Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 14 (2006) 4101–4109
Bioorganic & Medicinal Chemistry

New derivatives of xanthenone-4-acetic acid: Synthesis, pharmacological profile and effect on TNF-α and NO production by human immune cells

Silvia Gobbi,[a,*] Federica Belluti,[a] Alessandra Bisi,[a] Lorna Piazzi,[a] Angela Rampa,[a] Antonella Zampiron,[b] Mariagnese Barbera,[b] Anna Caputo[b] and Maria Carrara[b]

[a]*Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy*
[b]*Department of Pharmacology and Anaesthesiology, University of Padova, Largo Meneghetti 2, 35131 Padova, Italy*

Received 27 October 2005; revised 27 January 2006; accepted 3 February 2006
Available online 20 February 2006

Abstract—New derivatives of xanthenone-4-acetic acid, bearing an alkoxy chain of variable length and a basic moiety, were synthesised in order to test the influence of this additional function on antitumour activity. The introduction of bulky substituents carrying a basic nitrogen seems to be somewhat tolerated, since for some of the compounds the enhancement of lytic potential of human monocytes was comparable to that of the reference molecule DMXAA. The induction of the release of TNF-α and nitric oxide by human monocytes, as well as the hypothesis of a potentiation of the activity of lipopolysaccharide in the induction of those cytotoxic factors, was also evaluated. In this respect, the most interesting compound (**6a**) exhibited the same spectrum of biological activity shown by DMXAA and seems therefore to be endowed with the same mechanism of action of the reference compound.

1. Introduction

Xanthenone-4-acetic acid[1] (XAA, Chart 1) is a derivative of flavone-8-acetic acid[2] (FAA, Chart 1), a very interesting compound endowed with a peculiar antitumour profile in murine models, being remarkably active on solid tumours[3] although not extremely potent, which did not exhibit any significant activity in subsequent clinical trials.[4] XAA is structurally closely related to FAA and proved to be a more promising lead, showing considerably higher potency. Extensive SAR studies were performed on this molecule and the results of studies on mono- and di-substituted XAA analogues[5,6] suggested that activity was primarily dependent on the position of the substituents more than on their nature, being positions 5 and 6 the most favourable for substitution, especially with small lipophilic groups: 5,6-dimethyl-xanthenone-4-acetic acid (DMXAA, Chart 1) proved to be the most potent compound synthesised, showing activity on human models,[7] and it is now undergoing clinical trials.[8] The antitumour activity of these compounds is known to be due to indirect effects more than to direct cytotoxicity, involving both the host immune system (enhancement of lytic properties of macrophages and activity of NK cells) and the vascular system (haemorrhagic necrosis of tumour vasculature) through induction of a number of cytokines, such as tumour necrosis factor α (TNF-α) and interferons.[9,10] There is also evidence for a dose-dependent increase in plasma nitrite plus nitrate (NO_2^-/NO_3^-) concentrations in mice following administration of FAA and related drugs, which could contribute to tumour cell death by alteration of blood flow and direct cell killing.[11] In addition, DMXAA was shown to induce early changes in tumour vascular endothelial cells, which can be considered as an indication of apoptosis.[12] The mechanism of action of this class of compounds has not been elucidated yet

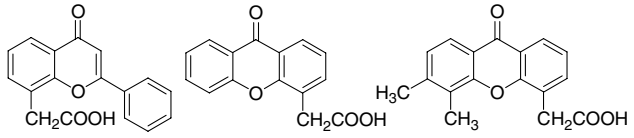


Chart 1. Reference structures.

Keywords: DMXAA; Human monocytes; TNF-α; Nitric oxide.
* Corresponding author. Tel.: +39 051 2099732; fax: +39 051 2099734; e-mail: silvia.gobbi@unibo.it

0968-0896/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2006.02.003

and their biological target is still unknown, but there is substantial evidence that the activation of nuclear-factor κB (NFκB) is involved. This is thought to be the main transcription factor leading to production of TNF and other cytokines[13] and DMXAA was shown to induce its activation in monocytes,[14] vascular endothelial cells[12] and different tumour cells.[15]

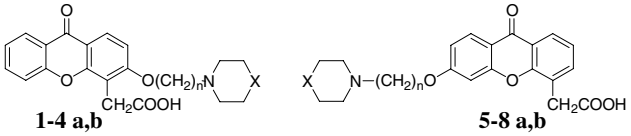
Our research group has been interested for some years in the synthesis and biological evaluation of analogues of FAA and XAA.[16] In a recent paper,[17] we reported the synthesis and biological activity of new analogues of XAA in which substituents in positions 5 and 6, the most favourable for an increase in activity, were grouped in cyclic structures, and of their synthetic intermediates bearing different alkoxy groups in position 6. In the present paper, we further investigate the SAR of this series and, in particular, the substitution in position 6 by introducing an alkoxy chain of variable length carrying a basic nitrogen (piperidine or morpholine) in order to test the influence of this additional function on the activity. For a deeper investigation of the influence of this addition on the whole molecule, the same functional groups were also introduced in position 3 of the xanthone nucleus.

The structures of the synthesised compounds are presented in Table 1.

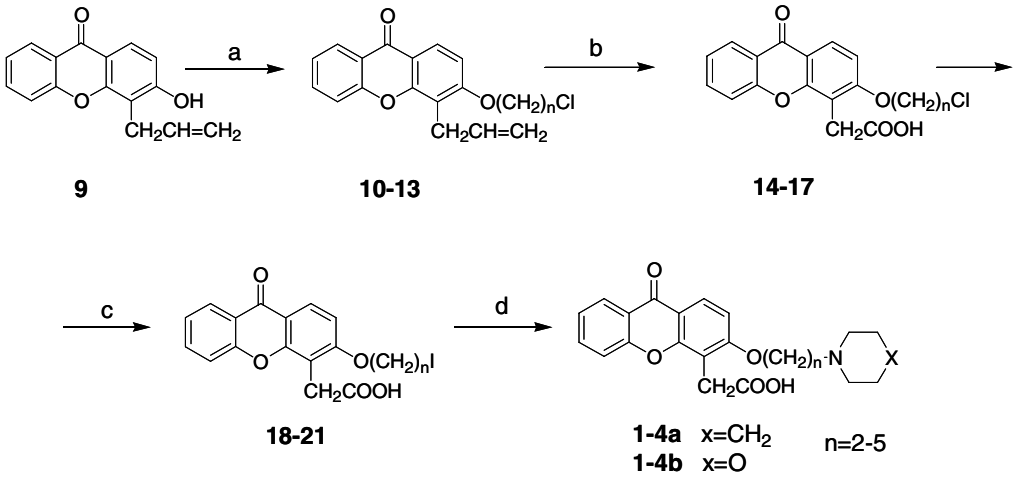
2. Chemistry

The synthesis of 3-alkoxyxanthones **1**–**4a**,**b** is reported in Scheme 1. 3-Hydroxy-4-allylxanthenone[18] was alkylated with the selected 1-bromo-ω-chloroalkane in the presence of potassium carbonate to obtain the corresponding ω-chloroalkoxy derivative, and the allyl group was oxidized to the carboxylic acid with $KMnO_4$. By heating with NaI the chlorine atom was substituted by an iodine and the compounds were then reacted with piperidine or morpholine to give the desired derivatives. 6-Alkoxyxanthones **5**–**8a**,**b** were prepared according to Scheme 2. The methyl ester of 6-hydroxyxanthone-4-acetic acid[17] was alkylated with the selected 1-bromo-ω-chloroal-

