Immune-modulating and anti-vascular activities of two xanthenone acetic acid analogues: A comparative study to DMXAA

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Abstract. In order to proliferate, solid tumours require the development and continuous expansion of an organised host-derived vascular network. The anti-vascular agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) emerged as derivative of the flavone-8-acetic acid (FAA) and xanthenone-4-acetic acid (XAA). Its anti-vascular activity is not based on direct cytotoxic effects, but is characterized by an immune-mediated component, through the activation of NF-κB pathway, and a direct anti-vascular action, involving the induction of endothelial cell apoptosis and changes in tumour vessel permeability. Despite promising pre-clinical results, DMXAA showed moderate anti-tumour activity in clinical trials. In this study, we compared to DMXAA the *in vitro* immune-modulating and the anti-vascular properties of two XAA analogues, AP/1649 and AP/1897. Their immune-stimulating activities were evaluated on a human monocyte cell line and their anti-vascular activities were studied by measuring the induction of HUVECs apoptosis and using DCE-MRI to determine tumour perfusion following drug treatment. Although the two molecules exerted an immune stimulation comparable to that produced by DMXAA, they showed reduced (AP/1649) or minimal (AP/1897) anti-vascular activity *in vitro*, and no anti-vascular effects *in vivo*. These results endorse the current theories concerning two independent actions exerted by DMXAA.

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Introduction

Tumours can develop in an avascular environment only to a maximum diameter of about 1 mm (1). Anti-vascular approaches have therefore been advanced as tumour treatments, which, by causing shutdown of the established tumour vasculature, can lead to tumour cell death (2). Anti-vascular strategies include the use of integrin-binding peptides conjugated to anticancer drugs (3), antibodies targeted to endothelial cell-specific proteins (4) and gene therapy approaches (5). Two classes of low molecular weight drugs are currently the closest to clinical application: the colchicine-related tubulin-binding agents, such as combretastatin, and drugs related to flavone acetic acid (FAA), such as dimethyl-xanthenone acetic acid (DMXAA). Both cause a rapid and selective shut-down of tumour blood vessels leading to haemorrhage and tumour necrosis (6).

DMXAA (Fig. 1c), is a more potent analogue of the anti-vascular agents flavone acetic acid (FAA, Fig. 1a) and xanthenone acetic acid (XAA, Fig. 1b) (7). The drug acts on a variety of murine tumours and induces extensive vascular collapse and tumour necrosis in human xenograft models (8). DMXAA inhibits tumour blood flow (9,10) supporting the theory that the induction of tumour necrosis results from cessation of blood flow. DMXAA exerts its anti-tumour action through an immune-modulating activity, based on the activation of Natural Killer cells and on the induction of TNF-α. It also induces a broad range of cytokines and other factors involved in immune activation, such as nitric oxide (NO) (11). Several lines of evidence indicate that DMXAA activates the nuclear factor-kappa B (NF-κB) by inactivating its inhibitory factor IκB-α (12). This event is likely a consequence of an allosteric interaction between DMXAA and the IKK kinase protein, which triggers the phosphorylation and destruction of IκB-α (12).

Despite promising pre-clinical results, DMXAA showed only moderate evidence of anti-tumour activity in clinical trials. We have been interested in the synthesis and biological

evaluation of analogues of FAA and XAA. However, among the approximately 80 molecules that have been tested, only a few showed comparable effects to DMXAA (13-19). In this study, we evaluated the activity of two selected XAA-derivatives AP/1649 (18) and AP/1897 (16) (Fig. 1d and e). Their immune-mediated action was studied by assessing their ability to stimulate a human monocyte cell line (MonoMac6 cells, MM6) to exert cytotoxic properties on tumour cells *in vitro*, and to release TNF-α. Their activation of NF-κB was also evaluated by assessing their effects on IκB-α phosphorylation. Their anti-vascular activities *in vitro* were assessed by measuring their ability to induce apoptosis in human umbilical vein endothelial cells (HUVECs). Magnetic resonance spectroscopy (MRS) and dynamic contrast agent enhanced imaging (DCE-MRI) techniques were employed to investigate their *in vivo* anti-tumour and anti-vascular properties in a murine sarcoma model (SaF).

