Synthesis and Biological Evaluation of 3-Alkoxy Analogues of Flavone-8-acetic Acid§

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New analogues of flavone-8-acetic acid were synthesized, bearing an alkoxy group in position 3 and different substituents on the benzene ring in position 2 of the flavone nucleus. The compounds were tested for direct cytotoxicity against four human tumor cell lines and for indirect antitumor effects by measuring their ability to enhance lytic properties of murine macrophages and human monocytes. Though direct toxicity was very low, the compounds were able to induce significant indirect toxicity. Notably, most of them (**4c**, **4d**, **4e**, **4f**, **4h**, **4i**, **4m**, **4n**, and **4o**) showed important activity on human monocytes and could be regarded as the first flavone derivatives endowed with such activity. Particularly interesting seem to be compounds **4m** and **4n**, which showed IC_{50} values up to 7 times higher than DMXAA, which has now completed phase I clinical trials.

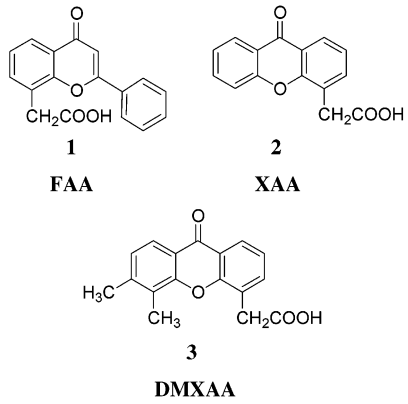
Introduction

Flavone-8-acetic acid[1] (FAA, **1**; Chart 1) has been considered a very interesting compound because of its peculiar antitumor profile, in particular its remarkable activity on solid tumors,[2] despite its low potency. Its activity is now known to be due to indirect effects more than to direct cytotoxicity, involving both the immune system (enhancement of lytic properties of macrophages[3] and activity of NK cells[4]) and the vascular system (hemorrhagic necrosis of tumor vasculature[5,6]) via induction of a number of cytokines.[7,8] Unfortunately, the antitumor activity seen both in vitro and in vivo in murine models was not confirmed in clinical trials, where FAA did not exhibit any significant activity.[9] Very interesting proved to be an early derivative of FAA, xanthenone-4-acetic acid[10] (XAA, **2**; Chart 1), structurally closely related to the lead molecule, which showed significantly higher potency. Extensive SAR studies performed on this molecule led to 5,6-dimethylxanthenone-4-acetic acid (DMXAA, **3**; Chart 1), which resulted the most potent compound synthesized, showing activity on human models.[11] DMXAA was therefore selected for further evaluation and has now completed phase I clinical trials.[12]

Despite extensive studies,[13,14] the mechanism of action of these molecules is still not clear and their biological target has not been identified yet. Moreover, structure−activity relationships for FAA derivatives have not been thoroughly examined and the modifications performed so far have shown very stringent structural requirements to maintain activity comparable to that of the parent compound.[15]

Our research group has been interested for some years in the synthesis and biological evaluation of analogues of FAA,[16] and in a recent paper[17] we reported the synthesis and biological activity of new analogues of XAA. In this paper we examine the effects of some more modifications in the lead molecule FAA on antitumor activity. In particular, we synthesized and evaluated derivatives bearing an alkoxy group in position 3 of the flavone nucleus and different substituents on the benzene ring in position 2 to further investigate the structural requirements for these parts of the molecule. The structures of the new compounds are shown in Table 1.

Chart 1. Reference Compounds



Chemistry

The synthesis of compounds **4a**−**l**,**o** is outlined in Scheme 1. 2′-Hydroxy-3′-allylacetophenone was condensed with a substituted benzaldehyde in KOH solution to give chalcones **5a**,**b**,**e**−**j**, which were oxidized by H_2O_2 in NaOH to the corresponding flavonols **6a**,**b**,**e**−**j**. These compounds were then alkylated with Me_2SO_4 or the selected alkyl halide to give **7a**,**b**,**e**−**l**, and the allyl group was oxidized by $KMnO_4$ to afford **4a**,**b**,**e**−**l**. Compounds **4c**,**d**,**o** were prepared in a similar way starting from 2′-hydroxy-3′-methylacetophenone to give

§ Dedicated to the memory of Professor Paolo Da Re.

