Synthesis and Antitumor Activity of New Derivatives of Xanthen-9-one-4-acetic Acid

Silvia Gobbi,[†] Angela Rampa,[†] Alessandra Bisi,[†] Federica Belluti,[†] Piero Valenti,*[,†] Anna Caputo,[‡] Antonella Zampiron,[‡] and Maria Carrara[‡]

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

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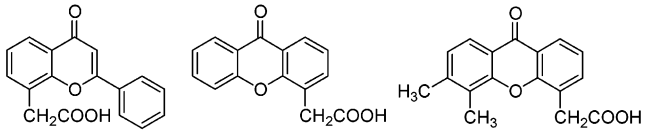
Xanthen-9-one-4-acetic acid (XAA) analogues in which the substituents in positions 5 and 6 are included in cyclic structures are described. Direct in vitro toxicity of the synthesized compounds against four tumor cell lines was evaluated, and their ability to stimulate mouse peritoneal macrophages and human monocytes in culture to become tumoricidal (indirect toxicity) was also studied. Despite low direct toxicity, almost all the compounds proved to be able to significantly enhance the lytic properties of both murine macrophages and human monocytes as well as the parent compound XAA and its most active derivative DMXAA taken as reference. In particular, compounds **4a**, **5a**, **7a**, **13a**,**b**, and **16a**,**b** showed higher activity than the lead compound on human monocytes, compound **7a** being 2.5 times more active than DMXAA, which is the most potent compound synthesized so far. Moreover, compounds **4a**, **5a**, **7a**, **13a**, **16a**, and **16b** proved to be able to induce TNF production in human immune cells.

Introduction

Flavone-8-acetic acid[1] (FAA, **1**; Chart 1) is a synthetic flavonoid that was identified as a lead anticancer compound among a broad series of molecules evaluated for antitumor activity on several murine models for some peculiar features. Actually, its main characteristics were low activity against fast-growing tumors such as leukemia, but striking, broad activity against slow-growing solid tumors[2] mostly insensitive to cytotoxic drugs, and absence of myelosuppression, usually observed with conventional antitumor agents.[3] On the other hand, its potency appeared to be low, since high doses and long exposure times were required for a direct cytotoxic effect.[4] On this basis, FAA was selected for further evaluation, but unfortunately the good activity seen both in in vitro and in vivo experiments was not confirmed in clinical trials.[5]

The unique pharmacological profile of **1** suggested an indirect effect of the drug, rather than direct toxicity, involving both a vascular component and the host immune system. FAA was shown to induce reduction of tumor blood flow and rapid hemorrhagic necrosis[6,7] due to vascular collapse in subcutaneous tumors. Moreover, this drug is now believed to act as a biological response modifier, since it stimulates the activity of NK cells[8] and enhances the lytic potential of macrophages in mice.[9] These effects are known to be mediated by the induction of different cytokines[10,11] such as tumor necrosis factor α (TNF-α) and interferons. 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,[12] which could contribute to tumor cell death by alteration of blood flow and direct cell killing.

Chart 1. Reference Compounds



1 **FAA** **2** **XAA** **3** **DMXAA**

Structurally related to FAA is xanthen-9-one-4-acetic acid[13] (XAA, **2**; Chart 1), where the xanthenone moiety can be regarded as a "fused" flavone. XAA showed higher potency than the parent compound and was taken as a new lead for the development of new derivatives. Structure−activity relationships of this molecule were amply investigated, and from studies on mono- and disubstituted XAA analogues,[14−16] it was concluded that activity was primarily dependent on the position of the substituents rather than on their nature, positions 5 and 6 being the most favorable for substitution, especially with small lipophilic groups.

5,6-Dimethyl-xanthen-9-one-4-acetic acid (DMXAA, **3**; Chart 1) proved to be the most potent XAA derivative, being able to induce cytokines (especially TNF) in human cells, an effect that was not seen with FAA.[17,18] DMXAA was then selected for further evaluation, and phase I clinical trials have now been completed.[19]

Extensive studies have been carried out to elucidate the mechanism of action of FAA at the molecular level. It was shown[20] that FAA and analogues can be decarboxylated by radicals (among them nitrogen oxides)[21] via one-electron oxidation leading to a benzyl type radical, which would rapidly react with oxygen to give peroxyl radicals, inducing cellular damage. Recent studies postulate a role for oxidative stress in the activity of this drug,[22] while its biological target is still under investigation.[23]

* To whom correspondence should be addressed. Phone: +39 051 2099701. Fax: +39 051 2099734. E-mail: pvalenti@alma.unibo.it.
[†] University of Bologna.
[‡] University of Padova.

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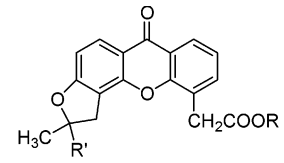
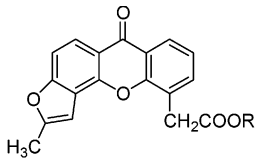
Chart 2. Structures of Target Compounds



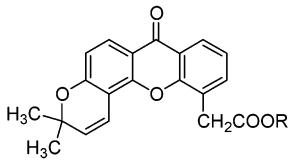
4a,b (R'=CH_3)

5a,b (R'=H)

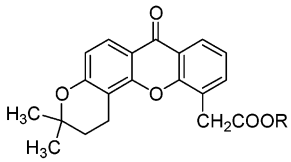


6a,b

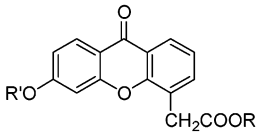
	R
a	CH_3
b	H



7a,b



8a,b



13a,b-16a,b

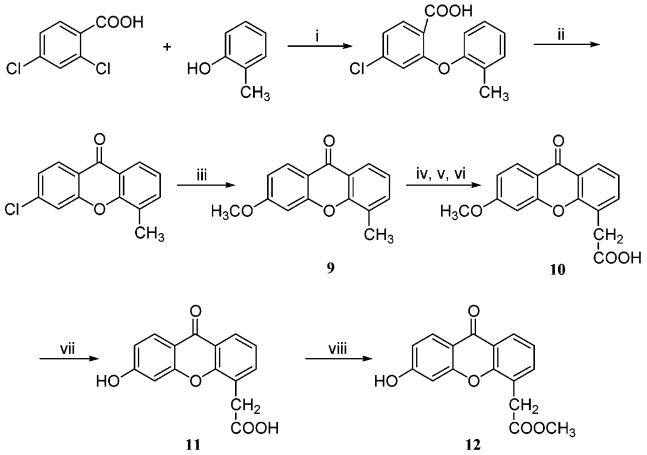
	R	R'
13a	CH_3	CH_2=C(CH_3)CH_2-
13b	H	CH_2=C(CH_3)CH_2-
14a	CH_3	CH_2=$CHCH_2$-
14b	H	CH_2=$CHCH_2$-
15a	CH_3	HC≡CCH_2-
15b	H	HC≡CCH_2-
16a	CH_3	(CH_3)$_2$C=$CHCH_2$-
16b	H	(CH_3)$_2$C=$CHCH_2$-

Continuing our SAR studies on this class of antitumor compounds,[24] in this paper we describe the synthesis of new XAA derivatives in which the substituents in positions 5 and 6 were included in cyclic structures, both saturated and unsaturated, to further investigate structural requirements and steric limitations in this part of the molecule. Since substitution with methoxy groups still maintained the same activity seen for **3**,[12] different oxygenated cycles, synthetically more convenient than nonoxygenated ones, were introduced in the lead structure. The synthetic intermediates, bearing different bulky alkoxy groups in position 6 of the xanthenone nucleus ("open intermediates"), were also taken into account and tested for their antitumor activity, since this position is considered one of the most favorable for substitution. Structures of the synthesized compounds are shown in Chart 2.