Materials and methods

*Cell lines. C13**. The human ovarian adenocarcinoma cells were provided by Professor C. Marverti (Department of Biomedical Sciences, University of Modena, Italy) and maintained in RPMI-1640 supplemented with FCS, penicillin/streptomycin (Biochrom K.G. Seromed).

MM6. The human monoblastic leukaemia MM6 cells, were supplied by the 'Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy', and maintained in RPMI-1640, supplemented with FCS, penicillin/streptomycin, sodium pyruvate, non-essential amino-acids and bovine insulin (Biochrom K.G. Seromed).

HUVEC. The human umbilical vein endothelial cells were provided by the gynaecological unit at Addenbrooke's hospital, Cambridge (UK) and were maintained in EBM-2® (endothelial cells basal medium-2, Cambrex).

SaF. Murine sarcoma F cells were obtained from the Gray Laboratory, Mount Vernon Hospital, Northwood, UK and maintained in MEM medium supplemented with FCS, glutamine and penicillin/streptomycin (Gibco).

Antiproliferative activity on MM6 cells. MM6 cells were seeded in triplicate ($2x10^4$ cells/well) in 96-well plates and exposed to 25, 50, and 100 μM DMXAA, AP/1649 or AP/1897. After 24 h the cell viability was assessed using a tetrazolium salts reduction-based assay (XTT), carried out with a commercially available kit (Cell proliferation kit II-XTT, Roche) according to manufacturer's instructions.

Stimulation of antiproliferative activity on C13 mediated by MM6 cells*. MM6 cells were seeded treated as above. After 24 h the culture media were removed and the monocytes were co-cultured for 24 h with C13* ($2x10^4$ cells/well). The viability of C13* cells in comparison to the control was assessed using the same XTT test performed in the previous experiment. The percentage inhibition of C13* cell growth was calculated as follows:

$$\frac{\text{Optical density (treated MM6+C13*) - Optical density (MM6)}}{\text{Optical density (C13*)}}$$

A further control of C13* cell co-cultivated with untreated MM6 cells was used.

Stimulation of TNF-α release. MM6 cells were seeded and treated with DMXAA, AP/1649 or AP/1897 at the same concentrations used in the previous experiment. After 4 or 24 h, culture media were collected and stored at -80˚C. A commercially available ELISA kit was used to determine the concentration of TNF-α (Human TNF-α Instant ELISA, Biosource) according to the manufacturer's instructions. The results were normalised to the protein content.

Induction of phosphorylation of IκB-α. MM6 cells were seeded as above and treated with 50 or 100 μM DMXAA or the two XAA-analogues. After 30 min, 1 or 4 h the cells were treated for 30 min with lysis buffer (50 mM Tris-HCl, 1 mM DTT, 1 mM Na_2 EDTA and 1% V/V Triton X-100) supplemented with a protease inhibitor cocktail and PMSF and shaken at 4˚C. After centrifuging the samples, a commercially available ELISA kit [Human IκB-α (pS32) Immunoassay Kit, Biosource] was used to determine the concentration of the S32-phosphorylated IκB-α in the supernatants, according to the manufacturer's instructions. The results were normalised to the protein content.

Protein assay. Protein concentration was determined using the Bio-Rad Protein Assay Dye (Bio-Rad), according to the manufacturer's instructions.

Detection of HUVEC apoptosis in vitro using flow cytometry. 10^6 cells/well were seeded in 6-cm petri dishes and, after 24 h, treated for 24 h with DMXAA, AP/1649 or AP/1897, at concentrations ranging from 25 to 200 μM. Cells were harvested, washed twice with PBS and re-suspended in HEPES-buffered saline [20 mM HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mM NaCl and 2 mM $CaCl_2$, pH 7.5]. 5% V/V Annexin V-FITC (Sigma) and 100 μg/ml propidium iodide (PI, Sigma) were added to each sample, which were then incubated in the dark for 10-15 min at room temperature. Samples were diluted with ice-cold HEPES-buffered saline and run in a FACSCalibur® (Beckton-Dickinson).