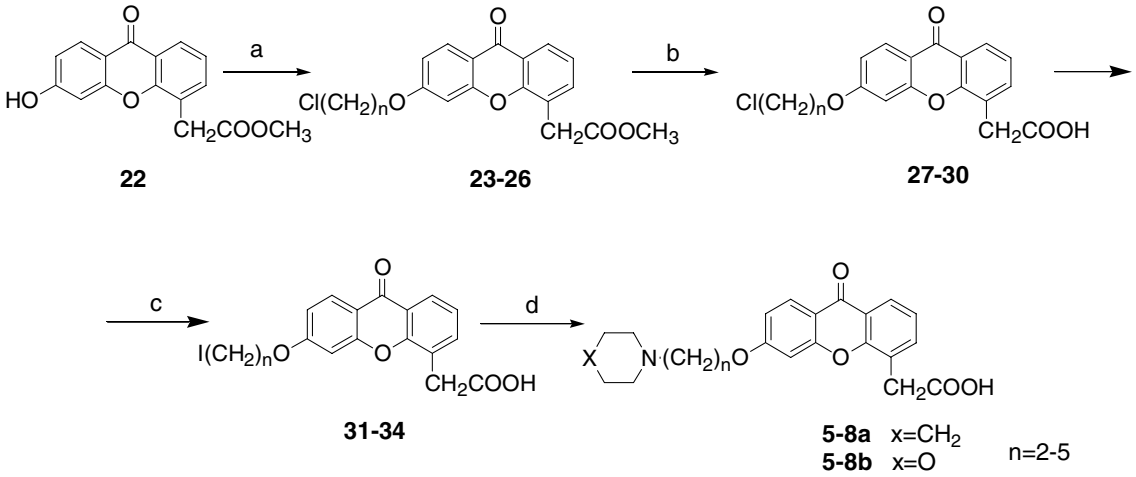
Table 1. Analytical data of the target compounds



Compound	*n*	X	Yield (%)	mp (°C)	^{1}H NMR δ (DMSO-d_6) and MS
1a	2	CH_2	50	217–219	1.35–1.40 (m, 2H); 1.50–1.55 (m, 4H); 2.45–2.50 (m, 4H); 2.75 (t, 2H); 3.85 (s, 2H); 4.35 (t, 2H); 7.25–8.20 (m, 6H). *m/z*: 381 (M^+, 5), 252 (29), 224 (30), 98 (100). Anal. C, H, N
1b	2	O	60	214–217	($CDCl_3$): 2.90–2.95 (m, 4H); 3.15 (t, 2H); 3.90–3.95 (m, 4H); 4.15 (s, 2H); 4.45 (t, 2H); 6.95–8.35 (m, 6H).*m/z*: 383 (M^+, 4), 252 (9), 100 (100). Anal. C, H, N
2a	3	CH_2	50	216–218	1.35–1.40 (m, 2H); 1.50–1.60 (m, 4H); 1.90–1.95 (m, 2H); 2.35–2.45 (m, 6H); 3.85 (s, 2H); 4.25 (t, 2H); 7.10–8.10 (m, 6H).*m/z*: 395 (M^+, 6), 252 (100), 224 (96), 98 (100). Anal. C, H, N
2b	3	O	65	212–214	($CDCl_3$): 2.10–2.15 (m, 2H); 2.80–2.85 (m, 6H); 3.75 (m, 4H); 3.90 (s, 2H); 4.15 (t, 2H); 6.90–8.25 (m, 6H). *m/z*: 397 (M^+, 5), 252 (18), 224 (15), 100 (100). Anal. C, H, N
3a	4	CH_2	50	162–165	1.45–1.50 (m, 2H); 1.65–1.70 (m, 4H); 1.80–1.85 (m, 4H); 2.65–2.75 (m, 6H); 3.85 (s, 2H); 4.25 (t, 2H); 7.25–8.20 (m, 6H). *m/z*: 409 (M^+, 4), 252 (28), 224 (25), 98 (100). Anal. C, H, N
3b	4	O	75	163–165	1.60–1.65 (m, 2H); 1.75–1.80 (m, 2H); 2.25–2.40 (m, 6H); 3.55–3.65 (m, 4H); 3.85 (s, 2H); 4.15 (t, 2H); 7.25–8.15 (m, 6H). *m/z*: 411 (M^+, 3), 252 (15), 226 (19), 100 (100). Anal. C, H, N
4a	5	CH_2	70	203–205	1.50–1.55 (m, 4H); 1.65–1.85 (m, 8H); 2.85 (t, 2H); 2.95–3.00 (m, 4H); 3.70 (s, 2H); 4.15 (t, 2H); 7.15–8.15 (m, 6H). *m/z*: 423 (M^+, 3), 154 (17), 98 (100). Anal. C, H, N
4b	5	O	60	212–214	1.50–1.55 (m, 2H); 1.65–1.85 (m, 4H); 3.05–3.15 (m, 6H); 3.75–3.85 (m, 6H); 4.25 (t, 2H); 7.25–8.15 (m, 6H). *m/z*: 425 (M^+, 3), 252 (37), 224 (32), 100 (100). Anal. C, H, N
5a	2	CH_2	10	188–190	1.40–1.45 (m, 6H); 2.40–2.45 (m, 4H); 2.60–2.65 (m, 2H); 3.95 (s, 2H); 4.20–4.25 (m, 2H); 7.05–8.10 (m, 6H). *m/z*: 381 (M^+, 25), 270 (86), 225 (100), 180 (78). Anal. C, H, N
5b	2	O	10	162–164	($CDCl_3$): 2.25–2.30 (m, 2H); 2.40–2.45 (m, 4H); 3.60–3.65 (m, 4H); 3.95 (s, 2H); 4.20–4.25 (m, 2H); 7.05–8.10 (m, 6H). *m/z*: 383 (M^+, 21), 323 (34), 251 (100), 225 (48). Anal. C, H, N
6a	3	CH_2	23	107–108	1.40–1.45 (m, 2H); 1.50–1.55 (m, 4H); 1.90–1.95 (m, 2H); 2.35–2.45 (m, 6H); 4.05 (s, 2H); 4.20–2.25 (m, 2H); 7.00–8.10 (m, 6H). *m/z*: 395 (M^+, 3), 226 (45), 197 (100). Anal. C, H, N
6b	3	O	10	108–110	1.90–1.95 (m, 2H); 2.40–2.50 (m, 6H); 3.60–3.65 (m, 4H); 3.95 (s, 2H); 4.20–4.25 (m, 2H); 7.05–8.10 (m, 6H). *m/z*: 397 (M^+, 44), 225 (100), 197 (60), 139 (86). Anal. C, H, N
7a	4	CH_2	10	177–179	1.45–1.80 (m, 10H); 2.40–2.50 (m, 6H); 4.05 (s, 2H); 4.20–4.25 (m, 2H); 7.05–8.10 (m, 6H). *m/z*: 409 (M^+, 3), 365 (100), 226 (87), 209 (91). Anal. C, H, N
7b	4	O	30	171–173	1.60–1.65 (m, 2H); 1.80–1.85 (m, 2H); 2.35–2.40 (m, 6H); 3.60–3.70 (m, 4H); 4.00 (s, 2H); 4.20–4.25 (m, 2H); 7.05–8.10 (m, 6H). *m/z*: 411 (M^+, 7), 380 (100), 366 (52). Anal. C, H, N
8a	5	CH_2	25	118–120	1.40–1.45 (m, 4H); 1.60–1.70 (m, 6H); 1.80–1.85 (m, 2H); 2.70–2.80 (m, 6H); 4.05 (s, 2H); 4.20–4.25 (m, 2H); 7.05–8.10 (m, 6H). *m/z*: 423 (M^+, 3), 197 (15), 98 (100). Anal. C, H, N
8b	5	O	78	86–88	($CDCl_3$): 1.35–1.40 (m, 2H); 1.70–1.80 (m, 4H); 2.60–2.65 (m, 2H); 2.80–2.90 (m, 4H); 3.80–3.90 (m, 4H); 3.95 (s, 2H); 4.00–4.05 (m, 2H); 6.90–8.20 (m, 6H). *m/z*: 425 (M^+, 3), 381 (100), 350 (85), 338 (56). Anal. C, H, N