Chemistry

The key intermediate for the synthesis was the methyl ester of 6-hydroxyxanthen-9-one-4-acetic acid (**12**). This compound was prepared as outlined in Scheme 1 following standard procedures. 4-Methyl-6-methoxyxanthen-9-one (**9**) was prepared by heating the 6-chloro derivative[13] with NaOMe in dioxane. Monobromination with *N*-bromosuccinimide using benzoyl peroxide as catalyst in CCl_4 gave the bromomethyl derivative, which was converted to cyanide and hydrolyzed to the desired xanthen-9-one-4-acetic acid (**10**).[13] This compound was demethylated to **11** by heating to 220−230 °C with pyridine hydrochloride and finally refluxed in MeOH in the presence of acid to afford **12**.

Scheme 1[a]



[a] Reagents: (i) Cu, CuI, K_2CO_3, pyridine, reflux, 2h; (ii) H_2SO_4, 100 °C, 1 h; (iii) NaOMe, dioxane, reflux, 100 h; (iv) NBS, benzoyl peroxide, CCl_4, reflux, 5 h; (v) NaCN, EtOH−H_2O, reflux, 20 h; (vi) AcOH, H_2O, H_2SO_4, reflux, 2 h; (vii) pyridine·HCl, 220−230 °C, 2.5 h; (viii) MeOH, H^+, reflux, 12 h.

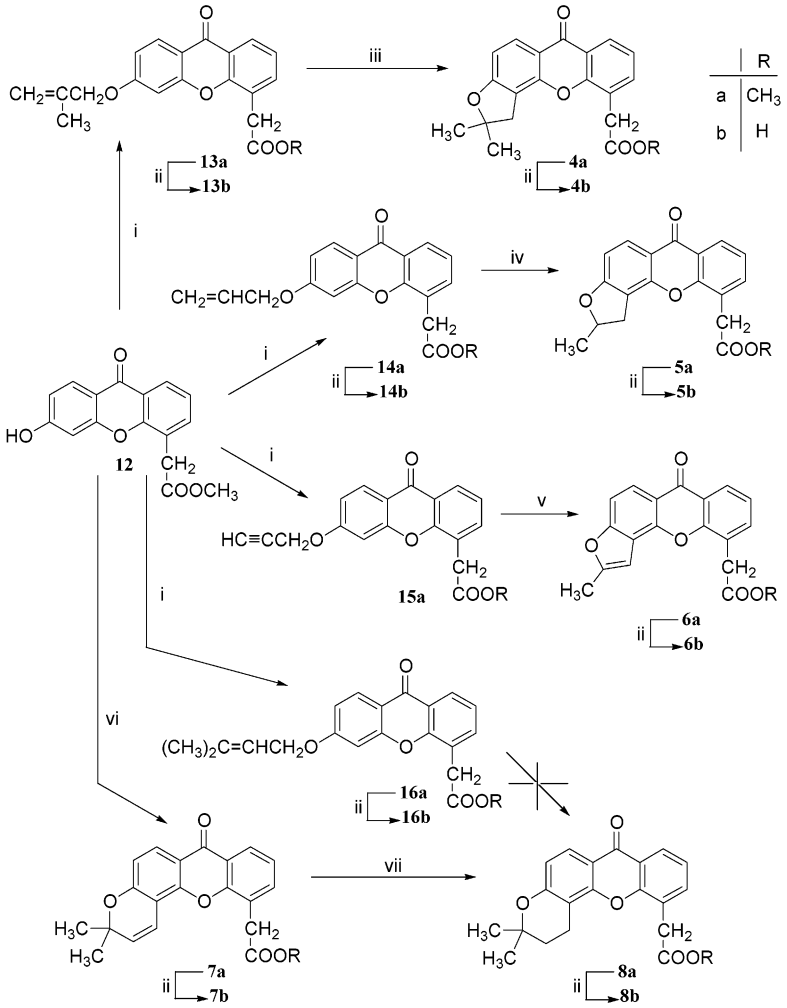
The final compounds were then prepared as shown in Scheme 2. The key intermediate **12** was treated with the selected alkyl halide to afford **13a**, **14a**, **15a**, and **16a**. These esters were hydrolyzed to the corresponding acids **13b**, **14b**, and **16b** (compound **15b** could not be obtained pure enough to be characterized and tested) or cyclized by heating, under different conditions, to afford **4a**, **5a**, and **6a**, which were then also hydrolyzed to **4b**, **5b**, and **6b**. Only **16a** did not cyclize under any of the conditions used, and compounds **7** and **8** were prepared using an alternative route. Compound **12** was heated in pyridine with 3-methyl-2-butenal to afford **7a**, which was hydrolyzed to **7b** or hydrogenated (Pd/C) to give **8a**, which was then hydrolyzed to **8b**.

Biological Assays

Since one of the peculiar features of the parent compound flavone-8-acetic acid was its remarkable preferential activity on solid tumors, all the target compounds **4**−**8a**,**b** and **13**−**16a**,**b** were tested for direct cytotoxicity in a preliminary in vitro assay against four human tumor cell lines: LoVo S and LoVo R, arising from human colon adenocarcinoma, and 2008 and C13*, arising from human ovarian adenocarcinoma. LoVo R cells differ from the parental line (LoVo S) because they are resistant to doxorubicin and express MDR, while C13* cells appear to be 10-fold more resistant to cisplatin than the original 2008 line, and furthermore, they show reduced cell membrane permeability to passive diffusion[25] and mitocondrial membrane functionality.[26]

The antitumor effects shown by both flavone-8-acetic acid and 5,6-dimethylxanthen-9-one-4-acetic acid seem

Scheme 2[a]



[a] Reagents: (i) R′X, K_2CO_3, reflux, 24 h; (ii) KOH, reflux, 4 h; (iii) 230−240 °C, 2,6-dimethylphenol, 8 h; (iv) 250−260 °C, 3 h; (v) CsF, *N*,*N*-diethylaniline, reflux, 4 h; (vi) 3-methyl-2-butenal, pyridine, 140 °C, 9 h; (vii) H_2, Pd/C.

to deeply involve the immune system by different pathways, including stimulation the activity of NK cells, increase in macrophage-mediated cytotoxicity, and induction of different cytokines. Thus, it was of primary importance to evaluate the indirect effects of the synthesized compounds to fully understand the biological meaning of the structural changes that had been introduced. Cytotoxicity induced on C13* cells cocultured with murine macrophages pretreated with the new compounds was therefore measured to test the new derivatives for their ability to improve lytic properties of those immune cells. Since studies performed in order to evaluate the effects of DMXAA on the human immune system gave stimulating results,[27] the ability of the synthesized compounds to enhance the lytic potential of human monocytes was also assessed. This model appeared particularly interesting because of the presence in these cells of the TLR2 receptor, which is involved in antiinflammatory cytokine activation,[28] and also because of the evidence of the role played by cytokines in the antitumor effects of this class of drugs.[27]