Evaluation of the effects on in vivo tumour growth. A suspension of 10^6 SaF cells in PBS was implanted subcutaneously in the dorsal flanks of female *CBA* mice (Charles River). Experiments were performed when the tumour diameter was 12-14 mm. The mice were injected daily i.p. with 27 mg/kg, corresponding to a peak circulating concentration of ~50 μM, DMXAA or analogues AP/1649 or AP/1897, firstly dissolved in DMSO and then diluted with saline to give a final DMSO concentration <5%. The tumour size was measured daily using callipers, and is reported as the product of the two largest perpendicular diameters. The mice were sacrificed 4 days after the beginning of the experiments.

Dynamic contrast agent-enhanced MRI and ^{31}P MR spectroscopy. The same type of mice used in the previous experiment were implanted with the SaF cells as above.

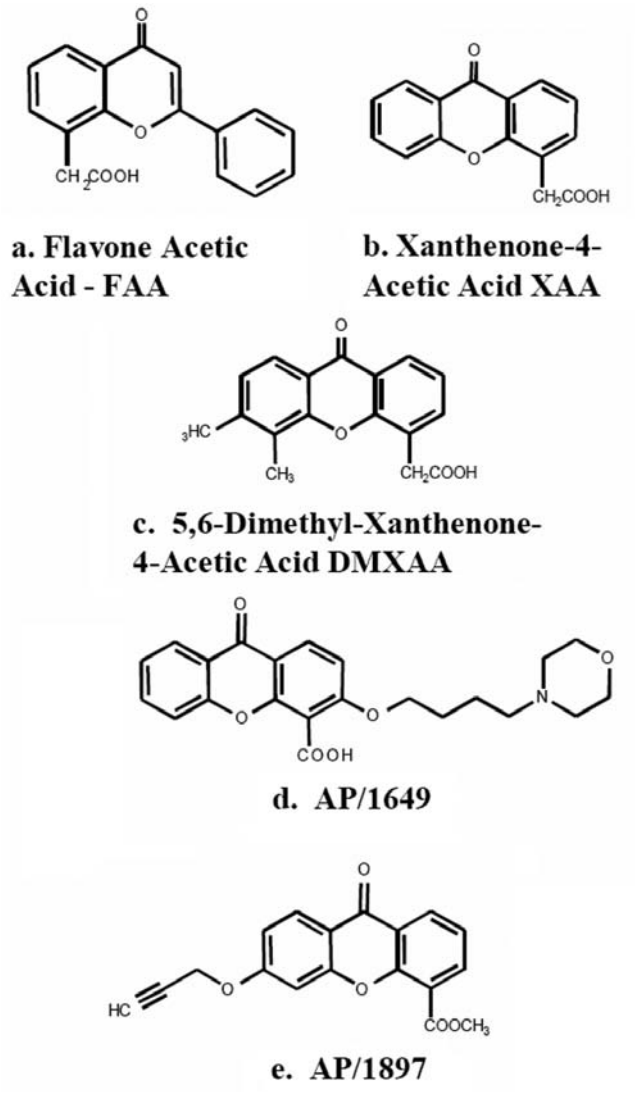

Figure 1. Molecular structures of FAA, XAA, DMXAA and the two XAA-derivatives subject of the study, AP/1649 and AP/1897.

When the tumour diameter was 12-14 mm, the mice were anaesthetised by i.p. injection of 10 ml/kg of a combination of Hypnorm (VetaPharma), Hypnovel (Roche) and dextrose-saline (4%:0.18%) in a ratio of 5:4:31, respectively. The ^{31}P MRS and the contrast agent inflow MRI experiments were carried out at 9.4 T before, 3 and 24 h after i.p. injection of 27 mg/kg DMXAA, or the derivatives AP/1649 or AP/1897. Control animals were treated with the drug solvent, 5% DMSO/saline. Animal procedures were carried out under the authority of project and personal licenses issued by the Home Office, UK.