Scheme 1. Reagents and conditions: (a) $Br(CH_2)_nCl$, K_2CO_3, acetone, reflux; (b) $KMnO_4$, H_2O/acetic acid/acetone, 0–5 °C, 6 h; (c) NaI, methylethylketone, reflux; (d) piperidine or morpholine, toluene, reflux.



Scheme 2. Reagents and conditions: (a) $Br(CH_2)_nCl$, K_2CO_3, acetone, reflux; (b) HCl 6 N; (c) NaI, methylethylketone, reflux; (d) piperidine or morpholine, toluene, reflux.

kane, the ester group was hydrolysed and the chlorine atom was further substituted by an iodine. Subsequent reaction with piperidine or morpholine gave the desired compounds.

3. Biological evaluation

In order to define the pharmacological profile of these compounds, their antiproliferative activity towards two human tumour cell lines was assessed, in particular the human ovarian adenocarcinoma cell line 2008 and the cisplatin-resistant subline C13*, considering the remarkable activity of this class of compounds on solid tumours. Their human monocytes-mediated cytotoxicity was also studied, pre-treating human peripheral blood mononuclear cells (HPBMC) with DMXAA or its analogues and, subsequently, considering the cytotoxicity on C13* cells.

Since cytokines' production is believed to be involved in host-mediated activity of DMXAA,[14] the induction of the release of TNF-α and NO by human monocytes was measured. It is well known that LPS stimulates TNF-α production and NO synthesis by various mechanisms, among which NF-κB translocation and NOS II induction.[19] LPS binds to a sieric protein named 'LPS binding protein' that in turn binds to the membrane receptor CD14, initiating a series of reactions which culminate in the nuclear translocation of NF-κB, which induces the transcription of various inflammatory cytokine genes.[20] In order to investigate deeper the effects of the new compounds, the hypothesis of a potentiation of the activity of lipopolysaccharide in the induction of those cytotoxic factors resulting from the association of selected compounds with LPS was considered.

4. Results

4.1. Antiproliferative activity

The new derivatives exhibited negligible inhibitory effects (Table 2). Only compounds **1a** and **6a** were able

Table 2. Antiproliferative activity on 2008 and C13* cells and indirect mediated activity on C13* cells co-cultured with human monocytes (HPBMC) pre-treated with the new compounds

Compound	IC_{50} (μM) 2008	PR versus IC_{50} DMXAA	IC_{50} (μM) C13*	PR versus IC_{50} DMXAA	IC_{50} (μM) C13* + HPBMC	PR versus IC_{50} DMXAA	PR versus IC_{50} C13*
DMXAA	448.1 (395.9–507.3)	1.0	641.8 (511.1–805.7)	1.0	136.6 (105.0–172.6)	1	4.7
1a	406.8 (316.3–523.0)	1.1	816.1 (642.7–1036.2)	0.8	nd	nd	nd
1b	815.1 (611.6–1086.3)	0.5	831.4 (628.3–1110.2)	0.8	156.5 (210.3–121.7)	0.9	5.3
2a	513.9 (421.0–627.3)	0.9	715.9 (557.8–918.8)	0.9	nd	nd	nd
2b	531.5 (437.1–646.3)	0.8	676.4 (536.1–853.4)	0.9	213.3 (290.9–156.4)	0.6	3.2
3a	466.8 (411.0–530.0)	0.9	567.3 (464.8–692.5)	1.1	140.9 (110.8–179.2)	1.0	4.0
3b	488.9 (427.4–559.1)	0.9	633.6 (567.4–799.5)	1.0	133.8 (108.7–164.9)	1.0	4.7
4a	611.4 (495.5–754.4)	0.7	610.1 (507.4–733.6)	1.0	nd	nd	nd
4b	534.2 (437.8–651.8)	0.8	592.6 (494.0–711.0)	1.1	nd	nd	nd
5a	599.6 (500.4–718.6)	0.7	nd	nd	nd	nd	nd
5b	640.1 (515.0–773.1)	0.7	nd	nd	nd	nd	nd
6a	438.6 (376.2–511.5)	1.0	767.5 (610.2–1143.8)	0.8	99.8 (83.1–119.9)	1.3	7.7
6b	640.1 (530.0–762.1)	0.7	nd	nd	nd	nd	nd
7a	565.1 (480.7–664.4)	0.8	882.0 (823.4–1679.8)	0.7	nd	nd	nd
7b	689.9 (566.1–840.7)	0.6	nd	nd	nd	nd	nd
8a	894.2 (695.9–1146.1)	0.5	nd	nd	517.1 (916.8–291.6)	0.3	nd
8b	566.8 (475.0–676.3)	0.8	nd	nd	nd	nd	nd

PR, Potency ratio; nd, not detectable.

to inhibit cell viability at a dose of 250 μM and only on 2008 cells, while for most of the derivatives (**7b**, **8a**, **8b** and **6b** on C13* cell line, **5b** and **5a** on both cell lines) a significant cytotoxic effect was only seen at the maximum tested dose (500 μM). Generally, the two cell lines showed different responses to the tested compounds, 2008 cells being more sensitive than C13* cells, and the antiproliferative capability of the compounds was comparable to that of DMXAA taken as reference.

4.2. Human monocytes-mediated toxicity

Indirect mediated activity was measured as cytotoxicity on C13* cells co-cultured with HPBMC pre-treated with the new compounds. Results of Table 2 showed that DMXAA was characterized by a significant ability to enhance the lytic properties of human monocytes, showing a 5-fold lower IC_{50}value with respect to direct toxicity. Like the reference compound, 5 out of 16 analogues induced an overall remarkable increase of the mediated toxicity, showing a decrease of the IC_{50} values ranging between 3- and 8-fold. In particular, considering the 3-alkoxyxanthones, compounds **1b**, **2b**, **3a** and **3b** proved to be able to significantly enhance the lytic properties of HPBMC, showing IC_{50} values 5.3, 3.2, 4.7 and 4.0 times lower than those of direct cytotoxicity, respectively. On the contrary, the analogues **1a**, **2a**, **4a** and **4b** were not able to influence the HPBMC activity. Among the 6-alkoxyxanthones, only derivative **6a** showed activity comparable to that of DMXAA. It is interesting to note that this compound was a little more potent (1.3 times) than the reference compound, but showed a significant enhancement of the lytic properties of HPBMC, showing an IC_{50} value 8.8 times lower than that of its direct cytotoxicity. Taking into account the other 6-alkoxyxanthone analogues, only **8a** was able to activate monocytes, but its activity became a lot lower than that of DMXAA.