Tumor necrosis factor α (TNF -α) appears to play a pivotal role in the antitumor effects of DMXAA in mice, and a strong correlation was observed between elevation of serum TNF and antitumor potential.[29] Moreover, Philpott's results[27] suggested that in vitro analysis of the response of human peripheral blood leucocytes to DMXAA could be a useful indicator of the activity of this class of agents. Therefore, the effects of some of the new compounds, i.e., **4a**, **5a**, and **7a** among cyclic compounds and **13a**, **16a** and **16b** among 6-alkoxy analogues, on TNF production by human peripheral blood mononuclear cells (HPBMC) were investigated. The responses obtained with these agents were also compared to those obtained with LPS, a known inducer of TNF synthesis.[30]

Results and Discussion

Direct Toxicity. The cytotoxic effect of the tested compounds on the selected tumor cell lines (LoVo S, LoVo R, 2008, and C13*) was generally quite low, as expected from the results obtained with DMXAA, taken as reference. Both the reference compound DMXAA and the new derivatives (except for **4b**, **6a**,**b**, **16a**,**b**, which resulted inactive) were able to inhibit cell growth in a dose-dependent manner. However, at least 250 μM was necessary to obtain significant cytotoxic activity, **4a** being the only active compound at 100 μM. The dose requested against C13* cells was even higher (500 μM). No other significant difference was seen in the activity against the selected cell lines (data not shown). From these data, it can be concluded that direct toxicity could

Table 1. Cytotoxicity on C13*cells, Toxicity Induced in Murine Macrophages, and Human Monocytes of 6-Alkoxy Derivatives (PR = Potency Ratio)

compound	C*13 cells, IC_{50} (μM)	murine macrophages, IC_{50} (μM)	PR vs DMXAA	human monocytes IC_{50} (μM)	PR vs DMXAA	PR vs macrophages
DMXAA	614.7 (511.1−805.7)	211.4 (155.4−287.6)	1	134.6 (105.0−172.6)	1	1.6[a]
13a	488.2 (384.2−620.4)	*b*		84.6 (67.8−105.7)	1.6[a]	
13b	443.1 (347.8−564.5)	77.3 (57.5−104.1)	2.7[a]	86.2 (69.2−107.3)	1.6[a]	0.9
14a	508.6 (382.6−676.0)	61.6 (36.8−103.1)	3.4[a]	113.8 (92.9−139.5)	0.8	0.5[c]
14b	510.4 (419.3−611.2)	217.6 (142.8−331.6)	1.0	*b*		
15a	410.2 (362.1−465.1)	371.8 (256.4−539.0)	0.6	*b*		
16a	702.5 (538.6−916.4)	223.8 (168.3−297.6)	1.0	73.6 (55.0−98.6)	1.8[a]	3.0[a]
16b	626.1 (475.7−816.2)	264.1 (211.9−329.1)	0.8	64.2 (48.4−85.3)	2.1[a]	4.1[a]

[a] More potent. [b] Not detected. [c] Less potent.

Table 2. Cytotoxicity on C13*cells, Toxicity Induced in Murine Macrophages, and Human Monocytes of Cyclic Derivatives (PR = Potency Ratio)

compound	C*13 cells, IC_{50} (μM)	murine macrophages, IC_{50} (μM)	PR vs DMXAA	human monocytes, IC_{50} (μM)	PR vs DMXAA	PR vs macrophages
DMXAA	614.7 (511.1−805.7)	211.4 (155.4−287.6)	1	134.6 (105.0−172.6)	1	1.6[a]
4a	548.7 (442.0−681.3)	201.3 (81.6−496.4)	1.0	73.7 (60.2−90.2)	1.8[a]	2.7[a]
4b	603.9 (446.2−817.5)	219.5 (77.2−62.0)	0.9	172.3 (131.4−225.8)	0.8	1.3
5a	*b*	82.8 (59.1−116.1)	2.5[a]	67.3 (56.2−80.7)	2.0[a]	1.2
5b	711.4 (509.0−994.2)	157.2 (113.6−217.6)	1.3	177.9 (126.7−241.0)	0.7	0.9
6a	*b*	80.9 (56.7−115.4)	2.6[a]	*b*		
6b	*b*	42.0 (23.2−76.1)	5.0[a]	*b*		
7a	*b*	30.5 (16.5−56.5)	6.9[a]	53.5 (46.0−62.2)	2.5[a]	0.6
7b	725.8 (543.4−969.3)	45.1 (29.5−69.0)	4.7[a]	258.6 (138.1−484.3)	0.5[c]	0.2[c]
8b	*b*	71.5 (46.5−109.9)	2.9[a]	107.9 (81.4−143.2)	1.2	0.7

[a] More potent. [b] Not detected. [c] Less potent.

be obtained with these compounds only with doses that are much higher than the usual doses of chemotherapeutic drugs.

Indirect Toxicity. Tumoricidal Activity. The evaluation of indirect cytotoxic effects on C13* cells cocultured with macrophages or monocytes provided more interesting results, which are summarized in Table 1 for the 6-alkoxy derivatives **13**−**16a**,**b** and in Table 2 for the cyclic compounds **4**−**8a**,**b**, taking in both cases DMXAA as the reference compound. Generally speaking, a remarkable increase in antitumor activity in comparison to direct cytotoxicity was seen with all tested compounds. It should also be noted that doses could be reduced to 50% (i.e., 25, 50, and 100 μM) when testing the compounds on cells cocultured with human monocytes with respect to murine macrophages.

Considering the 6-alkoxy derivatives (**13**−**16a**,**b**, Table 1), both DMXAA and the new derivatives proved to be able to significantly enhance the lytic properties of murine macrophages and showed an important increase in antitumor activity with respect to their direct toxicity. The new compounds showed activity comparable to or higher than the reference compound (compounds **13b** and **14a**, 2.7 and 3.4 times, respectively). In particular, it can be seen that insertion of an unsaturated, linear or branched alkoxy chain led to an increase of activity but the presence of a triple bond was less favorable, since compound **15a** showed slightly lower activity than DMXAA. For all compounds except **14b** and **15a**, the activity was maintained on human monocytes, derivatives **13a**,**b** and **16a**,**b** with branched unsaturated side chains proved to be 1.6, 1.6, 1.8, and 2.1 times more potent than DMXAA, respectively. Going from murine macrophages to human monocytes, the activity seemed to be lost for compounds **14b** and **15a**, but it is interesting to note that compound **13a** showed higher activity than DMXAA in the human model while it proved to be inactive on murine macrophages.

All cyclic compounds (**4**−**8a**,**b**, Table 2) showed remarkable activity on murine immune cells, generally higher than their direct toxicity. Compounds bearing an ester function seemed to be more active than the

corresponding acids, and both five- and six-membered rings seemed to lead to increased activity. In particular, compounds **6b** and **7a** proved to be the most active derivatives, being 5.0 and 6.9 times more active than DMXAA, respectively. Interestingly, compounds **5a**, **6a**, **6b**, **7a**, and **8b** were able to induce indirect toxicity, even though no direct cytotoxicity was detected. Activity on human monocytes was maintained for almost all tested derivatives except **6a**,**b**, which proved to be inactive. The introduction of a cyclic moiety therefore seemed to be favorable for activity on human immune cells also. Compounds **4a**, **5a**, and **7a** seem to be of particular interest, being 1.8-, 2.0-, and 2.5-fold more active than DMXAA, respectively.