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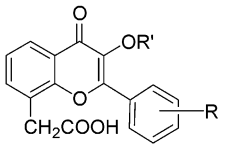
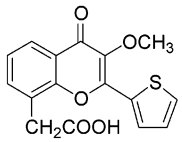
Table 1. Physicochemical and Analytical Data of Studied Compounds



4a-n

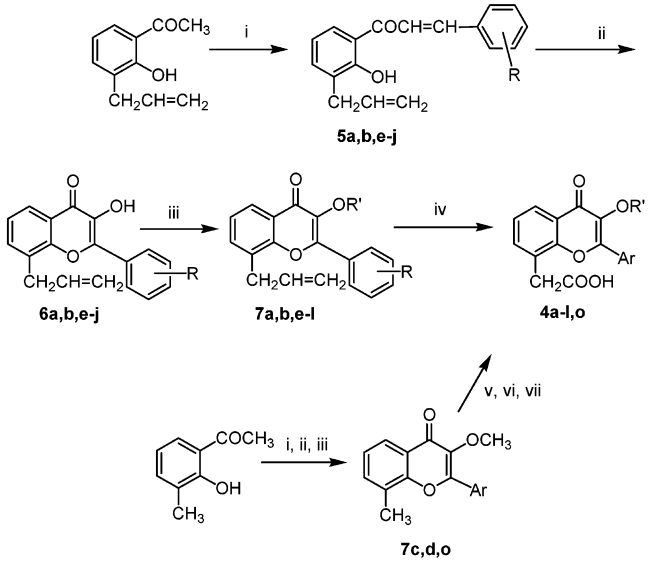


4o

compd	R	R′	mp[a] (°C)	yield (%)	formula	anal.
4a	H	CH_3	198−199	20	$C_{18}H_{14}O_5$	C, H
4b	4′-CH_3	CH_3	233−234	19	$C_{19}H_{16}O_5$	C, H
4c	4′-F	CH_3	204−206	45	$C_{18}H_{13}FO_5$	C, H
4d	4′-OCH_3	CH_3	223−226	40	$C_{19}H_{16}O_6$	C, H
4e	3′-CH_3	CH_3	163−164	19	$C_{19}H_{16}O_5$	C, H
4f	2′-CH_3	CH_3	149−151	15	$C_{19}H_{16}O_5$	C, H
4g	3′-OCH_3	CH_3	142−144	13	$C_{19}H_{16}O_6$	C, H
4h	2′-OCH_3	CH_3	205−206	48	$C_{19}H_{16}O_6$	C, H
4i	4′-Cl	CH_3	224−226	10	$C_{18}H_{13}ClO_5$	C, H
4j	2′-Cl	CH_3	210−212	12	$C_{18}H_{13}ClO_5$	C, H
4k	H	CH_2CH_3	195−196	24	$C_{19}H_{16}O_5$	C, H
4l	H	$CH(CH_3)_2$	209−210	28	$C_{20}H_{18}O_5$	C, H
4m	3′-CF_3	CH_3	200−202	10	$C_{19}H_{13}F_3O_5$	C, H
4n	3′-Cl	CH_3	156−158	10	$C_{18}H_{13}ClO_5$	C, H
4o			245−247	30	$C_{16}H_{12}O_5S$	C, H

[a] Recrystallization solvent: ethanol.

Scheme 1[a]



[a] Reagents: (i) substituted benzaldehyde, KOH; (ii) H_2O_2, NaOH; (iii) Me_2SO_4 or R′X, K_2CO_3, reflux; (iv) $KMnO_4$, 0−5 °C, 6 h; (v) NBS; (vi) NaCN; (vii) H_2SO_4, AcOH, H_2O.

the different 2-substituted 3-methoxy-8-methylchromones; these were then brominated, converted to cyanide, and hydrolyzed to give the desired compounds.

Since compounds **4m** and **4n** could not be prepared in acceptable yields with these methods, an alternative route was selected (Scheme 2). Starting from 2′-hydroxyacetophenone and using the procedure shown in Scheme 1, 3-methoxyflavone **10** was obtained, which was hydrolyzed by heating it for 4 h with KOH.[18] 2′-Hydroxy-2-methoxyacetophenone **11** was then alkylated with allyl bromide and by subsequent Claisen rearrangement gave 2′-hydroxy-3′-allyl-2-methoxyacetophenone **12**. This compound was then condensed with a substituted benzoyl chloride in the presence of the corresponding sodium salt to give compounds **7m** and **7n**, which were oxidized by $KMnO_4$ to **4m** and **4n**, respectively.