When the same assay was performed in association with LPS (Fig. 1 and Table 3), only DMXAA improved the HPBMC lytic activity, becoming 3 times more potent than it was when used alone. Among the selected compounds, **6a** displayed the same ability to increase its indirect cytotoxicity when used in association, while the activity of **3a** and **3b** was remarkably reduced. In particular, **3a** completely lost its activity, whereas the indirect cytotoxicity of derivative **3b** was considerably reduced.

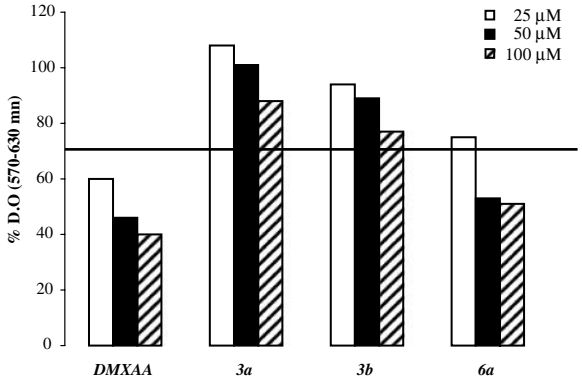


Figure 1. Proliferation of C13* cells co-cultivated on HPBMC pre-treated with DMXAA or new compounds with LPS. $*P < 0.05$; $**P < 0.01$.

Table 3. Human monocytes-mediated toxicity on C13* cells of DMXAA or new compounds with LPS

Compound	IC_{50} (μM)	PR versus DMXAA
DMXAA	45.6 (29.5–70.6)	1
3a	nd	nd
3b	366.6 (229.7–585.3)	0.1
6a	86.8 (61.8–121.8)	0.5

PR, Potency ratio; nd, not detectable.

4.3. TNF-α production

The HPBMCs' TNF production and release after 24 h exposure to DMXAA and selected compounds **3a**, **3b**

and **6a**, with or without LPS, was measured (Fig. 2a). When used alone, neither DMXAA nor the two derivatives substituted in position 3 (**3a** and **3b**) were able to stimulate TNF-α production, but cytokine production was also lower than control. It should be noted that, while compounds **3a** and **3b** seemed to inhibit TNF-α release in a dose-dependent manner, the inhibition induced by DMXAA was inversely dose-correlated. Very interesting was the effect seen with 6-alkoxyxanthone **6a,** which significantly enhanced the capability of HPBMC to produce and release cytokines, especially at the two higher tested doses: the levels of TNF-α obtained in response to **6a** at 50 and 100 μM were significantly larger (about 50%) than the level of the control (Fig. 2a).

As expected from Philpott's results,[14] when DMXAA was tested in association with LPS, a remarkable TNF production with respect to the control was stimulated (Fig. 2b). However, this stimulation was not significantly different from that obtained with LPS alone. Otherwise, the capability of HPBMC treated with the selected compounds associated with LPS to produce and release TNF-α was significantly lower than that induced by LPS alone. On the other hand, considering the 3-alkoxyxanthones, **3a** remained completely inactive, while **3b** stimulated human mononuclear cells' TNF-α production with respect to the control, even if only at the lowest tested dose (25 μM). The ability of analogue **6a** to induce TNF seemed not to be influenced by the association with LPS. In fact, at the two higher doses used (50 and 100 μM), it maintained its capability to induce an increase of TNF production with respect to the control, but this production was still significantly lower than that obtained with LPS alone.

4.4. Nitrite quantitation

The nitrite assay was used as reliable indicator of HPBMCs' nitric oxide production and its release after 24 h exposure to DMXAA and selected compounds **3a**, **3b** and **6a**, with or without LPS, was measured (Fig. 3). When used alone, DMXAA was not able to provide significant levels of nitrite at any tested dose (Fig. 3a). The pattern obtained with two 3-alkoxyxanthones **3a** and **3b** used alone was different than that provided by the reference. At 25 μM both compounds showed a very important inhibition of nitric oxide production (about 60%), but this result completely changed after 50 μM exposure. Results obtained with HPBMC treatment with the highest concentration (100 μM) of **3a** and **3b** showed that, with respect to the control, they significantly increased the amount of nitrite (about 50%) released. Analogue **6a** was able to stimulate a significant nitric oxide production only at the highest tested dose, while at both 25 and 50 μM it seemed not to influence nitrite production.

When associated with LPS, DMXAA led to an increase of nitric oxide production with respect to the control (Fig. 3b). However, the remarkable HPBMC stimulation resulting from treatment with 100 μM DMXAA was not significantly higher than that obtained with LPS alone. Very interesting is the LPS influence on monocytes' capability to produce and release nitric oxide when co-exposed to two 3-alkoxyxanthones **3a** and **3b**. As reported in Fig. 3, both the inhibition effect

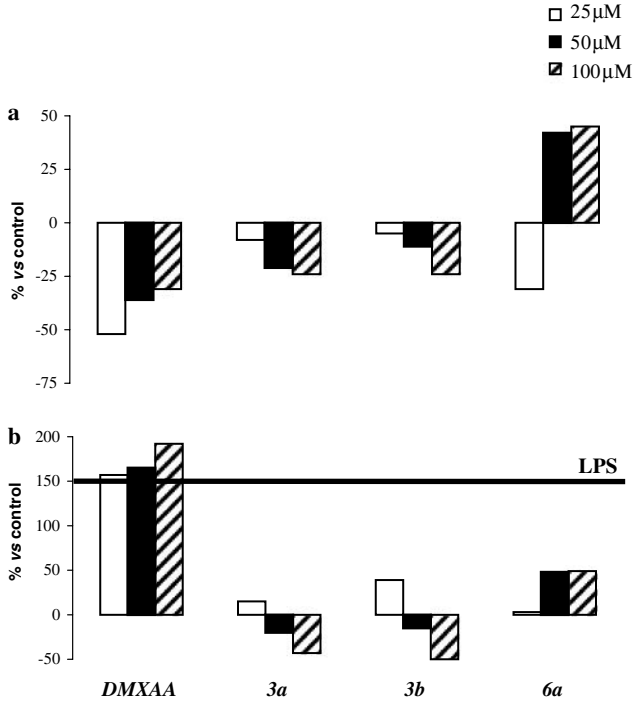


Figure 2. TNF-α released by HPBMC treated with selected compounds alone (a) or in association with LPS (b).