The results obtained can be summarized as follows. (1) All the new compounds, except for **13a**, were able to enhance the lytic properties of murine macrophages. In particular, the introduction of a five- or six-membered ring seemed to be the most favorable, since almost all the compounds in this series were significantly more active than DMXAA, particularly compounds **6a**,**b**, **7a**,**b**, and **8b**, whereas most of them did not show any direct cytotoxicity versus C*13 cells. Among 6-alkoxy derivatives, **13b** and **14a** proved to be 2.7 and 3.4 times more active than the reference compound.

(2) Most of the new derivatives except for **6a**,**b**, **14b**, and **15a** showed significant toxicity on C*13 cells cocultured with human monocytes. Among 6-alkoxy derivatives, **13a**,**b** and **16a**,**b** with branched side chains proved to be 1.6, 1.6, 1.8, and 2.1 times more active than DMXAA, respectively. Considering the cyclic derivatives, it should be noted how esters seemed to be more active than free acids, since compounds **4a**, **5a**, and **7a** showed activity up to 2.5 times higher than DMXAA; however, this could partly be due to better cell uptake of these molecules with respect to the corresponding free acids.

(3) By comparison of the activities on murine macrophages and human monocytes, a certain degree of species specificity could be noted. In particular, compound **13a**, bearing a branched side chain, was able to stimulate the lytic potential of human immune cells while it did not show any such activity on murine macrophages. Compound **4a**, with a saturated five-membered ring, and **16a**,**b**, also with a branched side chain, also proved to be respectively 2.7, 3.0, and 4.1 times more active on human cells with respect to murine cells. On the other hand, compounds **6a**,**b**, with an unsaturated five-membered ring, and **14b** and **15a**, with a linear side chain, were active only on murine cells.

Tumor Necrosis Factor α (TNF-α) Production. The effects of the selected compounds (**13a**, **16a**,**b**, **4a**, **5a**, **7a**), tested at three different concentrations, on TNF-α production by HPBMC are shown in Figures 1 and 2. The results showed that no significant cytokine production was stimulated by DMXAA alone, as expected from Philpott's results,[31] at any of the concentrations tested. On the contrary, although the response of HPBMC to the new derivatives was at all times lower than the response to LPS, after 4 h of incubation the levels of TNF-α obtained in response to 100 μM of all tested compounds (and especially **13a**) were significantly larger than the level of the negative control (Figure 1). In particular, the levels of TNF-α obtained

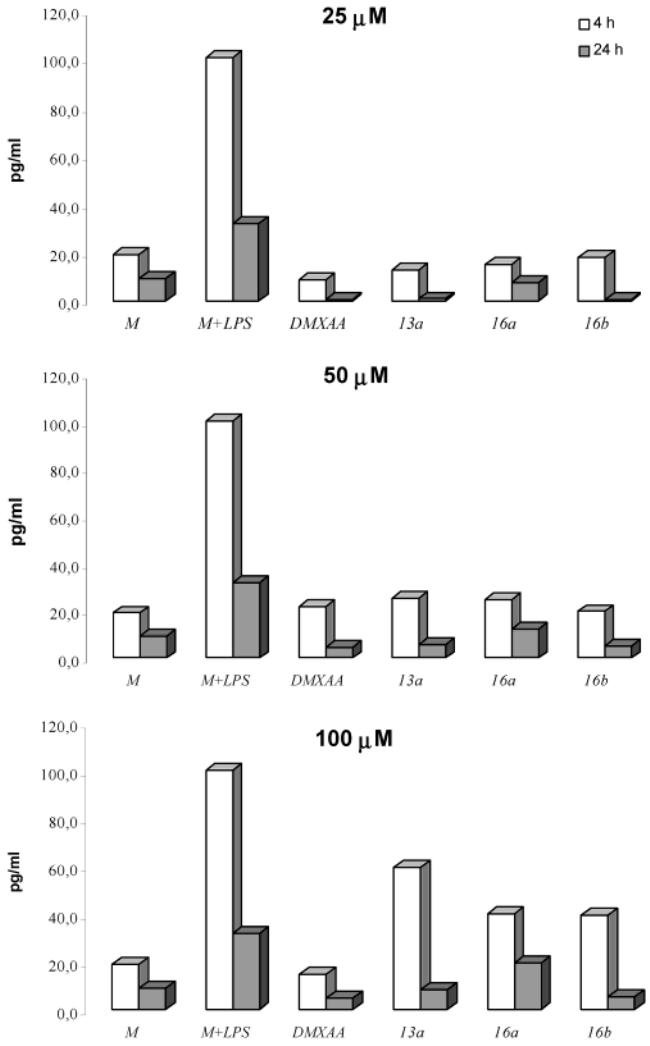


Figure 1. Effect of DMXAA and analogues tested at different concentrations on TNF-α production by human monocytes. The first two bars represent medium from unstimulated cells.

in response to **13a** were 60% of those obtained in response to LPS (59.5 pg/mL versus 100.2 pg/mL). LPS is known to form complexes with LPS binding proteins (LBP), which interact with the CD14 receptor, a membrane-bound glycoprotein[32] linked to the TNF and IL-1 kinase cascades by the TLR2 protein on the plasma membrane,[28,33] inducing NF-κB activity.[28] Because TNF is synthesized by human peripheral blood mononuclear cells in response to the tested compounds, it is possible that the activation pathways are the same for the two agents.

Conclusions

A new series of derivatives of the drug xanthen-9-one-4-acetic acid (XAA), bearing a rather large lypophilic substituent in position 6 or a cyclic moiety in positions 5 and 6, was synthesized. The compounds were tested both for direct cytotoxicity on four human tumor cell lines and for indirect activity, evaluating their ability to stimulate mouse peritoneal macrophages and human monocytes in culture to become tumoricidal. Despite quite low direct toxicity, almost all the compounds were able to induce an increase in the lytic properties of murine macrophages in the same way as that for the reference compound DMXAA. Most of the new derivatives showed remarkable activity on human monocytes

