ml⁻¹ (Figure 2D).

Inhibition of tube formation by XAA analogues in human ECV304 cells. The anti-vascular activity is another key component of the antitumour action of this class of agents, and the ability of the mono-methyl analogues to inhibit tube formation on matrigel layers was also examined. We used human ECV304 cells as a model for endothelial cells in previous investigations of the role of NF-κB in apoptosis induction by DMXAA, when primary human umbilical cord vein cells were found to be non-responsive to DMXAA (Woon et al, 2007), and we have used ECV304 cells here also. A representative field showing tube formation by ECV304 cells on matrigel after 18-h culture with each XAA analogue at 100 µg ml⁻³ is shown in Figure 3A. 5-MeXAA, 7-MeXAA and DMXAA inhibited tube formation by approximately 50% compared with control cultures, whereas 1-MeXAA, 2-MeXAA, 3-MeXAA and 6-MeXAA all showed < 20% inhibition.
Human-selective DMXAA analogues

BRITISH JOURNAL OF CANCER

100

m

compared with 7,8-MeXAA, which gave only 30% inhibition at 100 \( \mu \text{g ml}^{-1} \).

The concentration of drug required to achieve 50% (IA\( \text{SO}_5 \)) tube inhibition was 40 \( \mu \text{g ml}^{-1} \) for 8-MeXAA, and 65 \( \mu \text{g ml}^{-1} \) for 7,8-MeXAA compared with 200 \( \mu \text{g ml}^{-1} \) required for DMXAA (Figure 3C). The five-fold increase in potency of 8-MeXAA over DMXAA to inhibit tube formation, was not due to increased cytotoxicity of 8-MeXAA, as there was no loss in viability of the ECV304 cells when cultured with 8-MeXAA at concentrations up to 1000 \( \mu \text{g ml}^{-1} \) (Figure 4C). 7,8-MeXAA appeared to be the most cytotoxic of the three analogues, reducing viability of ECV304 cells by 50% at 250 \( \mu \text{g ml}^{-1} \) (Figure 3C).

8-MeXAA and 7,8-MeXAA show no antitumour activity in mice compared with DMXAA. Substitution at the 7- and 8-positions provided the most active XAA analogues for human cells. These human-selective analogues, however, appear not to be very active for stimulating murine leukocytes to produce cytokines as DMXAA. As the ability to induce cytokines appears to be a critical aspect of its long term antitumour effects, we would not expect the 7- and 8-substituted analogues to show much antitumour activity in mice, even against human tumour xenografts. These agents target predominantly the stromal components of the tumour which, including the tumour vasculature, are derived from the murine host. For proof-of-principle, activity of 8-MeXAA, 7,8-MeXAA and DMXAA was measured against A375 human melanoma xenografts. We have used A375 xenograft model...

www.bjcancer.com | DOI:10.1038/bjc.2013.101

1309
previously for DMXAA studies (Henare et al., 2012) and we have found it to be one of the more responsive xenograft model for studying DMXAA. Xenografts from mice that had been administered 24 h prior with DMXAA at its maximum tolerated dose (MTD) of 25 mg kg\(^{-1}\), showed widespread necrosis (Figures 4B and E), whereas degree of necrosis of xenografts excised from mice 24 h after treatment with the same dose of 8-MeXAA (Figures 4C and E) or 7,8-MeXAA (Figures 4D and E) was no greater than that of untreated controls (Figures 4A and E). A single injection of DMXAA at 25 mg kg\(^{-1}\) delayed the re-growth of A375 xenografts implanted subcutaneously in CD1 nude mice significantly (\(P = 0.01\)) by 16 days (Figure 5A). As predicted, treatment with 8-MeXAA at 25 mg kg\(^{-1}\) or at its MTD of 50 mg kg\(^{-1}\) gave only a minimal growth delay of only 5 days (\(P = 0.03\)) and 3 days (\(P = 0.19\)), respectively (Figure 5B). No significant difference in tumour growth was observed with 7,8-MeXAA at 25 mg kg\(^{-1}\) (\(P = 0.56\)), although a higher dose of 50 mg kg\(^{-1}\) gave a 3 days growth delay and a significant difference in growth parameters compared with untreated tumours (\(P = 0.05\); Figure 5C). In HCT116 xenografts, DMXAA (25 mg kg\(^{-1}\)) induced a significant 4-day growth delay (\(P = 0.03\)), but 8-MeXAA at 25 mg kg\(^{-1}\) (\(P = 0.27\)) or 50 mg kg\(^{-1}\) (\(P = 0.06\)) did not cause a significant difference in growth, confirming the inferior antitumour activity of 8-MeXAA compared with that of DMXAA in another xenograft model (Supplementary Figure 1).