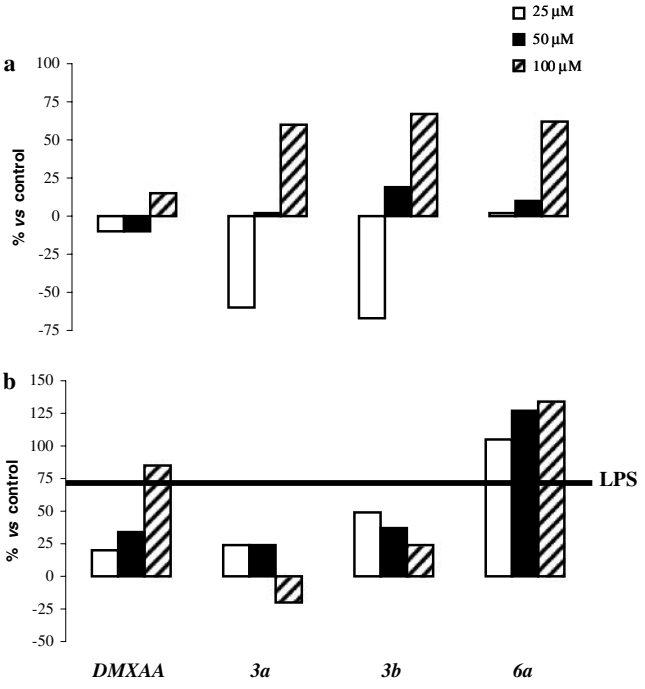


Figure 3. NO released by HPBMC treated with selected compounds alone (a) or in association with LPS (b).

obtained at the lowest dose and the stimulation effect at the highest dose were completely antagonized. On the contrary, when HPBMC were co-exposed to LPS and compound **6a** a potent stimulation was provided. In fact, not only the levels of nitrite measured were significantly higher with respect to the control, but they were also significantly higher than those induced by LPS alone. In particular, an increase of 48, 79 and 89%, with respect to the LPS alone, was pointed out at 25, 50 and 100 μM, respectively.

5. Discussion

As expected, all new derivatives exerted direct cytotoxicity only when tested at very high concentrations, which are very far from those commonly used in chemotherapy.

In order to define the real biological meaning of the modifications brought to the molecule of DMXAA, the ability of the new compounds to stimulate the tumouricidal activity of human monocytes was considered. For this evaluation concentrations lower than those used for the cytotoxicity assays (25, 50 and 100 μM) were employed and some of the new compounds showed a remarkable indirect activity (Table 2). In particular, among the 3-alkoxy derivatives, only compounds bearing a morpholine moiety seem to be able to enhance the lytic properties of monocytes, except for **4b** which is inactive and **3a** and **3b**, having a four carbon-atom chain, which exhibited the same activity as DMXAA, the most potent XAA derivative. Considering derivatives with the chain in position 6, the most interesting compound seems to be **6a**, with a three carbon-atom chain carrying a piperidine ring. This compound proved to be 1.3 times more potent than the reference molecule, whereas the others were only poorly or not active.

On the basis of the results obtained, the study continued with the evaluation of the LPS ability to potentiate the host-mediated cytotoxicity of DMXAA and derivatives **3b**, **3a** and **6a**, inducing TNF-α and NO synthesis. The analogues were selected considering their biological activity and their structural analogies, being **6a** and **3b** the most active compounds of the two different series, while **3a** differs from **3b** only for a piperidine instead of a morpholine ring.

When associated with LPS, the sole combinations to remain significantly active in the monocytes-mediated cytotoxicity assays were those with DMXAA and derivative **6a**. The comparison of the results of the associations with those obtained with the compounds alone revealed different responses. In particular, when associated with LPS, DMXAA became 3 times more potent in stimulating the lytic properties of human monocytes, while the derivatives were either not influenced by the presence of the LPS as compound **6a**, or significantly or totally inactivated as compound **3b** and compound **3a**, respectively.

As regards TNF-α production, no significant cytokine's release was determined by the reference compound alone, while its combination with LPS induced considerable levels of TNF-α with respect to the control, though not higher than that obtained with LPS alone. This seems to be in contradiction with the results described by Philpott and co-workers,[14] who used DMXAA concentrations at least 30 times higher (3 mM). The 3-alkoxyxanthones **3a** and **3b**, according to host-mediated cytotoxicity results, reduced the cytokine's production when used alone, and even more in association with LPS. On the contrary, derivative **6a** used at 50 and 100 μM was able to stimulate human monocytes to release TNF-α both alone and in association with LPS.

As far as the nitric oxide production is concerned, it was influenced by the scheme of treatment in an important manner. DMXAA alone was able to stimulate NO release only at the highest concentration tested, while in combination with LPS it induced greater levels of NO with respect to the control, anyhow lower than that obtained with LPS alone. Derivatives **3a** and **3b** alone influenced in a concentration-dependent manner the NO production, the lowest concentration significantly reducing the NO level with respect to the control, the highest one being particularly active in stimulating it. When associated to LPS, both of them induced NO production in the inverse manner. The 6-alkoxyxanthone **6a** was able to stimulate NO release both alone, if used at a concentration of 100 μM, and in association with LPS. In the latter case, the two agents interacted synergically when **6a** was used at the two lower concentrations, or at least additively at the highest one.

6. Conclusions

New derivatives of xanthenone-4-acetic acid, bearing an alkoxy chain of variable length and a basic moiety in positions 3 or 6, were synthesised in order to test the influence of this additional function on antitumour activity. From an overall view of the obtained results, it can be seen that the introduction of an alkoxy chain carrying an additional function, for example, a basic nitrogen, did not lead to a significant increase in activity, although it seems to be better tolerated in position 3 with respect to position 6. We already showed[18] that the introduction of a bulky lipophilic group in position 6 could lead to a slight increase in activity with respect to DMXAA and it was interesting to note that compound **6a** still maintained the activity of the parent compound. This seems to indicate that the possibility of introducing substituents which are bulkier than the small lipophilic groups previously reported,[5,6] and carrying additional functions in different positions on the xanthenone nucleus, could still be explored. In particular derivative **6a**, carrying a substituent in position 6 like DMXAA, showed the most interesting spectrum of biological activity. It can be supposed for this molecule to be endowed with a mechanism of action similar to that of DMXAA, as far as NF-κB activation and NOS II induction are concerned. The 3-alkoxy-derivatives **3a** and **3b**, though maintaining interesting activities, differ

substantially from derivative **6a**, in particular regarding the TNF and NO production, suggesting that their cytotoxic effects could be due to different factors not considered in this study.

7. Experimental

7.1. Chemistry

7.1.1. General methods. Melting points were determined in open glass capillaries using a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were recorded in DMSO-d_6 (unless otherwise indicated) on a Varian Gemini 300 spectrometer. Chemical shifts are reported in δ values relative to tetramethylsilane and spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet) or m (multiplet). Mass spectra were recorded on a V.G. 7070 E spectrometer. Silica gel (Merck, 230–400 mesh) was used for purification with flash chromatography. Elemental analyses were within 0.4% of the theoretical values. Compounds were named following IUPAC rules as applied by AUTONOM, Beilstein-Institute and Springer.

7.1.2. Synthesis of chloroalkoxy-4-substituted-xanthenones (10–13 and 23–26). A mixture of 3-hydroxy-4-allylxanthenone **9**[18] or methyl ester of 6-hydroxyxanthenone-4-acetic acid **22**[17] (20 mmol), K_2CO_3 (30 mmol) and 1-bromo-ω-chloroalkane (40 mmol) in 50 mL acetone was refluxed for 20 h and hot filtered. The solvent was evaporated and the residue was resuspended in CH_2Cl_2. The organic layer was washed with 5% NaOH solution, then with water and then dried on Na_2SO_4. After evaporation of the solvent and crystallization from EtOH, the following compounds were obtained:

7.1.2.1. 3-(2-Chloroethoxy)-4-allylxanthenone (10). Yield 61%, mp 146–148 °C. ^{1}H NMR ($CDCl_3$): δ 3.70-3.80 (m, 2H, CH_2Ar), 3.90 (t, J = 6.6 Hz, 2H, CH_2Cl), 4.45 (t, J = 6.6 Hz, 2H, CH_2O), 5.10–5.15 (m, 2H, CH_2 allyl), 6.05–6.15 (m, 1H, CH allyl), 7.05–8.40 (m, 6H, aromatic).