Biological Assays

All the synthesized compounds were tested for direct cytotoxicity in a preliminary in vitro assay against four tumor cell lines, LoVo S and LoVo R, arising from human colon adenocarcinoma, and 2008 and C13*, arising from human ovarian adenocarcinoma, since one of the peculiar features of the parent compound flavone-8-acetic acid was its remarkable preferential activity on solid tumors. LoVo R cells differ from the parental LoVo S line 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[19] and mitochondrial membrane functionality.[20]

The antitumor effects shown by both flavone-8-acetic acid and xanthenone-4-acetic acid seem to deeply involve the immune system by different pathways, including stimulation of 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 or human monocytes pretreated with the new compounds was therefore measured to test them for their ability to improve lytic properties of those immune cells.

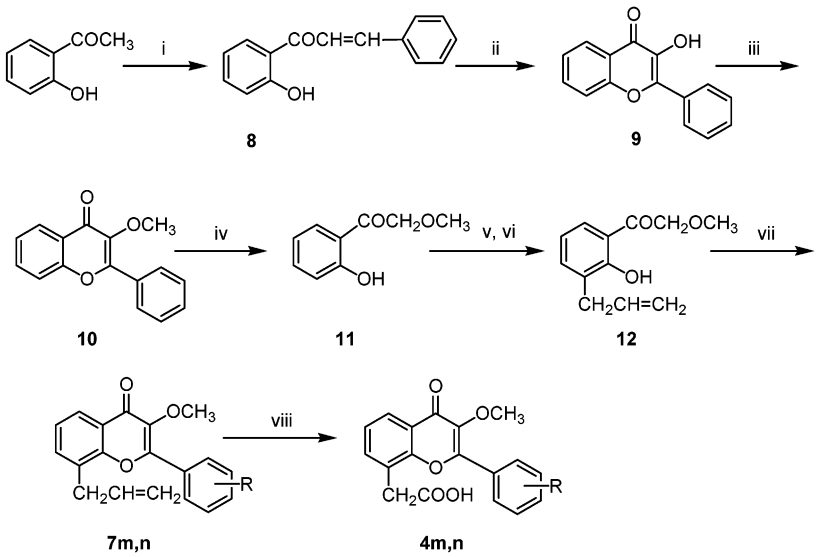
Since tumor necrosis factor α (TNF-α) appears to play a pivotal role in the antitumor effects of DMXAA in mice[21] and since the in vitro analysis of the response of human peripheral blood leucocytes to DMXAA could be a useful indicator of the activity of this class of agents,[22] the effects of selected compounds (**4e**, **4f**, **4i**, **4m**, and **4n**) 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.[23]

Results and Discussion

Direct Toxicity. Some of the synthesized compounds were able to induce direct cytotoxicity against the selected tumor cell lines (LoVo S, LoVo R, 2008, and C13*) but only at very high concentrations, since significant toxicity was only seen at the maximum tested dose (500 μM) and measured IC_{50} values were lower than the IC_{50} of the parent compound FAA (Table 2). The four cell lines showed different responses to FAA, ovarian cells being more sensitive than colon cells. Thus, the structural modifications introduced in the lead molecule caused a significant reduction of the direct cytotoxic effect, especially on ovarian adenocarcinoma derived cells (2008 and C13*).

Indirect Toxicity. Tumoricidal Activity. Indirect antitumor effect was measured as cytotoxicity induced on C13* cells cocultured with murine macrophages or human monocytes pretreated with the new compounds. The IC_{50} values for the tested compounds are shown in Table 3, where both FAA and DMXAA were taken as

Scheme 2[a]



[a] Reagents: (i) PhCHO, KOH; (ii) H_2O_2, NaOH; (iii) Me_2SO_4, K_2CO_3, reflux; (iv) KOH, EtOH, reflux; (v) allyl bromide, acetone, K_2CO_3; (vi) 250 °C, 3 h; (vii) RPhCOONa, RPhCOCl, EtOH, 180−190 °C, 7 h; (viii) $KMnO_4$, 0−5 °C, 6 h.