DMXAA treatment increased stromal-cell derived, murine IL-6, IP-10, MCP-1, MIP-1\(\alpha\) and TNF-\(\alpha\) in A375 xenografts (Figure 5D), but significant induction of murine cytokines were not observed following treatment with 8-MeXAA (Figure 5E) or 7,8-MeXAA (Figure 5F). Low but significant increases in melanoma cell-derived human IL-6, MCP-1, and MIP-1\(\alpha\) were also observed after DMXAA treatment, but not TNF-\(\alpha\) (Figure 5G), in contrast to the increases in murine TNF-\(\alpha\) observed in the xenografts. The addition of DMXAA at 300 \(\mu\)g ml\(^{-1}\) to A375 human melanoma cells in culture did not result in significant increases in cytokine levels (Supplementary Table 2). Rather, DMXAA reduced constitutive production of MCP-1 and VEGF by A375 cells in culture, but had no significant effect on constitutively produced IL-8, and did not induce TNF-\(\alpha\) (Supplementary Table 2). A significant increase in IL-8 only was observed in xenografts...
from mice treated with 8-MeXAA (Figure 5H), and xenografts treated with 7,8-MeXAA showed no increase in any of the human cytokines (Figure 5I).

The toxicity of the murine inactive analogues in mice differs from that described for DMXAA. Mice treated with 8-MeXAA or 7,8-MeXAA at doses above 50 mg kg\(^{-1}\) begin losing weight 14 days after treatment and reach humane ethical endpoint shortly thereafter, and 50 mg kg\(^{-1}\) was used as the MTD for long-term survival experiments. The acute dose-limiting toxicities observed with DMXAA at doses above its MTD (diarrhoea, conjunctivitis, hunching, ruffled fur and hypothermia that require the mice to be euthanised 6–8 h after treatment) appear to be linked to TNF-\(\alpha\) production, which peaks 3–4 h after treatment (Philpott et al., 1995). These acute, cytokine-related toxicities are not observed with 8-MeXAA or 7,8-MeXAA consistent with the lack of cytokine induction by these analogues in mice.

**DISCUSSION**

We have conducted structure-activity studies with a subset of xanthenone analogues that have a methyl-group substituted systematically at each available position of the parent compound. The primary objectives were to determine if the SAR of XAA analogues were different for mice and men, and whether we could identify analogues with better activity in humans than DMXAA. We showed marked differences in the SAR of the XAA analogues in murine and human leukocytes. The SAR of the analogues for murine IL-6 induction is consistent with that observed for natural killer cell activation (Ching et al., 1991), NF-\(\kappa\)B activation (Woon et al., 2005) and plasma nitrate production (Thomsen et al., 1991; Veszelovszky et al., 1993) in mice, and correlates with the ability of the XAA analogues to induce haemorrhagic necrosis of tumours in mice (Ching et al., 1991; Rewcastle et al., 1991; Thomsen et al., 1991; Veszelovszky et al., 1993; Woon et al., 2005). In mice, the 3-, 5- and 6-substituted analogues are more active than the parent XAA, with DMXAA, the 5, 6-disubstituted analogue being even more potent than the mono-substituted analogues. The 2-, 7- and 8-substituted analogues, generally are inactive in murine systems. In this study, we showed that DMXAA induced the highest amount of IL-6 in murine leukocytes in culture, but DMXAA was not very effective in stimulating HPBLs in culture to produce IL-6. The selectivity of DMXAA for murine cells over human cells could explain in part the disappointing results of DMXAA in phase III clinical trials (Lara et al., 2011), when preclinical studies in mice were so promising.