7.1.2.2. 3-(3-Chloropropoxy)-4-allylxanthenone (11). Yield 70%, mp 172–175 °C. ^{1}H NMR ($CDCl_3$): δ 2.25–2.35 (m, 2H, CH_2), 3.70–3.80 (m, 4H, CH_2Ar + CH_2Cl), 4.30 (t, J = 6.6 Hz, 2H, CH_2O), 5.00–5.10 (m, 2H, CH_2 allyl), 6.00–6.05 (m, 1H, CH allyl), 7.05–8.35 (m, 6H, aromatic).

7.1.2.3. 3-(4-Chlorobutoxy)-4-allylxanthenone (12). Yield 50%, mp 188–190 °C. ^{1}H NMR ($CDCl_3$): δ 1.55–1.65 (m, 4H, 2× CH_2), 3.65–3.75 (m, 4H, CH_2Ar + CH_2Cl), 4.25 (t, J = 6.6 Hz, 2H, CH_2O), 5.10–5.20 (m, 2H, CH_2 allyl), 6.00–6.05 (m, 1H, CH allyl), 7.05–8.45 (m, 6H, aromatic).

7.1.2.4. 3-(5-Chloropentoxy)-4-allylxanthenone (13). Yield 70%, mp 90–92 °C. ^{1}H NMR ($CDCl_3$): δ 1.70–1.75 (m, 2H, CH_2), 1.80–1.90 (m, 4H, 2× CH_2), 3.65 (t, J = 6.6 Hz, 2H, CH_2Cl), 3.65–3.70 (m, 2H, CH_2Ar), 4.15 (t, J = 6.6 Hz, 2H, CH_2O), 5.10–5.15 (m, 2H, CH_2 allyl), 6.00–6.05 (m, 1H, CH allyl), 6.90–8.30 (m, 6H, aromatic).

7.1.2.5. 6-(2-Chloroethoxy)xanthenone-4-acetic acid methyl ester (23). Yield 70%, mp 125–128 °C. ^{1}H NMR ($CDCl_3$): δ 3.75 (s, 3H, $COOCH_3$), 3.90 (t, J = 6.6 Hz, 2H, CH_2Cl), 4.00 (s, 2H, CH_2CO–), 4.35 (t, 2H, CH_2O), 6.90–8.35 (m, 6H, aromatic).

7.1.2.6. 6-(3-Chloropropoxy)xanthenone-4-acetic acid methyl ester (24). Yield 95%, mp 115–118 °C. ^{1}H NMR ($CDCl_3$): δ 2.30–2.35 (m, 2H, CH_2), 3.75–3.85 (m, 5H, CH_2Cl + $COOCH_3$), 4.05 (s, 2H, CH_2CO–), 4.30–4.35 (m, 2H, CH_2O), 6.95–8.30 (m, 6H, aromatic).

7.1.2.7. 6-(4-Chlorobutoxy)xanthenone-4-acetic acid methyl ester (25). Yield 74%, mp 115 °C. ^{1}H NMR ($CDCl_3$): δ 2.05–2.15 (m, 4H, 2× CH_2), 3.70–3.75 (m, 2H, CH_2Cl), 3.80 (s, 3H, $COOCH_3$), 4.05 (s, 2H, CH_2CO–), 4.10–4.15 (m, 2H, CH_2O), 6.90–8.30 (m, 6H, aromatic).

7.1.2.8. 6-(5-Chloropentoxy)xanthenone-4-acetic acid methyl ester (26). Yield 95%, mp 116–118 °C. ^{1}H NMR ($CDCl_3$): δ 1.70–1.75 (m, 2H, CH_2), 1.85–1.95 (m, 4H, 2× CH_2), 3.75–3.85 (m, 5H, CH_2Cl + $COOCH_3$), 4.05 (s, 2H, CH_2CO–), 4.10–4.15 (m, 2H, CH_2O), 6.85–8.35 (m, 6H, aromatic).

7.1.3. Synthesis of 3-alkoxyxanthenone-4-acetic acids (14–17). A mixture of the allyl derivative (**10**–**13**, 20 mmol), acetic acid (75 mL), acetone (75 mL) and water (50 mL) was cooled to 0–5 °C and $KMnO_4$ (0.1 mol) was added in portions over 6 h. The reaction mixture was then stirred at rt for 1 h, poured into water and H_2O_2 was added until it became colourless. The precipitate formed was filtered, resuspended in $NaHCO_3$ and the aqueous solution was acidified and filtered to obtain the following compounds:

7.1.3.1. 3-(2-Chloroethoxy)xanthenone-4-acetic acid (14). Yield 57%, mp 222–225 °C. ^{1}H NMR: δ 3.90 (s, 2H, CH_2CO–), 4.05 (t, J = 6.6 Hz, 2H, CH_2Cl), 4.50 (t, J = 6.6 Hz, 2H, CH_2O), 7.25–8.25 (m, 6H, aromatic), 12.50 (broad, 1H, COOH).

7.1.3.2. 3-(3-Chloropropoxy)xanthenone-4-acetic acid (15). Yield 55%, mp 185–188 °C. ^{1}H NMR: δ 2.20–2.25 (m, 2H, CH_2), 3.70–3.75 (m, 2H, CH_2Cl), 3.95 (s, 2H, CH_2CO–), 4.20 (t, J = 6.6 Hz, 2H, CH_2O), 7.25–8.20 (m, 6H, aromatic), 12.50 (broad, 1H, COOH).

7.1.3.3. 3-(4-Chlorobutoxy)xanthenone-4-acetic acid (16). Yield 50%, mp 188–190 °C. ^{1}H NMR: δ 1.85–1.95 (m, 4H, 2× CH_2), 3.65–3.70 (m, 2H, CH_2Cl), 3.95 (s, 2H, CH_2CO–), 4.30 (t, J = 6.6 Hz, 2H, CH_2O), 7.25–8.25 (m, 6H, aromatic).

7.1.3.4. 3-(5-Chloropentoxy)xanthenone-4-acetic acid (17). Yield 50%, mp 201–204 °C. ^{1}H NMR: δ 1.50–1.55 (m, 2H, CH_2), 1.75–1.85 (m, 4H, 2× CH_2), 3.65 (t, J = 6.6 Hz, 2H, CH_2Cl), 3.85 (s, 2H, CH_2CO–), 4.20 (t, J = 6.6 Hz, 2H, CH_2O), 7.25–8.20 (m, 6H, aromatic).

7.1.4. Synthesis of 6-alkoxyxanthenone-4-acetic acids (27–30). The ester derivative (**23**–**26**, 15 mmol) was

refluxed in 6 N HCl for 4 h, hot filtered and the precipitate formed was collected and crystallized from toluene to give the following compounds:

7.1.4.1. 6-(2-Chloroethoxy)xanthenone-4-acetic acid (27). Yield 46%, mp 203–206 °C. ^{1}H NMR: δ 4.00 (s, 2H, CH_2CO–), 4.15 (t, J = 6.6 Hz, 2H, CH_2Cl), 4.45–4.50 (t, J = 6.6 Hz, 2H, CH_2O), 7.05–8.10 (m, 6H, aromatic).