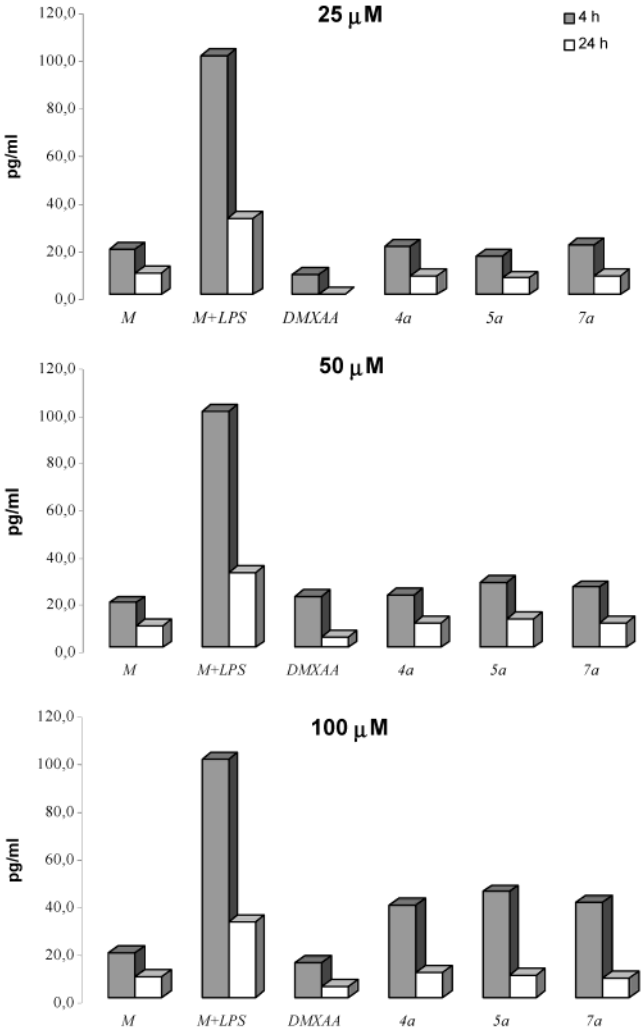


Figure 2. Effect of DMXAA and analogues tested at different concentrations on TNF-α production by human monocytes. The first two bars represent medium from unstimulated cells.

as well, compounds **13a**,**b** and **16a**,**b**, with branched side chains, and **4a**, **5a**, and **7a**, esters of cyclic compounds, being significantly more potent than DMXAA. Since the antitumor effects of DMXAA are known to be associated with TNF production and since the TLR2 receptor for the induction of inflammatory cytokines was found in lymphoid tissues with the highest expression in peripheral blood leukocytes,[28] studies were carried out to investigate if compounds **4a**, **5a**, **7a**, **13a**, **16a**, and **16b** were able to induce TNF production in HPBMC cultures. For all tested compounds, a significant response could be noticed after 4 h of incubation. These compounds proved to be particularly promising and were therefore selected for further evaluation.

Experimental Section

Chemistry. General Methods. All melting points were determined in open glass capillaries using a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were recorded in solution on a Varian Gemini 300 spectrometer with Me_4Si as the internal standard. Mass spectra were recorded on a V.G. 7070 E spectrometer. Silica gel (Merck, 230−400 mesh) was used for purification with flash chromatography. Wherever analyses are only indicated with element symbols, analytical results obtained for those elements are within 0.4% of the theoretical values. The names of compounds were obtained using AUTONOM, PC software for nomenclature in organic chemistry from Beilstein-Institut and Springer.

4-Methyl-6-methoxyxanthen-9-one (9). A mixture of 2,4-dichlorobenzoic acid (85 g, 0.4 mol), *o*-cresol (104 g, 0.8 mol), K_2CO_3 (110.4 g, 0.8 mol), pyridine (15.8 g, 0.2 mol), Cu (2 g), and CuI (2 g) in water (250 mL) was refluxed for 2 h. The reaction mixture was basified with Na_2CO_3 and washed with ether and then acidified with HCl, and the resulting precipitate was filtered and resuspended in dilute NaOH. The solution was filtered and acidified with acetic acid. The separated solid was then collected by filtration and crystallized from MeOH/H_2O to give 73.5 g (70%) of 4-chloro-2-(2-methylphenoxy)-benzoic acid, mp 151−154 °C (lit. mp[13] 158−159 °C). ^{1}H NMR ($CDCl_3$): δ 2.2 (s, 3H, CH_3), 6.6−8.2 (m, 7H, aromatic).

The compound (70 g, 0.27 mol) was then added portionwise to concentrated H_2SO_4 (700 mL), and the solution was heated to 100 °C for 1 h. The reaction mixture was then poured into ice and the separated solid was filtered, washed with water, dried, and crystallized from EtOH to give 39.5 g (60%) of 4-methyl-6-chloroxanthen-9-one, mp 148−149 °C (lit. mp[13] 145−146 °C). ^{1}H NMR ($CDCl_3$): δ 2.5 (s, 3H, CH_3), 7.3−8.25 (m, 6H, aromatic).

A suspension of the above 4-methyl-6-chloroxanthen-9-one (20 g, 0.08 mol) in dioxane (400 mL) was added to a solution of Na (38 g) in MeOH (500 mL), and the mixture was refluxed for 100 h. After removal of the solvent, the residue was taken up in water and filtered to give 18.2 g (95%) of **9**, mp 152−155 °C (EtOH) (lit. mp[13] 151−152 °C). ^{1}H NMR ($CDCl_3$): δ 2.5 (s, 3H, CH_3), 3.95 (s, 3H, CH_3O), 6.9−8.25 (m, 6H, aromatic). Anal. ($C_{15}H_{12}O_3$) C, H.

6-Methoxyxanthen-9-one-4-acetic Acid (10). A mixture of **9** (10 g, 0.04 mol), *N*-bromosuccinimide (7.6 g, 0.04 mol), and a catalytic amount of benzoyl peroxide in CCl_4 (300 mL) was refluxed under intense illlumination for 5 h. The mixture was then hot-filtered, and the precipitate was filtered and crystallized from toluene to give 10 g of 4-(bromomethyl)-6-methoxyxanthen-9-one (82%), mp 177−180 °C. ^{1}H NMR ($CDCl_3$): δ 3.95 (s, 3H, CH_3O), 4.85 (s, 2H, CH_2Br), 6.95−8.4 (m, 6H, aromatic).

To a solution of this compound (4 g, 13 mmol) in EtOH (400 mL) NaCN (0.66 g, 13 mmol) in water (10 mL) was added, and the mixture was refluxed for 20 h. After evaporation of the solvent, the residue was resuspended in water and extracted with CH_2Cl_2. The organic layer was then dried over Na_2SO_4 and was evaporated to dryness, and the resulting solid was crystallized from toluene to give 2.75 g (80%) of 6-methoxyxanthen-9-one-4-acetonitrile, mp 208−210 °C. ^{1}H NMR ($CDCl_3$): δ 3.95 (s, 3H, CH_3O), 4.05 (s, 2H, CH_2CN), 6.9−8.35 (m, 6H, aromatic).

The above acetonitrile (1.5 g, 6 mmol) was dissolved in a mixture of acetic acid (6 mL), water (6 mL), and concentrated H_2SO_4, and the mixture was refluxed for 2 h. Water was then added, and a precipitate was formed, which was filtered and dissolved in aqueous $NaHCO_3$. The solution was filtered and acidified with HCl, and the separated solid was filtered, dried, and crystallized from EtOH to give 1.25 g (73%) of **10**, mp 196−197 °C (lit. mp[13] 205−207 °C). ^{1}H NMR (DMSO-d_6): δ 3.95 (s, 3H, CH_3O), 4.0 (s, 2H, CH_2COOH), 6.95−8.1 (m, 6H, aromatic). Anal. ($C_{16}H_{12}O_5$) C, H.

6-Hydroxyxanthen-9-one-4-acetic Acid (11). A mixture of 6-methoxyxanthen-9-one-4-acetic acid[13] **10** (1 g, 3.5 mmol) and dry pyridine hydrochloride was heated to 210−220 °C for 2.5 h. After cooling, the reaction mixture was poured into H_2O and the separated solid was collected by filtration and dried to give 0.85 g (90%) of solid, mp 283−285 °C (EtOH) (lit. mp 303−305 °C). ^{1}H NMR (DMSO-d_6): δ 3.95 (s, 2H, CH_2COOH), 6.9−8.05 (m, 6H, aromatic), 11.0 (s, 1H, OH). Anal. ($C_{15}H_{10}O_5$) C, H.