The studies here identified that methyl substitution at the 8-position produced an analogue that was considerably more selective for activity in human cells than DMXAA. 8-MeXAA was...
the most active of the monomethyl-XAA for stimulating IL-6 (Figures 1D and E) and IL-8 (Figures 1F and 2A) production by HPBLs. 8-MeXAA was also the most effective of the mono-methyl-XAA analogues at inhibiting tube formation by ECV304 cells on matrigel (Figures 3A and B). It was five-fold more potent than DMXAA at inhibiting ECV304 tube formation as well as being less cytotoxic to the cells (Figure 3C). Previously, 8-substituted analogues have been shown not to exert any antitumour effects in mice (Rewcastle et al., 1991), and 8-MeXAA was frequently used in studies as the negative control compound for DMXAA (Ching et al., 1991; Rewcastle et al., 1991; Thomsen et al., 1991; Veszelovszky et al., 1993). Moreover, 8-MeXAA was shown not to exert any direct anti-vascular effects in mice, as no apoptosis was induced in CD31-positive vascular endothelial cells in colon 38 tumours from 8-MeXAA-treated mice compared with those from DMXAA-treated mice (Ching et al., 2002). The findings here showing that 8-MeXAA is the most active in human cells are surprising and unexpected. Their lack of activity in mice however hampers the advancement of the 8-substituted analogues into in vivo evaluations. Preliminary studies with rat and dog leukocyte cultures, carried out as part of our efforts to find a suitable animal model for establishing the in vivo activity of human-selective XAA analogues, have indicated that neither the response from the dog or the rat mimics the response of human leukocytes to the XAA analogues (unpublished).

In addition to inter-species differences in SAR, the cytokine response stimulated by the XAA compounds in leukocytes also differs between species and could lead to inter-species differences in the antitumour activity of this class of compounds. For example, IFN-β is abundantly induced by DMXAA in murine macrophages (Perera et al., 1994; Roberts et al., 2007), and the antitumour activity of DMXAA in IFN-β knock-out mice was severely attenuated (Roberts et al., 2008). However, IFN-β is not detected in HPBL cultures treated with DMXAA or 8-MeXAA (data not shown). IL-8 is the most abundant cytokine produced by HPBLs in culture in response to DMXAA (Wang et al., 2009) or 8-MeXAA (Figures 1 and 2). This CXC chemokine has been ascribed a number of pro-tumour activities (Waugh and Wilson, 2008), such as increasing tumour cell proliferation and survival; inducing endothelial cell angiogenesis (Huang et al., 2000; Kline et al., 2007); and more recently, inducing the epithelial–mesenchymal transition that promotes metastasis of carcinoma cells (Fernando et al., 2011).

On the other hand, human ovarian cancer lines that express IL-8 have a reduced tumourigenicity, mediated by an increased accumulation of neutrophils at the inoculation site (Lee et al., 2000). That study suggested that IL-8 may have a positive role in controlling cancer growth (Lee et al., 2000). As there is no exact IL-8 homologue in mice, the effect of increased IL-8 production to the antitumour activity of the XAAs is difficult to assess without an appropriate animal model, although KC, a functional homologue of IL-8 in mice, is induced by DMXAA in spleen and tumour tissue, but not in serum or in murine leukocyte cultures (Wang et al., 2009).

The results in Figure 5 confirm previous studies that the antitumour activity of an XAA analogue is linked with its ability to elicit a vigorous cytokine response (Cao et al., 1999; Joseph et al.,

Figure 4. Haemorrhagic necrosis in A375 xenografts 24 h after treatment with DMXAA, 8-MeXAA or 7,8-MeXAA. (A) Representative montage of haematoxylin and eosin H&E-stained section from A375 xenografts untreated; (B) At 24 h after treatment with DMXAA; (C) 8-MeXAA; or (D) 7,8-MeXAA at 25 mg kg⁻¹. (E) Quantitative data of degree of haemorrhagic necrosis in montaged sections. Mean ± s.e.m. from n = 3 tumours per treatment group; *Denotes statistical significance between DMXAA-treated tumours and -untreated controls compared by one-way ANOVA.
Human-selective DMXAA analogues

BRITISH JOURNAL OF CANCER

Figure 5. Tumour growth and cytokine production in A375 human melanoma xenografts in nude mice following treatment with DMXAA (left panels), 8-MeXAA (middle panels) or 7,8-MeXAA (right panels). (A–C) Tumour growth in mice without treatment (filled circles), and following treatment with DMXAA (A), 8-MeXAA (B) or 7,8-MeXAA (C) at 25 mg kg$^{-1}$ (open circles), or 50 mg kg$^{-1}$ (open triangles). Mean ± s.e.m. of five mice per group. *P < 0.05 between treated and untreated group compared using Student’s t-tests. (D–F) Concentration of indicated murine cytokines measured in tumour homogenates from mice without treatment (black bars) or from mice 4 h after treatment with analogues at 25 mg kg$^{-1}$ (grey bars). (G–I) Concentration of indicated human cytokines measured in tumour homogenates from mice without treatment (black bars) or from mice 4 h after treatment (hatched bars). Mean ± s.e.m. of three tumours per group. *P < 0.05 between tumours from treated and untreated mice compared using Student’s t-tests.