7.1.4.2. 6-(3-Chloropropoxy)xanthenone-4-acetic acid (28). Yield 39%, mp 160–162 °C. ^{1}H NMR: δ 2.35–2.40 (m, 2H, CH_2), 3.85 (m, 2H, CH_2Cl), 4.05 (s, 2H, CH_2CO–), 4.30–4.35 (m, 2H, CH_2O), 7.05–8.15 (m, 6H, aromatic).

7.1.4.3. 6-(4-Chlorobutoxy)xanthenone-4-acetic acid (29). Yield 95%, mp 159–160 °C. ^{1}H NMR: δ 1.85–1.95 (m, 4H, 2× CH_2), 3.75–3.80 (m, 2H, CH_2Cl), 4.00 (s, 2H, CH_2CO–), 4.20–4.25 (m, 2H, CH_2O), 7.05–8.10 (m, 6H, aromatic).

7.1.4.4. 6-(5-Chloropentoxy)xanthenone-4-acetic acid (30). Yield 83%, mp 151 °C. ^{1}H NMR: δ 1.60–1.65 (m, 2H, CH_2), 1.75–1.85 (m, 4H, 2× CH_2), 3.70–3.75 (m, 2H, CH_2Cl), 4.05 (s, 2H, CH_2CO–), 4.15–4.25 (m, 2H, CH_2O), 7.05–8.15 (m, 6H, aromatic).

7.1.5. Synthesis of iodoalkoxy-4-acetic acids (18–21 and 31–34). A mixture of the chlorine derivative (**14**–**17** and **27**–**30**, 5 mmol) and NaI (5 mmol) in methylethylketone (40 mL) was refluxed for 5–7 h. The reaction was allowed to cool to rt and the precipitate formed was filtered and crystallized from toluene to give the following compounds:

7.1.5.1. 3-(2-Iodoethoxy)xanthenone-4-acetic acid (18). Yield 78%, mp 232–235 °C. ^{1}H NMR: δ 3.55 (t, J = 6.6 Hz, 2H, CH_2I), 3.95 (s, 2H, CH_2CO–), 4.45 (t, J = 6.6 Hz, 2H, CH_2O), 7.25–8.20 (m, 6H, aromatic).

7.1.5.2. 3-(3-Iodopropoxy)xanthenone-4-acetic acid (19). Yield 80%, mp 192–195 °C. ^{1}H NMR: δ 2.20–2.25 (m, 2H, CH_2), 3.40–3.45 (m, 2H, CH_2I), 3.95 (s, 2H, CH_2CO–), 4.20 (t, J = 6.6 Hz, 2H, CH_2O), 7.25–8.25 (m, 6H, aromatic).

7.1.5.3. 3-(4-Iodobutoxy)xanthenone-4-acetic acid (20). Yield 90%, mp 196–198 °C. ^{1}H NMR: δ 2.00–2.10 (m, 4H, 2× CH_2), 3.40–3.45 (m, 2H, CH_2I), 3.95 (s, 2H, CH_2CO–), 4.20 (t, J = 6.6 Hz, 2H, CH_2O), 7.25–8.25 (m, 6H, aromatic), 12.45 (broad, 1H, COOH).

7.1.5.4. 3-(5-Iodopentoxy)xanthenone-4-acetic acid (21). Yield 75%, mp 180–182 °C. ^{1}H NMR: δ 1.50–1.55 (m, 2H, CH_2), 1.75–1.85 (m, 4H, 2× CH_2), 3.30–3.35 (m, 2H, CH_2I), 3.85 (s, 2H, CH_2CO–), 4.15 (t, J = 6.6 Hz, 2H, CH_2O), 7.20–8.25 (m, 6H, aromatic), 12.45 (broad, 1H, COOH).

7.1.5.5. 6-(2-iodoethoxy)xanthenone-4-acetic acid (31). Yield 98%, mp 198–199 °C. ^{1}H NMR: δ 3.60 (t, J = 6.6 Hz, 2H, CH_2I), 3.95 (s, 2H, CH_2CO–), 4.45 (t, J = 6.6 Hz, 2H, CH_2O), 7.15–8.10 (m, 6H, aromatic).

7.1.5.6. 6-(3-Iodopropoxy)xanthenone-4-acetic acid (32). Yield 98%, mp 169–171 °C. ^{1}H NMR: δ 2.30–2.35 (m, 2H, CH_2), 3.40–3.45 (m, 2H, CH_2I), 4.00 (s, 2H, CH_2CO–), 4.20–4.25 (m, 2H, CH_2O), 6.90–8.30 (m, 6H, aromatic).

7.1.5.7. 6-(4-Iodobutoxy)xanthenone-4-acetic acid (33). Yield 98%, mp 168–172 °C. ^{1}H NMR: δ 1.85–1.95 (m, 4H, 2× CH_2), 3.30–3.35 (m, 2H, CH_2I), 4.05 (s, 2H, CH_2CO–), 4.20–4.25 (m, 2H, CH_2O), 7.05–8.10 (m, 6H, aromatic).

7.1.5.8. 6-(5-Iodopentoxy)xanthenone-4-acetic acid (34). Yield 90%, mp 167–169 °C. ^{1}H NMR: δ 1.50–1.55 (m, 2H, CH_2), 1.80–1.85 (m, 4H, 2× CH_2), 3.20–3.25 (m, 2H, CH_2I), 4.00 (s, 2H, CH_2CO–), 4.20–4.25 (m, 2H, CH_2O), 7.10–8.15 (m, 6H, aromatic).

7.1.6. Synthesis of ω-piperidine- and ω-morpholinealkoxyxanthenone-4-acetic acids (1–4 and 5–8). A mixture of the iodine derivative (**18**–**21** and **31**–**34**, 0.01 mol) and piperidine or morpholine (0.02 mol) in toluene was refluxed for 8 h. The solvent was evaporated and the residue was resuspended in water, the pH was adjusted to 7 and the precipitate formed was filtered and crystallized from ethanol (toluene for compounds **2a**, **2b**, **3a**, **3b** and **4a**) to give the title compounds collected in Table 1.

7.2. Biological evaluation

7.2.1. Cell culture. The human ovarian adenocarcinoma cell line 2008 and the cisplatin-resistant subline C13*, kindly supplied by Prof. G. Marverti (Department of Biomedical Sciences—University of Modena), were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1% antibiotics (all products of Biochrom KG Seromed) and 200 mM glutamine (Merck).