In the contrast agent inflow experiments, a series of T_1-weighted spin-echo images (repetition time 130 msec, echo time 8 msec, field of view 60x30 mm, data matrix 256x128, slice thickness 1.5 mm) were acquired from the tumours before and for 20 min after injection of 200 μl Gd-DTPA (0.2 mM/kg Magnevist, Schering) in the tail vein. Maps of T_1 relaxation times were acquired at before and after the T_1-weighted MRI (IR-FLASH pulse sequence, 11 inversion times between 50 msec and 10 sec, 10-sec delay between images). Paramagnetic relaxation enhancement (R1p), indicator of the contrast agent uptake following Gd-DTPA injection, was estimated from the observed signal intensities and pre-contrast T_1 values. Contrast agent uptake was quantified as the area under the inflow curve (AUC) for the tumour during the first 20 min after contrast agent injection (20). The ratios of inorganic phosphate (Pi) to nucleoside triphosphate (NTPs) were estimated from tumour-localized ^{31}P MRS spectra (ISIS pulse sequence, 256 averages, 3-sec repetition time, spectral width 10 kHz, 6144 data-points).

Table I. Stimulation of antiproliferative activity on C13* mediated by MM6 cells.

Compound	25 μM	50 μM	100 μM
DMXAA	80%	66%[b]	59%[b]
AP/1649	67%[a]	60%[b]	55%[b]
AP/1897	71%	61%[b]	55%[b]

Percentage versus control of viable C13* cells after 24-h co-culture with MM6 pre-treated with 25, 50 or 100 μM DMXAA, AP/1649 or AP/1897. As control, C13* cells not co-cultivated with MM6 were used. [a]$p<0.05$. [b]$p<0.01$, p-values were calculated vs the control.

Statistical analysis. For experiments *in vitro*, at least three different experiments were performed for each drug dose. For experiments *in vivo* at least five animals for each compound or control were used. Results were analysed using Student's t-test for comparisons between two groups and analysis of variance (ANOVA) with Dunnett's *post hoc* correction for multiple comparisons when more than two groups were compared.

Results

Antiproliferative activity on MM6 cells. Neither DMXAA nor the two XAA-analogues showed any significant cytotoxic effect on MM6 cells in comparison to non-treated cells (data not shown).

Stimulation of antiproliferative activity on C13 mediated by MM6 cells*. The percentage viabilities of C13* cells co-cultivated with pre-treated MM6 monocytes are shown in Table I. A similar dose-dependent decrease of C13* cell proliferation resulted from the treatment of human MM6 cells with 50 and 100 μM DMXAA or the XAA-analogues, AP/1649 and AP/1897. The analogues also significantly inhibited C13* cell proliferation at 25 μM.

Induction of TNF-α release by MM6. The TNF-α released into the culture medium of MM6 cells that had been treated with the three compounds for 4 h was inversely related to the dose (Fig. 2a). MM6 cells that had been treated with 25 μM DMXAA, AP/1649 or AP/1897 led to levels of the cytokine that were 3.6-, 2.1- and 5.5-fold higher, respectively, than the control. Only derivative AP/1897 maintained a significant activity at 50 μM. When the cells were treated for 24 h, the levels of TNF-α after exposure to DMXAA decreased

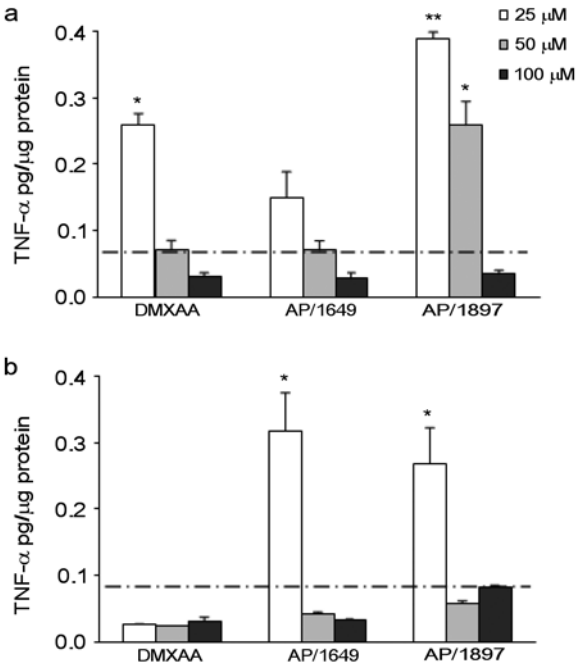

Figure 2. Concentration of TNF-α (pg TNF-α/mg proteins) in MM6 supernatants after 4- (a) or 24- (b) h treatment with 25, 50 or 100 μM DMXAA AP/1649 or AP/1897. Dotted lines, TNF-α content in control samples (not treated MM6 cells). $^{*}p<0.05$; $^{**}p<0.01$; p-values were calculated vs the control.