Methyl 6-Hydroxyxanthen-9-one-4-acetate (12). A solution of **11** (0.65 g, 2.4 mmol) in MeOH (20 mL) and some drops of H_2SO_4 was refluxed for 12 h and then was evaporated to dryness. The residue, on crystallizing from MeOH, afforded 0.5 g (70%) of product, mp 239−240 °C. ^{1}H NMR (DMSO-d_6): δ 3.65 (s, 3H, CH_3), 4.0 (s, 2H, CH_2COO-), 6.9−8.5 (m, 6H, aromatic), 11.0 (s, 1H, OH). Anal. ($C_{16}H_{12}O_5$) C, H.

General Procedure for the Synthesis of Methyl-6-alkyloxyxanthen-9-one-4-acetates (13a−16a). A mixture of **12** (5 mmol), K_2CO_3 (6 mmol), and the selected alkyl halide (7 mmol) in acetone was refluxed for 24 h and was hot-filtered. The solvent was then evaporated, and the residue was dissolved in CH_2Cl_2, washed with 5% NaOH solution and with H_2O, dried over Na_2SO_4, and evaporated to dryness. The resulting solid was crystallized from toluene.

13a: starting from **12** and 3-chloro-2-methylpropene, yield 60%, mp 85−87 °C. ^{1}H NMR ($CDCl_3$): δ 1.9 (s, 3H, CH_3), 3.75 (s, 3H, COO*CH*$_3$), 4.0 (s, 2H, *CH*$_2$COOH), 4.6 (s, 2H, CH_2O), 5.1 (s, 1H), 5.2 (s, 1H) [*CH*$_2$═], 6.9−8.3 (m, 6H, aromatic). ^{13}C NMR ($CDCl_3$): δ 19.4, 35.2, 52.2, 72.2, 101.1, 113.4, 113.6, 115.6, 122.0, 123.2, 123.4, 125.9, 128.1, 135.5, 139.6, 154.1, 157.4, 164.1, 171.0, 175.0. MS *m*/*z* (rel abundance): 338 (M^+, 9.78), 91 (100), 56 (53.30). Anal. ($C_{20}H_{18}O_5$) C, H.

14a: starting from **12** and allyl bromide, yield 95%, mp 97−100 °C. ^{1}H NMR ($CDCl_3$): δ 3.75 (s, 3H, COO*CH*$_3$), 4.0 (s, 2H, *CH*$_2$COOH), 4.7 (m, 2H, *CH*$_2$OAr), 5.4 (m, 2H, *CH*$_2$═CH), 6.1 (m, 1H, CH_2═*CH*−CH_2), 6.9−8.25 (m, 6H, aromatic). ^{13}C NMR ($CDCl_3$): δ 35.2, 52.1, 69.2, 101.0, 113.6, 115.5, 118.3, 121.9, 123.1, 123.4, 125.8, 128.1, 131.9, 135.5, 154.0, 157.4, 163.8, 170.9, 174.6. MS *m*/*z* (rel abundance): 324 (M^+, 100), 283 (41.46), 265 (38.75). Anal. ($C_{19}H_{16}O_5$) C, H.

15a: starting from **12** and propargyl bromide, yield 100%, mp 167−169 °C. ^{1}H NMR ($CDCl_3$): δ 2.6 (m, 1H, CH), 3.75 (s, 3H, COO*CH*$_3$), 4.0 (s, 2H, *CH*$_2$COOH), 4.8 (m, 2H, CH_2O), 7.0−8.3 (m, 6H, aromatic). ^{13}C NMR ($CDCl_3$): δ 35.2, 52.2, 56.2, 76.4, 77.3, 101.5, 113.5, 116.1, 121.9, 123.2, 123.5, 125.9, 128.3, 135.6, 154.1, 157.3, 162.7, 171.0, 176.0. MS *m*/*z* (rel abundance): 322 (M^+, 100), 294 (26.32), 262 (44.26). Anal. ($C_{19}H_{14}O_5$) C, H.

16a: starting from **12** and 3,3-dimethylallyl bromide, yield 75%, mp 95−96 °C. ^{1}H NMR ($CDCl_3$): δ 1.85 (d, 6H, 2 × CH_3), 3.75 (s, 3H, COO*CH*$_3$), 4.0 (s, 2H, *CH*$_2$COOH), 4.68 (d, 2H, CH_2O), 5.55 (m, 1H, ═CH−), 6.95−8.3 (m, 6H, aromatic). ^{13}C NMR ($CDCl_3$): δ 18.2, 25.8, 35.2, 52.2, 65.5, 100.9, 113.9, 115.4, 118.6, 122.1, 123.2, 123.5, 126.0, 128.1, 135.5, 139.3, 154.2, 157.6, 164.4, 171.1, 176.1. MS *m*/*z* (rel abundance): 352 (M^+, 4.70), 284 (100), 69 (86.79). Anal. ($C_{20}H_{20}O_5$) C, H.

Methyl (2,2-Dimethyl-6-oxo-1,2-dihydro-6*H*-3,11-dioxacyclopenta[*a*]anthracen-10-yl)acetate (4a). A mixture of **13a** (0.8 g, 2.3 mmol) and 2,6-dimethylphenol (0.7 g, 5.5 mmol) was heated to 230−240 °C for 8 h. After cooling, the mixture was dissolved in diethyl ether, washed with 10% NaOH solution and with H_2O, dried over Na_2SO_4, and evaporated to dryness. The residue was crystallized from ligroin to give **4a** (52%), mp 189−190 °C. ^{1}H NMR ($CDCl_3$): δ 1.6 (s, 6H, CH_3), 3.2 (s, 2H, *CH*$_2$Ar), 3.7 (s, 3H, COO*CH*$_3$), 3.9 (s, 2H, *CH*$_2$COO−), 6.8−8.3 (m, 5H, aromatic). ^{13}C NMR ($CDCl_3$): δ 28.4, 35.8, 39.4, 52.2, 90.2, 108.0, 112.5, 114.6, 120.7, 123.2, 123.5, 126.2, 128.6, 135.4, 152.7, 153.5, 163.8, 173.9, 174.6. MS *m*/*z* (rel abundance): 338 (M^+, 100), 278 (35.20), 263 (43.90). Anal. ($C_{20}H_{18}O_5$) C, H.

Methyl (2-Methyl-6-oxo-1,2-dihydro-6*H*-3,11-dioxacyclopenta[*a*]anthracen-10-yl)acetate (5a). Compound **14a** (0.5 g, 1.5 mmol) was heated to 250−260 °C for 3 h. The residue was dissolved in CH_2Cl_2, washed with dilute NaOH, dried over Na_2SO_4, evaporated to dryness, and crystallized from ethanol to give **5a** (41%), mp 134−136 °C. ^{1}H NMR ($CDCl_3$): δ 1.6 (d, 3H, CH_3), 3.00−3.55 (m, 2H, *CH*$_2$Ar), 3.7 (s, 3H, COO*CH*$_3$), 3.9 (s, 2H, *CH*$_2$COO−), 5.2 (m, 1H, CH), 6.8−8.3 (m, 5H, aromatic). ^{13}C NMR ($CDCl_3$): δ 21.9, 29.7, 33.6, 35.8, 52.2, 82.3, 107.8, 113.1, 116.0, 122.0, 123.3, 123.6, 126.2, 128.7, 135.5, 154.8, 157.6, 162.0, 171.4, 174.9. MS *m*/*z* (rel abundance): 324 (M^+, 89.55), 265 (100), 59 (39.12). Anal. ($C_{19}H_{16}O_5$) C, H.