7.2.2. Antiproliferative activity. For growth inhibition assays 4 times 10^4 cells/mL were plated out in 96-well culture plates (Falcon) and 24 h later cells were changed into the appropriate medium with or without the freshly dissolved test compounds. Each XAA derivative was dissolved in DMSO and distilled water to 1000 μM and then diluted to the highest concentration (500 μM) in medium, filter-sterilized, diluted and used immediately. DMSO amount in sample solutions was lower than 1% v/v. After 24 h of incubation, cells' growth was determined by a tetrazolium salt reduction assay (MTT).[21] Twenty microlitres of MTT solution (5 mg/mL in PBS) was added to each well, and plates were incubated for 4 h at 37 °C. DMSO (150 μL) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The absorbance was measured on a micro-culture plate reader (Titertek Multiscan) using a test wavelength of 570 nm and a reference wavelength of 630 nm. DMXAA was taken as reference because of its potency on human models.[7]

7.2.3. Human mononuclear cells. Human peripheral blood mononuclear cells (HPBMC) were isolated from heparinized whole blood by centrifugation over Ficoll–Paque (Pharmacia), plated (10^4 cells/well)in 96-well plates and allowed to adhere at 37 °C. Viability of the cells was assessed by Trypan blue dye exclusion and was always more than 95%. After 2 h, the medium and the non-adherent cells were discarded, the plates were vigorously washed three times with RPMI 1640 medium and further incubated in medium supplemented with 5% FCS in the presence of different concentrations (25, 50 and 100 μM) of XAA analogues or DMXAA as reference compound. Furthermore, the same assay was performed treating HPBMC with the selected compounds **3a**, **3b** and **6a** or DMXAA in association with LPS 10 ng/mL (lipopolysaccharide from *Escherichia coli* serotype 0127; F8, Sigma). After 24 h, the medium was drawn and the C13∗ cells (10^4 cells/mL) were plated above. The optimal macrophage/C13∗ cell ratio has been previously determined (results not reported). Cells were co-cultivated for 24 h and then lysis of C13∗ cells was assessed by MTT test.[22] The percentages of specific cytotoxicity were calculated as follows:

$$\frac{OD_{(HPBMC+C13*)} - OD_{(HPBMC)}}{OD_{(C13*)}}$$

7.2.4. TNF-α production and nitrite assay. Human peripheral blood mononuclear cells (HPBMC) were isolated and treated with compounds **3a**, **3b** and **6a** or DMXAA, with or without LPS as described above. After 24 h incubations, culture media were carefully collected and stored at −70 °C until assayed. Commercially available enzyme-linked immunosorbent assay kit (Biotrak ELISA System—Amersham Pharmacia Biotech) was used to determine the concentration of TNF-α, according to manufacturer's instructions. The collected culture media were also used to evaluate the concentration of nitrite, as a reliable indicator of nitric oxide production and release by the 'Griess reaction.'[23] Five hundred microlitres of each sample was mixed with 250 μL of Griess A (1% sulfanilamide in 5% phosphoric acid) and Griess B (0.1% naphthylethylenediamine dihydrochloride in water) and then the absorbance was photometrically determined at 543 nm. The nitrite concentration in each culture medium was extrapolated from the standard curve.

7.2.5. Reagents. DMSO, dimethylsulfoxide (J.T. Baker); MTT (Sigma); LPS (lipopolysaccharide from *E. coli* serotype 0127; F8, Sigma); Ficoll–Paque (Pharmacia); sulfanilamide and naphthylethylenediamine dihydrochloride (Sigma).

7.2.6. Statistical analysis. For each assay three different experiments were performed in triplicate. Results were statistically evaluated by Student's *t*-test. The IC_{50}, 95% confidence limits, and the potency ratio between DMXAA and each XAA analogue (IC_{50}DMXAA/IC_{50} derivative) were estimated using the Litchfield and Wilcoxon method.[24]

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.02.003.

References and notes

1. Rewcastle, G. W.; Atwell, G. J.; Baguley, B. C.; Calveley, S. B.; Denny, W. A. *J. Med. Chem.* **1989**, *32*, 793–799.
2. Atassi, G.; Briet, P.; Berthelon, J. J.; Collonges, F. *Eur. J. Med. Chem. Chim. Ther.* **1985**, *20*, 393–402.
3. Plowman, J.; Narayanan, V. L.; Dykes, D.; Szarvasi, E.; Briet, P.; Yader, O. C.; Paull, K. D. *Cancer Treat. Rep.* **1986**, *70*, 631–635.
4. Bibby, M. C.; Double, J. A. *Anti-Cancer Drugs* **1993**, *4*, 3–17.
5. Atwell, G. J.; Rewcastle, G. W.; Baguley, B. C.; Denny, W. A. *J. Med. Chem.* **1990**, *33*, 1375–1379.
6. Rewcastle, G. W.; Atwell, G. J.; Zhuang, L.; Baguley, B. C.; Denny, W. A. *J. Med. Chem.* **1991**, *34*, 217–222.
7. For review see: Baguley, B. C. *Lancet Oncol.* **2003**, *4*, 141–148.
8. Rustin, G. J.; Bradley, C.; Galbraith, S.; Stratford, M.; Loadman, P.; Waller, S.; Bellenger, K.; Gumbrell, L.; Folkes, L.; Halbert, G. Phase I/II trials committee of cancer research UK. *Br. J. Cancer* **2003**, *88*, 1160–1167.
9. Ching, L. M.; Goldsmith, D.; Joseph, W. R.; Korner, H.; Sedgwick, J. D.; Baguley, B. C. *Cancer Res.* **1999**, *59*, 3304–3307.
10. Wang, L. C.; Reddy, C. B.; Baguley, B. C.; Kestell, P.; Sutherland, R.; Ching, L. M. *Biochem. Pharmacol.* **2004**, *67*, 937–945.
11. Thomsen, L. L.; Ching, L. M.; Zhuang, L.; Gavin, J. B.; Baguley, B. C. *Cancer Res.* **1991**, *51*, 77–81.
12. Ching, L. M.; Cao, Z.; Kieda, C.; Zwain, S.; Jameson, M. B.; Baguley, B. C. *Br. J. Cancer* **2002**, *86*, 1937–1942.
13. Karin, M.; Ben-Neriah, Y. *Annu. Rev. Immunol.* **2000**, *18*, 621–663.
14. Philpott, M.; Ching, L. M.; Baguley, B. C. *Eur. J. Cancer* **2001**, *37*, 1930–1937.
15. Woon, S. T.; Baguley, B. C.; Palmer, B. D.; Fraser, J. D.; Ching, L. M. *Oncol. Res.* **2002**, *13*, 95–101.
16. Gobbi, S.; Rampa, A.; Bisi, A.; Belluti, F.; Piazzi, L.; Valenti, P.; Caputo, A.; Zampiron, A.; Carrara, M. *J. Med. Chem.* **2003**, *46*, 3662–3669, and references therein.
17. Gobbi, S.; Rampa, A.; Bisi, A.; Belluti, F.; Valenti, P.; Caputo, A.; Zampiron, A.; Carrara, M. *J. Med. Chem.* **2002**, *45*, 4931–4939.
18. Sato, H.; Dan, T.; Onuma, E.; Tanaka, H.; Koga, H. *Chem. Pharm. Bull.* **1990**, *38*, 1266–1277.
19. Tsai, S. H.; Lin-Shiau, S. Y.; Lin, J. K. *Br. J. Pharmacol.* **1999**, *126*, 673–680.
20. Yamamoto, H.; Hanada, K.; Nishijima, M. *Biochem. J.* **1997**, *325*, 223–228.
21. Mosmann, T. J. *Immunol. Methods* **1983**, *65*, 55–63.
22. Garbin, F.; Eckert, K.; Immenschuh, P.; Kreuser, E. D.; Maurer, H. R. *Int. J. Immunopharmacol.* **1997**, *19*, 323–332.
23. Green, L. C.; Wagner, D. A.; Glogorwki, J.; Skipper, P. L.; Wisnok, J. S.; Tannenbowm, S. R. *Anal. Biochem.* **1982**, *126*, 131–138.
24. Tallarida, R. J.; Murray, R. B. *Manual of Pharmacological Calculations with Computer Programs*; Springer: Berlin, 1987, p 153.