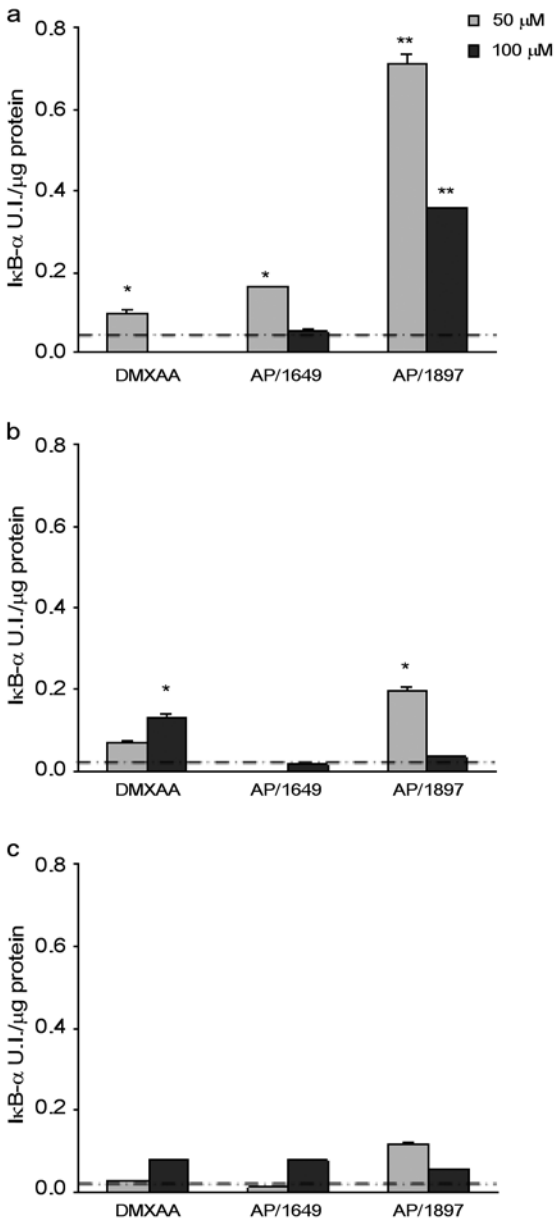

Figure 3. Concentration of phosphorylated IκB-α (U.I. p-IκB-α/mg proteins) in MM6 lysates, after ½- (a), 1- (b) or 4- (c) h treatment with 50 or 100 μM DMXAA AP/1649 or AP/1897. Dotted lines, phosphorilated IκB-α content in control samples (not treated MM6 cells). $^{*}p<0.05$; $^{**}p<0.01$.

dramatically, whereas in the supernatants of the cells treated with 25μM AP/1649 and AP/1897 significant levels of cytokine were still detected.

Induction of IκB-α phosphorylation in MM6 cells. Generally, the results showed differences in the activities of the two XAA-analogues in comparison to DMXAA. At 50 μM AP/1649 rapidly and significantly induced IκB-α phosphorylation, but this effect also faded quickly within the first hour after exposure (Fig. 3a and b). AP/1897 produced the largest and most rapid induction of IκB-α phosphorylation, and this effect faded away more slowly in comparison to AP/1649, being still detectable after 4 h of treatment (Fig. 3). DMXAA showed lower activity in comparison to the XAA-analogues and the maximum effect was detected after 1-h exposure, but decreased within 4 h.

Detection of HUVEC apoptosis in vitro using flow cytometry. Both DMXAA and AP/1649 induced significant levels of apoptosis versus the control, at concentrations ranging from 50 to 200 μM (Fig. 4). The maximum effect (~30% apoptotic cells) was obtained, for both compounds, at the highest concentration tested. DMXAA at 25 μM was able to exert an apoptotic/toxic effect on HUVECs and was significantly more effective than AP/1649 at 50 and 150 μM. No significant induction of apoptosis was detected after the treatment with AP/1897 at concentrations up to 150 μM.