Despite considerable effort and interest, the biochemical target(s) for this class of compounds have not been elucidated, posing another challenge to the advancement of a second generation analogue, or of DMXAA itself in different clinical settings, as the molecular mechanism of action is not completely understood. The NF-κB signalling pathway (Joseph et al., 1999; Woon et al., 2003; Wang et al., 2006), the TBK1-IRF-3 signalling axis (Roberts et al., 2007), the NOD signalling pathway (Cheng et al., 2010), and at least three members of the MAPK superfamily (Sun et al., 2011) are involved in one or more of the pleiotrophic effects of DMXAA in mice. Studies from our own laboratory showed that more than thirty oxidisable proteins were photo-affinity labelled with an azido-analogue of DMXAA in cellular extracts from murine leukocytes, implicating a role for redox signalling (Palmer et al., 2007; Brauer et al., 2010). The yet unidentified cellular enzymes that catalyse the first-step, one-electron oxidation of DMXAA to form the benzyl radical initiating the generation of reactive oxygen species and redox signalling could also be regarded as biochemical target(s) for this class of compounds. More recently, Prantner et al. (2012) showed that absence of Stimulator of Interferon Gene (STING) impaired...
DMXAA-induced IFN-β production by murine macrophages. Although we have not been able to demonstrate induction of IFN-β by the methyl-XAA analogues in cultured HPBLs, it would be of interest to evaluate the binding of the analogues to murine and human STING. Structural differences in the murine and human homologues of the targets could provide an explanation for the inter-species differences in the SAR of the XAA analogues.

In summary, we show major differences in the SAR of XAA analogues for stimulating cytokines in human or murine leukocyte cultures. We identify that the 8-substituted XAA analogue is more active in human cell systems, whereas 5- or 6-substituted analogues, such as DMXAA, are the most active in murine systems. The 8-substituted analogues, however, are not active in mice, highlighting the need to identify or establish appropriate animal models to demonstrate their human potential in vivo. Humanised mice with reconstituted human lymphoid and myeloid components (Melkus et al., 2006; Strowig et al., 2009; Jaiswal et al., 2012) would be helpful in evaluating the cytokine stimulatory activity of the XAA analogues in human leukocytes in vivo. However, in order to be able to comprehensively assess the multitude of anti-vascular, cytokine-modulatory, immune-modulatory and the antitumour effects of this class of agents, humanised mice with reconstituted human immune and human vascular components together with autologous histocompatible tumour cells for implantation would be required, and currently such mice are not widely available.

ACKNOWLEDGEMENTS

This work was funded by grants to LMC from Auckland and National Divisions of the Cancer Society of New Zealand and grant # P01 CA66726 to SMA.

REFERENCES

Baguley BC, Ching L-M (2002) DMXAA: an antivascular agent with multiple host responses. *Int J Radiation Oncol Biol Phys* 54(5): 1503–1511.

Bibby M, Phillips R, Double J, Pratesi G (1991) Anti-tumour activity of flavone acetic acid (NSC 347512) in mice–influence of immune status. *Br J Cancer* 64(1): 57.

Brauer RD, Vistisen KS (2012) Modelling human choroidal melanoma xenograft growth in immunocompromised rodents to assess treatment efficacy. *Invest Ophthalmic Vis Sci* 53(60): 2693–2701.

Cao Z, Joseph W, Browne W, Mountjoy K, Palmer B, Baguley B, Ching L (1999) Thalidomide increases both intra-tumoural tumour necrosis factor-α production and anti-tumour activity in response to 5, 6-dimethylxanthene-4-acetic acid. *Br J Cancer* 80(S5): 716.

Cheng G, Sun J, Fridlander ZG, Wang LCS, Ching LM (2010) Activation of the nuclear factor-κB signaling pathway by the non-bacterially derived xanthone drug 5, 6-dimethylxanthene-4-acetic acid (Vadimezan). *J Biol Chem* 285(14): 10553.

Ching L, Cao Z, Kieda C, Zwaan S, Jameson MB, Baguley B (2002) Induction of endothelial cell apoptosis by the antivascular agent 5, 6-dimethylxanthene-4-acetic acid. *Br J Cancer* 86(12): 1937–1942.