Methyl (2-Methyl-6-oxo-6*H*-3,11-dioxacyclopenta[*a*]anthracen-10-yl)acetate (6a). A mixture of **15a** (0.7 g, 2 mmol), CsF (0.43 g, 3 mmol), and *N*,*N*-diethylaniline (10 mL) was refluxed under N_2 for 4 h. The reaction mixture was then diluted with diethyl ether and filtered. A precipitate was formed, which was crystallized from toluene to give **6a** (66%), mp 187−189 °C. ^{1}H NMR ($CDCl_3$): δ 2.58 (s, 3H, CH_3), 3.75 (s, 3H, COO*CH*$_3$), 4.0 (s, 2H, *CH*$_2$COO−), 6.8 (s, 1H, CH), 7.4−8.35 (m, 5H, aromatic). ^{13}C NMR ($CDCl_3$): δ 14.2, 36.1, 52.3, 100.2, 108.7, 116.7, 118.4, 121.6, 122.1, 123.6, 123.7, 126.2, 135.5, 150.5, 153.9, 156.5, 158.6, 171.1, 176.7. MS *m*/*z* (rel abundance): 322 (M^+, 78.43), 263 (100), 59 (10.75). Anal. ($C_{19}H_{14}O_5$) C, H.

Methyl (3,3-Dimethyl-7-oxo-3*H*,7*H*-4,12-dioxabenzo[*a*]anthracen-10-yl)acetate (7a). 3-Methyl-2-butenal (1 mL, 10 mmol) was slowly added to a suspension of **12** (2 g, 7 mmol) in anhydrous pyridine (2 mL, 25 mmol) that had been heated to 140 °C, and the mixture was then heated for 3 h. Another 1 mL of 3-methyl-2-butenal was added, and heating was continued for another 6 h. The solvent was then evaporated, and the residue was separated by flash chromatography (toluene/ethyl acetate 7:3) to give **7a** (10%), mp 138−140 °C (toluene). ^{1}H NMR ($CDCl_3$): δ 1.52 (d, 6H, CH_3), 3.7 (s, 3H, COO*CH*$_3$), 3.95 (s, 2H, *CH*$_2$COO−), 5.75−8.25 (m, 7H, olefinic and aromatic). ^{13}C NMR ($CDCl_3$): δ 28.4, 36.1, 52.3, 78.1, 109.1, 114.2, 115.0, 115.1, 121.9, 123.6, 124.6, 126.1, 127.3, 130.1, 135.6, 151.1, 153.6, 158.2, 171.4, 174.5. MS *m*/*z* (rel abundance): 350 (M^+, 8.84), 335 (100), 276 (19.38). Anal. ($C_{21}H_{18}O_5$) C, H.

Methyl (3,3-Dimethyl-7-oxo-2,3-dihydro-1*H*,7*H*-4,12-dioxabenzo[*a*]anthracen-10-yl)acetate (8a). A solution of **7a** (0.15 g, 0.43 mmol) in THF was hydrogenated at room temperature and pressure over Pd/C. The reaction mixture was filtered, and the solvent was evaporated. The residue was hydrolyzed without further purification.

General Procedure for the Synthesis of Compounds 4b−8b and 13b−16b. A mixture of the methyl ester (0.8 mmol) in ethanol and KOH (1.2 mmol) in H_2O was refluxed for 4 h. The solvent was evaporated, and the residue was dissolved in H_2O and acidified with HCl. The precipitate was then collected by filtration and crystallized from toluene.

4b: yield 51%, mp 235−236 °C. ^{1}H NMR (DMSO-d_6): δ 1.55 (s, 6H, CH_3), 3.2 (s, 2H, *CH*$_2$Ar), 3.9 (s, 2H, *CH*$_2$COOH), 6.92−8.05 (m, 5H, aromatic). ^{13}C NMR (DMSO-d_6): δ 28.0, 35.2, 90.4, 107.7, 113.1, 114.8, 120.8, 123.5, 124.6, 124.8, 127.6, 136.0, 152.6, 153.4, 164.4, 171.7, 174.5. MS *m*/*z* (rel abundance): 324 (M^+, 3.53), 91 (100), 65 (12.64). Anal. ($C_{19}H_{16}O_5$) C, H.

5b: yield 10%, mp 238−240 °C. ^{1}H NMR (DMSO-d_6): δ 1.5 (d, 3H, CH_3), 2.95−3.55 (m, 2H, *CH*$_2$Ar), 3.9 (s, 2H, *CH*$_2$-COOH), 5.25 (m, 1H, CH), 6.95−8.05 (m, 5H, aromatic). ^{13}C NMR (DMSO-d_6): δ 21.6, 32.7, 35.2, 82.3, 107.6, 113.4, 115.0, 120.9, 123.1, 123.6, 124.7, 127.7, 136.1, 153.5, 157.1, 162.3, 171.9, 174.7. MS *m*/*z* (rel abundance): 310 (M^+, 69.66), 309 (100), 77 (47.22). Anal. ($C_{18}H_{14}O_5$) C, H.

6b: yield 15% (ligroin), mp 275−276 °C (dec). ^{1}H NMR (DMSO-d_6): δ 2.55 (s, 3H, CH_3), 4.0 (s, 2H, *CH*$_2$COOH), 6.92 (s, 1H, *CH*Ar), 7.45−8.15 (m, 5H, aromatic). ^{13}C NMR (DMSO-d_6): δ 14.2, 36.4, 100.3, 108.5, 116.5, 118.2, 121.4, 122.2, 123.0, 123.9, 126.2, 135.4, 150.1, 153.5, 156.6, 158.4, 171.3, 176.6. MS *m*/*z* (rel abundance): 308 (M^+, 94.76), 263 (100), 131 (9.54). Anal. ($C_{18}H_{12}O_5$) C, H.

7b: yield 35%, mp 202−204 °C. ^{1}H NMR (DMSO-d_6): δ 1.5 (s, 6H, CH_3), 3.97 (s, 2H, *CH*$_2$COOH), 5.95−8.1 (m, 7H, olefinic and aromatic). ^{13}C NMR (DMSO-d_6): δ 27.8, 35.6, 78.0, 108.6, 113.8, 114.0, 114.8, 120.7, 123.7, 124.5, 125.0, 126.6, 130.7, 136.2, 151.0, 153.5, 157.7, 171.7, 174.7. MS *m*/*z* (rel abundance): 336 (M^+, 5.62), 321 (100), 276 (10.06). Anal. ($C_{20}H_{16}O_5$) C, H.