Evaluation of the effects on in vivo tumour growth. Only the treatment with DMXAA led to a significant delay in tumour growth, in comparison to the control tumours. The growth of the tumours treated with the two derivatives was generally comparable to that obtained for the control (data not shown).

In vivo ^{31}P MRS on SaF tumours. Changes in tumour energy levels were detected by investigating the levels of phosphorous metabolites in SaF tumours, before and at 3 or 24 h after drug treatment. Example spectra of an untreated mouse (Fig. 5a) and three mice treated with DMXAA (Fig. 5b), AP/1649 (Fig. 5c) or AP/1897 (Fig. 5d) are shown in Fig. 5. The effect of the drugs on tumour cell energy status was assessed from measurements of the Pi-to-NTP ratio (Fig. 6a), which increases when cellular energy status deteriorates.

Only treatment with DMXAA was able to exert significant effects on the energy status of the tumour tissue. There were significant increases in the Pi/NTP ratios after 3 and 24 h of

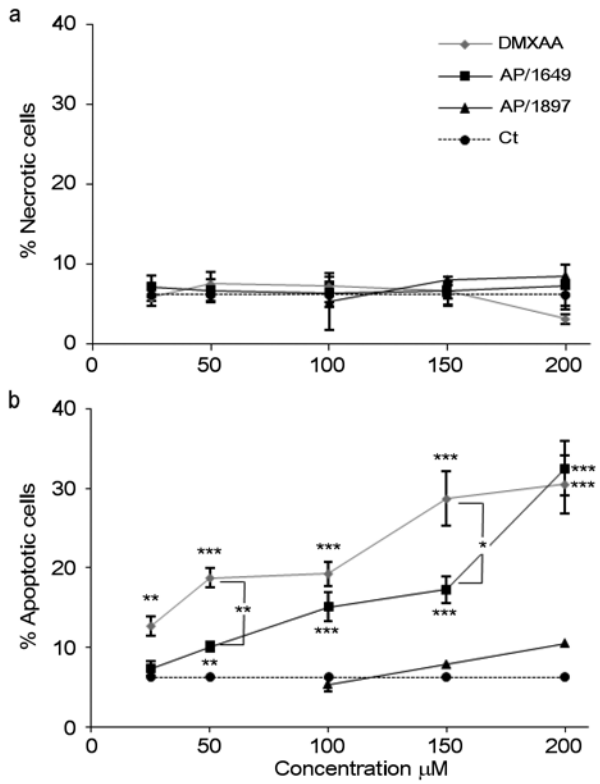

Figure 4. Percentage of necrotic (a) and apoptotic (b) HUVECs cells after 24-h exposure to DMXAA, AP/1649 or AP/1897 ranged from 25 to 200 μM. As control, non treated cells were used. *p<0.05; **p<0.01; ***p<0.001. If not specified, p-values were calculated vs the control.

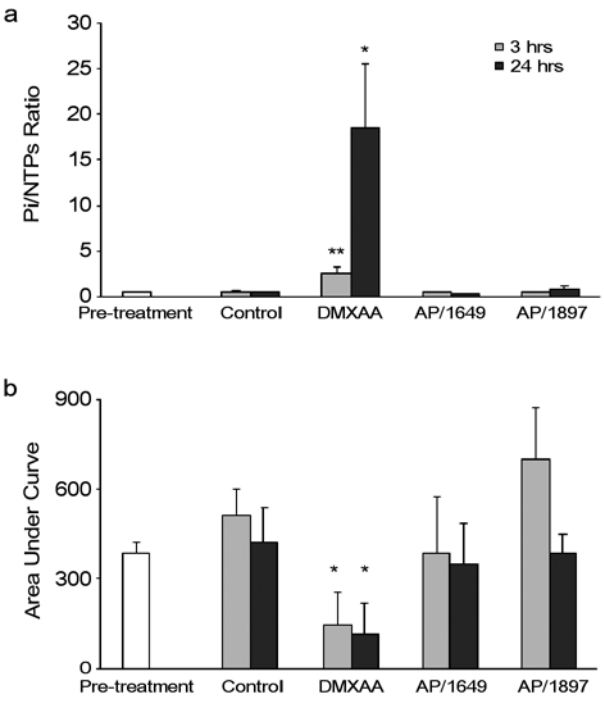

Figure 6. Pi to NTPs ratio of the peaks area in ^{31}P NMR spectra (a) and area under the curve (AUC) of the Gd-DTPA inflow data (change in R_{1p} over time) during the first 20 min after injection (b) in mice-implanted SaF tumors 3 and 24 h after treatment with 27 mg/kg animal, DMXAA, AP/1649 or AP/1897 dissolved in 5% DMSO saline. Control tumours were treated with 5% DMSO saline only. *p<0.05; **p<0.01. p-values were calculated vs the control.