Ching L, Joseph W, Baguley B (1992) Antitumour responses to flavone-8-acetic acid and 5, 6-dimethylxanthene-4-acetic acid in immune deficient mice. *Br J Cancer* 66(1): 128.

Ching LM, Goldsmith D, Joseph WR, Körner H, Sedgwick JD, Baguley BC (1999) Induction of intratumoral tumour necrosis factor (TNF) synthesis and hemorrhagic necrosis by 5, 6-dimethylxanthene-4-acetic acid (DMXAA) in TNF knockout mice. *Cancer Res* 59(14): 3304.

Ching LM, Joseph WR, Zhuang L, Atwell GJ, Rewcastle GW, Denny WA, Baguley BC (1991) Induction of natural killer activity by xanthene analogues of flavone acetic acid: relation with antitumour activity. *Eur J Cancer* 27(1): 79–83.

Fernando RI, Castillo MD, Litzinger M, Hamilton DH, Palena C (2011) IL-8 signaling plays a critical role in the epithelial–mesenchymal transition of human carcinoma cells. *Cancer Res* 71(15): 5296.

Henare K, Wang L, Wang LC, Thomsen L, Tijomo SM, Chen CJ, Winkler S, Dunbar PR, Print CG, Ching LM (2012) Dissection of stromal and cancer cell-derived signals in melanoma xenografts before and after treatment with DMXAA. *Br J Cancer* 106: 1134–1147.

Huang S, Robinson JB, DeGuzman A, Bucana CD, Fidler II (2000) Blockade of nuclear factor-κB signaling impairs angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. *Cancer Res* 60(19): 5334.

Jaiswal S, Pazoles P, Woda M, Schultz LD, Greiner DL, Brehm MA, Mathew A (2012) Enhanced humoral and HLA-A2-restricted dengue virus-specific T-cell responses in humanized BLT NSG mice. *Immunology* 136: 334–343.

Jassar A5, Suzuki E, Kapoor V, Sun J, Silverberg MB, Cheung L, Burdick MD, Striter RM, Ching LM, Kaiser LR (2005) Activation of tumor-associated macrophages by the vascular disrupting agent 5, 6-dimethylxanthene-4-acetic acid induces an effective CD8 + T-cell-mediated antitumor immune response in murine models of lung cancer and mesothelioma. *Cancer Res* 65(24): 11752–11761.

Joseph WR, Cao Z, Mountjoy KG, Marshall ES, Baguley BC, Ching L-M (1999) Stimulation of tumors to synthesize tumor necrosis factor-α in situ using 5,6-dimethylxanthene-4-acetic acid. *Cancer Res* 59(3): 633–638.

Kanwar JR, Kanwar RK, Pandey S, Ching LM, Krissansen GW (2001) Vascular attack by 5, 6-dimethylxanthene-4-acetic acid combined with B7. 1 (CD80)-mediated immunotherapy overcomes immune resistance and leads to the eradication of large tumors and multiple tumor foci. *Cancer Res* 61(5): 1948–1956.

Kline M, Donovan K, Wellick L, Lutz C, Jin W, Moom-Tasson L, Xiong Y, Wirtzg TE, Kumar S, Rajkumar SV (2007) Cytokine and chemokine profiles in multiple myeloma; significance of stromal interaction and correlation with disease progression. *Leukemia* 31(5): 591–598.

Lara PN, Douillard JY, Nakagawa K, von Pawel J, McKeage MJ, Albert I, Losonczy G, Reck M, Heo DS, Fan X (2011) Randomized phase III placebo-controlled trial of carboplatin and paclitaxel with or without the vascular disrupting agent vadamexin (ASA404) in advanced non-small-cell lung cancer. *J Clin Oncol* 29(22): 2965.

Lash C, Li A, Rutland M, Baguley B, Zwi L, Wilson W (1998) Enhancement of the anti-tumour effects of the antisvascular agent 5, 6-dimethylxanthene-4-acetic acid (DMXAA) by combination with 5-hydroxytryptamine and bioreductive drugs. *Br J Cancer* 78(4): 439.

Lee LF, Hellendall RP, Wang Y, Haskell JS, Mukaida N, Matsushima K, Ting JPY (2000) IL-8 reduced tumorigenicity of human ovarian cancer in vivo due to neutrophil infiltration. *J Immunol* 164(5): 2769.