8b: yield 30%, mp 208−210 °C. ^{1}H NMR (DMSO-d_6): δ 1.5 (s, 6H, CH_3), 1.95 (m, 2H, *CH*$_2$CH$_2$Ar), 2.95 (m, 2H, CH$_2$*CH*$_2$-Ar), 4.0 (s, 2H, *CH*$_2$COOH), 6.9−8.1 (m, 5H, aromatic). ^{13}C NMR (DMSO-d_6): δ 12.9, 27.2, 35.4, 48.4, 73.7, 108.3, 113.6, 114.6, 120.5, 123.6, 124.2, 125.1, 126.2, 130.6, 151.3, 154.0, 157.8, 171.6, 174.5. MS *m*/*z* (rel abundance): 338 (M^+, 94.37), 283 (100), 255 (96.03). Anal. ($C_{20}H_{18}O_5$) C, H.

13b: yield 68%, mp 217−218 °C. ^{1}H NMR ($CDCl_3$): δ 1.8 (s, 3H, CH_3), 4.0 (s, 2H, *CH*$_2$COOH), 4.52 (s, 2H, CH_2O), 5.05−5.12 (d, 2H, *CH*$_2$═), 6.92−8.25 (m, 6H, aromatic). ^{13}C NMR (DMSO-d_6): δ 19.1, 34.6, 71.5, 101.2, 112.6, 114.0, 114.6, 121.0, 123.6, 124.5, 124.6, 127.4, 136.2, 140.0, 153.7, 157.0, 163.7, 171.7, 174.7. MS *m*/*z* (rel abundance): 324 (M^+, 53.11), 309 (14.18), 55 (100). Anal. ($C_{19}H_{16}O_5$) C, H.

14b: yield 33%, mp 195−197 °C. ^{1}H NMR (DMSO-d_6): δ 3.95 (s, 2H, CH_2COOH), 4.78 (m 2H, CH_2O), 5.4 (m, 2H, CH_2=CH−), 6.1 (m, 1H, CH_2=CH−CH_2−), 7.1−8.1 (m, 6H, aromatic). ^{13}C NMR (DMSO-d_6): δ 34.6, 69.0, 101.1, 114.1, 114.7, 118.1, 121.1, 123.6, 124.6, 124.7, 127.5, 132.7, 136.3, 153.8, 157.1, 163.8, 171.8, 174.9. MS *m*/*z* (rel abundance): 310 (M^+, 100), 77 (16.50). Anal. ($C_{18}H_{14}O_5$) C, H.

16b: yield 75%, mp 197−200 °C. ^{1}H NMR (DMSO-d_6): δ 1.78 (d, 6H, CH_3), 3.98 (s, 2H, CH_2COOH), 4.72 (d, 2H, CH_2O), 5.5 (m, 1H, CH), 7.05−8.1 (m, 6H, aromatic). ^{13}C NMR (DMSO-d_6): δ 18.0, 25.4, 34.6, 65.4, 100.8, 114.2, 114.4, 118.8, 121.0, 123.5, 124.5, 124.6, 127.3, 136.1, 138.1, 153.6, 157.0, 164.0, 171.7, 174.7. MS *m*/*z* (rel abundance): 338 (M^+, 12.99), 270 (45.88), 69 (100). Anal. ($C_{19}H_{18}O_5$) C, H.

Biological Assays. Compounds. The compounds were dissolved in DMSO and stored in the dark as stock solutions (1000 μM) at −20 °C. For experimental use, all compounds were prepared from stock solutions, diluted with growth medium, filtered, sterilized, and used immediately.

Cell Lines. The human colon adenocarcinoma cells LoVo R (doxorubicin-resistant and multidrug-resistant cells) and LoVo S (sensitive cells), kindly supplied by the Centro di Riferimento Oncologico (Aviano, Pordenone, Italy), were cultured in Ham F 12, plus 10% heat-inactivated fetal calf serum, 1% antibiotics (all products of Biochrom KG Seromed), and 1% 200 mM glutamine (Merck). For LoVo R cells, the medium was supplemented with 100 ng/mL doxorubicin.

The human ovarian adenocarcinoma cell line 2008 and the *cis*-DDP-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 2 mM L-glutamine (Merck).

Direct Cytotoxicity. The cells were seeded in 96-well tissue plates (Falcon) and treated 24 h later with each agent at different concentrations. Viable cell growth was determined by tetrazolium salts reduction assay (MTT) after 24 h of incubation.[34]

An amount of 20 μL 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 microculture plate reader (Titertek Multiscan) using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Indirect Toxicity. Murine Resident Macrophages. The ability of the new derivatives to stimulate mouse macrophages in culture to become tumoricidal was evaluated using resident peritoneal macrophages.[35] Resident peritoneal cells were isolated by two injections of 5 mL of PBS containing 10 U/mL heparin into the peritoneal cavity. The cavity was gently massaged for 2 min, and the cells were removed by drawing fluid out with a syringe.[36]

The recovered cell suspension was centrifuged, and the pellet was washed twice in sterile PBS. The cells were then resuspended in RPMI 1640 plus 5% FCS and plated in a culture flask left to adhere at 37 °C. After 2 h, medium and nonadherent cells were discarded, the flask was washed with sterile PBS, adhering cells (macrophages) were resuspended, centrifuged, counted using 0.5% trypan blue, resuspended in complete medium, and then plated in 96-well plates (Falcon) at a concentration of 1×10^4 cells/well in the presence of different concentrations of DMXAA and analogues, using triplicate wells per drug dose. After 24 h, the medium was discarded and the C13* cells (2×10^3 cells/well) were plated as above. The optimal macrophages/C13* cells ratio was determined in preliminary experiments (results not reported). The cells were cocultured for 24 h. Lysis of C13* cells was assessed by the MTT test,[37] and the percentages of specific cytotoxicity were calculated as follows:

$$\frac{\text{OD(macrophages+C13*)} - \text{OD(macrophages)}}{\text{OD(C13*)}}$$

Human Mononuclear Cells. Human peripheral blood mononuclear cells (HPBMC) were isolated from heparinized whole blood by centrifugation over Ficoll-Paque (Pharmacia), plated in 96-well plates, and allowed to adhere at 37 °C. After 2 h, medium and nonadherent cells were discarded and the plates were vigorously washed three times with RPMI 1640 medium supplemented with 5% FCS and further incubated in complete medium in the presence of different concentrations (25, 50, 100 μM) of DMXAA and analogues, using triplicate wells per drug dose. After 24 h, the medium was discarded and the C13* cells (2×10^3 cells/well) were plated as above. The cells were cocultured for 24 h and then assessed by MTT test.

Quantification of TNF-α Production. Human peripheral blood mononuclear cells (HPBMC) were isolated as described above and treated with culture medium or DMXAA or the selected compounds at the concentrations of 25, 50 and 100 μM. LPS (lipopolysaccharide from *E. coli* serotype 0127;B8, SIGMA) was used as a positive control at the final concentration of 1 μg/mL. After 4 and 24 h incubations, the medium was carefully collected and stored at −70 °C until assayed. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine the concentration of TNF-α (Biotrak ELISA System, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Statistical Analysis. For each assay, three different experiments were performed in triplicate. The results were statistically evaluated by the Student's *t* test.[38] The IC_{50}, 95% confidence limits, and the potency ratio between DMXAA and each analogue (IC_{50}(DMXAA)/IC_{50}(derivative)) were estimated using the Litchfield and Wilcoxon method.[38]

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