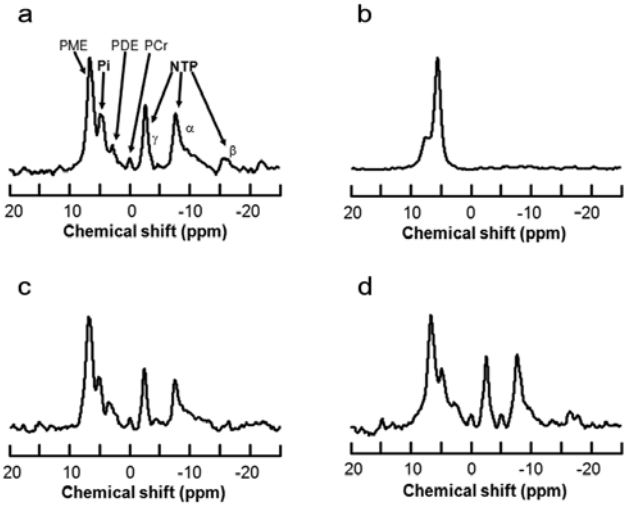

Figure 5. Examples of ^{31}P spectra of SaF tumours not treated (a) or 24 h after the treatment with DMXAA (b), AP/1649 (c) or AP/1897m (d) and location of the metabolite peaks (spectra not in scale).

drug treatment. In particular, compared to the pre-treated tumours, DMXAA led to Pi/NTP ratios that were 6-fold higher at 3 h after treatment and 40-fold higher at 24 h. The tumours treated with AP/1649 or AP/1897 did not show significant changes in energy status.

Measurement of in vivo contrast agent inflow in SaF tumours. Changes in tumour perfusion were detected by analysis of contrast agent inflow in SaF tumours, before and after drug treatment. The post-treatment investigation was carried out on the same animals used in the previous experiment and the results are expressed as the AUC of the contrast agent inflow versus time.

Treatment with DMXAA led, after both time-points, to a decrease in contrast agent inflow, corresponding to AUC values of 145.35 and 113.20 after 3 and 24 h, respectively, versus 383.70, for the pre-treatment tumours (Fig. 6b). No significant differences in the AUC values were detected following treatment with AP/1649 or AP/1897.

Discussion

The anti-vascular approach, as a potential strategy for cancer treatment, was based on the observation that physical obstruction of tumour blood vessels led to tumour regression in mice (2). Vascular targeting (VTAs) and disrupting (VDAs) agents lead to a rapid and extensive shutdown of established tumour vasculature and to tumour cell death. A number of new VDAs have been developed which show activity but are not too toxic (21,22). The interest in structure activity relationship (SAR) studies with FAA and XAA derivatives arose from their unusual anti-tumour effects, which involve activation of the immune system and damage to the tumour vascular network. Since FAA failed in clinical trials (23), research focused on the development of derivatives that are effective in humans (24). The potential effectiveness of this approach was confirmed by DMXAA (25-27) which is the most potent XAA analogue discovered and which is

currently in phase II clinical trials (28). DMXAA has been the most widely studied derivative, although its molecular target and mechanism of action are not completely clear. In order to find molecules with an improved anti-tumour and anti-vascular activity we have studied several series of FAA and XAA derivatives (13-19). Here we describe the *in vitro* immune-modulating activity and the *in vitro* and *in vivo* anti-vascular properties of two XAA-analogues, AP/1649 AP/1897, using DMXAA as a reference.