McKeage MJ, Reck M, Jameson MB, Rosenthal MA, Gibbs D, Mainwaring PN, Freitag L, Sullivan R, Von Pawel J (2009) Phase II study of ASA404 (vadamexin, 5,6-dimethylxanthene-4-acetic acid/DMXAA) 1800 mg/m² combined with carboplatin and paclitaxel in previously untreated advanced non-small-cell lung cancer. *Lung Cancer* 65(2): 192–197.

Melkus MW, Estes JD, Padgett-Thomas A, Gatlin J, Denton PW, Othoieno FA, Geve AJ, Haase AT, Garcia JV (2006) Humanized mouse mount specific adaptive and innate immune responses to EBV and TSST-1. *Nat Med* 12(11): 1316–1322.

Palmer BD, Henare K, Woon ST, Sutherland R, Reddy C, Wang LCS, Kieda C, Ching LM (2007) Synthesis and biological activity of azido analogues of 5, 6-dimethylxanthene-4-acetic acid for use in photoaffinity labeling. *J Med Chem* 50(16): 3757–3764.

Pang JH, Cao Z, Joseph W, Baguley BC, Ching LM (1998) Antitumour activity of the novel immune modulator 5, 6-dimethylxanthene-4-acetic acid (DMXAA) in mice lacking the interferon-gamma receptor. *Eur J Cancer* 34(8): 1282–1289.

Perera PY, Barber SA, Ching LM, Vogel SN (1994) Activation of LPS-inducible genes by the antitumor agent 5, 6-dimethylxanthene-4-acetic acid in primary murine macrophages. Dissection of signaling pathways leading to gene induction and tyrosine phosphorylation. *J Immunol* 153(10): 4684–4697.

Philpott M, Baguley BC, Ching LM (1995) Induction of tumour necrosis factor-α by single and repeated doses of the antitumour agent 5,6-dimethylxanthene-4-acetic acid. *Cancer Chemother Pharmacol* 36: 143–148.

Prantner D, Perkins DJ, Lai W, Williams MS, Sharma S, Fitzgerald KA, Vogel SN (2012) 5,6-Dimethylxanthene-4-acetic acid (DMXAA) activates
Stimulator of Interferon Gene (STING)-dependent innate immune pathways and is regulated by mitochondrial membrane potential. J Biol Chem 287: 39776–39788.

Rewcastle GW, Atwell GJ, Zhuang L, Baguley BC, Denny WA (1991) Potential antitumor agents. 61. Structure-activity relationships for in vivo colon 38 activity among disubstituted 9-oxo-9H-xanthene-4-acetic acids. J Med Chem 34(1): 217–222.

Roberts ZJ, Ching LM, Vogel SN (2008) IFN-β-dependent inhibition of tumor growth by the vascular disrupting agent 5, 6-dimethylxanthenone-4-acetic acid (DMXAA). J Interferon Cytokine Res 28(3): 133–139.

Roberts ZJ, Goutagny N, Perera PY, Kato H, Kumar H, Kawai T, Akira S, Savan R, Van Echo D, Fitzgerald KA (2007) The chemotherapeutic agent DMXAA potently and specifically activates the TBK1–IRF-3 signaling axis. J Exp Med 204(7): 1559.

Sun J, Wang LCS, Fridlender ZG, Kapoor V, Cheng G, Ching LM, Albelda SM (2011) Activation of mitogen-activated protein kinases by 5, 6-dimethylxanthenone-4-acetic acid (DMXAA) plays an important role in macrophage stimulation. Biochem Pharmacol 82: 1175–1185.

Waugh DJ, Wilson C (2008) The interleukin-8 pathway in cancer. Clin Cancer Res 14(21): 6733–6741.

Woo ST, Hung SSC, Wu DCF, Schooltink MA, Sutherland R, Baguley BC, Chen Q, Chamley LW, Ching LM (2005) NF-κB-independent induction of endothelial cell apoptosis by the vascular disrupting agent DMXAA. Anticancer Res 27(1A): 327.

Woo ST, Reddy CB, Drummond CJ, Schooltink MA, Baguley BC, Kieda C, Ching LM (2005) A comparison of the ability of DMXAA and xanthenone analogues to activate NF-κB in murine and human cell lines. Oncology Res 15(6): 351–364.

Zhuang L, Ching LM, Kestell P, Baguley BC (2002) The antitumour activity of 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in TNF receptor-1 knockout mice. Br J Cancer 87(4): 465–470.

Zwi LJ, Baguley BC, Gavin JB, Wilson WR (1994) Correlation between immune and vascular activities of xanthenone acetic acid antitumor agents. Oncology Res 6(2): 79–85.

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)