Generally, both molecules showed a similar ability to modulate the activity of human monocytes and the results obtained were at least comparable to those obtained with DMXAA. In particular, both showed an ability to induce cytotoxic activity of MM6 towards human ovarian adeno-carcinoma cells at non-toxic concentrations for MM6 cells. Both were able to stimulate the NF-κB pathway but with different net effects. AP/1897 caused MM6 cells to produce an increased release of TNF-α in comparison to DMXAA, which correlated with the strongly enhanced induction of IκB phosphorylation. AP/1649 led to only a moderate release of TNF-α, which correlated with a slightly increased induction of IκB phosphorylation. The maximum release of cytokine was obtained with the exposure to the lowest concentration in a dose-inverse manner. The results confirmed what obtained in a previous study on murine splenocytes which showed that 35 μM was the dose exerting the maximum effect (29). This apparently contradictory result could be explained by the negative feedback exerted by both TNF-α and IκB on NF-κB pathway when over-stimulated, and with the evidence that TNF-α interacts with his receptors triggering a maximal cellular response when as little as 10% of the cell membrane receptors are occupied (30).

Both compounds were able to induce significant levels of IκB phosphorylation in comparison to DMXAA, AP/1897 being particularly effective in this respect. Both of the selected XAA analogues are able to activate the mechanisms that induce synthesis of TNF-α in human monocytes. Furthermore, in the supernatants of the cells treated for 24 h with 25 μM AP/1649 and AP/1897, significant levels of TNF-α were detected, whereas after exposure for 24 h to DMXAA the levels of the cytokine decreased dramatically. This suggests that the two derivatives could sustain the induction of the cytokine for a longer period compared to DMXAA, and that after 24 h most of the TNF-α induced by DMXAA undergoes degradation.

The investigation of the anti-tumour and anti-vascular properties of the two molecules produced conflicting results. Whilst DMXAA had significant anti-vascular effects, both *in vitro* and *in vivo*, the two XAA analogues showed only partial or even no evidence of any ability to target the tumour vessels. They were not able to inhibit the tumour growth, neither did they have any effect on tumour energy status, as assessed using ^{31}P MRS, nor on vascular function, as determined using DCE-MRI. In contrast, DMXAA delayed the tumour growth, lowered tumour NTP levels, and damaged the tumour vascular network, within 3 h of drug injection. The early inhibition of tumour blood flow, together with the large changes in tumour energy status, suggest that DMXAA causes shutdown of the tumour blood vessels and later a consequent generalised damage of the tumour tissue.

Given the significant immune-stimulation detected *in vitro* for both AP/1649 and AP/1897, their lack of activity in the *in vivo* tumour model may be explained by their relative failure to induce apoptosis in HUVECs, when compared with DMXAA. AP/1897 was almost completely inactive, and caused only a slight induction of apoptosis at 200 μM, and although AP/1649 induced effective apoptotic effects at 50 μM, DMXAA was significantly more effective than AP/1649 at 50 and 150 μM. These results suggest that the XAA-derivative should be able to exert the same anti-vascular effect as DMXAA, but only if used at remarkably higher concentrations. Although the immune-mediated effects exerted by DMXAA play a pivotal role in its anti-tumour action, the direct anti-vascular component of its mechanism of action has been demonstrated as being independent of immune-stimulation, as DMXAA showed significant activity in TNF-α receptor-1 knock-out mice (31). The partial or complete lack of activity shown by AP/1649 and AP/1897 in the induction of HUVEC apoptosis *in vitro*, and their failure to disrupt tumour blood vessels *in vivo* support and strengthen this hypothesis. However, several external factors, not strictly related to the mechanism of action of the two molecules, but affecting drug action *in vivo*, should be considered. Variations in their pharmacokinetic could lead, for example, to decreased absorption, incomplete distribution or an increased metabolism, affecting their concentration in the tumour tissue.

In conclusion, although promising results were obtained on the *in vitro* immune-stimulation, the two XAA-analogues did not show any improvement in the anti-vascular and anti-tumour activities when compared to DMXAA. This loss of effectiveness is probably due to the partial or total loss of an anti-vascular action, for AP/1649 and AP/1897 respectively. This result shows that immune-stimulation *per se* is not enough to cause damage of the tumour tissue, and endorses the theory that the activity of DMXAA is based on two different actions, immune-mediated toxicity and vascular disruption, which are independent and both are necessary to efficiently induce tumour regression.

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