Table 2. Cytotoxicity of New Compounds toward the Studied Tumor Cell Lines and Potency Ratio (PR) versus FAA

compd	LoVo S IC_{50} (μM)	PR vs FAA	LoVo R IC_{50} (μM)	PR vs FAA	2008 IC_{50} (μM)	PR vs FAA	C13* IC_{50} (μM)	PR vs FAA
FAA	563.9 (464.9−684.1)	1	378.3 (310.7−460.6)	1	421.3 (369.3−480.7)	1	233.3 (183.4−297.0)	1
4a	719.4 (575.4−899.5)	0.8	626.6 (470.8−834.1)	0.6[b]	*a*		757.0 (581.5−985.3)	0.3[b]
4b	632.0 (450.5−886.6)	0.9	570.0 (436.0−745.3)	0.7	*a*		806.2 (600.9−1081.7)	0.3[b]
4c	*a*		*a*		*a*		*a*	
4d	601.2 (449.1−804.8)	0.9	555.5 (413.1−747.0)	0.7[b]	624.6 (455.3−901.8)	0.7[b]	548.0 (443.5−676.8)	0.4[b]
4e	696.6 (535.2−906.7)	0.8	648.5 (518.2−811.6)	0.6[b]	*a*		*a*	
4f	*a*		*a*		*a*		524.2 (389.3−705.9)	0.4[b]
4g	829.4 (580.2−1185.6)	0.7	513.6 (423.0−623.6)	0.7[b]	*a*		*a*	
4h	979.7 (716.9−1338.9)	0.6[b]	794.1 (575.7−995.3)	0.5[b]	*a*		*a*	
4i	*a*		*a*		*a*		350.0 (331.5−369.5)	0.7
4j	604.9 (465.6−785.9)	0.9	780.3 (594.7−953.9)	0.5[b]	*a*		*a*	
4k	611.4 (495.5−754.4)	0.9	*a*		*a*		*a*	
4l	815.1 (611.6−1086.3)	0.7[b]	406.8 (316.3−523.0)	0.9	*a*		*a*	
4m	*a*		531.5 (437.1−646.3)	0.7	*a*		*a*	
4n	534.2 (437.8−651.8)	1	*a*		*a*		*a*	
4o	*a*		*a*		*a*		*a*	

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

references. A general increase in antitumor activity with respect to direct toxicity was observed for the new compounds except for **4a**, which did not show any indirect effect.

While compounds **4c**, **4h**, **4m**, and **4o** were inactive, the other derivatives showed activity comparable to or higher than FAA on murine macrophages. In particular, compounds **4b** and **4g** proved to be able to significantly enhance the lytic properties of murine macrophages, showing IC_{50} values 1.7 and 1.6 times higher than FAA, respectively.

Very interesting seem to be the effects seen on human immune cells. As expected, FAA was not able to enhance the lytic properties of monocytes whereas DMXAA showed activity 2-fold higher in this test system than on murine macrophages. Considering the new derivatives, it should be noted that, while compounds **4b**, **4g**, **4j**, **4k**, and **4l** showed no activity on human monocytes, for compounds **4d**, **4e**, **4f**, **4i**, and **4n** the activity was enhanced going from murine to human immune cells. Interesting enough, both **4b** and **4g**, which were the most active compounds on murine macrophages, did not

Table 3. Toxicity Induced in Murine Macrophages and Human Monocytes by New Compounds (PR = Potency Ratio)

compd	murine macrophages, IC_{50} (μM)	PR vs FAA	human monocytes, C_{50} (μM)	PR vs DMXAA	PR vs macrophages
FAA	285.4 (210.9−386.2)	1	*a*		
DMXAA	211.4 (155.4−287.6)		134.6 (105.0−172.6)	1	1.6[b]
4a	*a*		*a*		
4b	164.5 (118.8−227.7)	1.7[b]	*a*		
4c	*a*		436.1 (257.5−738.8)	0.3[c]	
4d	238.5 (159.2−357.1)	1.2	181.0 (136.4−240.1)	0.7	1.3
4e	234.5 (160.8−342.0)	1.2	113.1 (88.6−144.4)	1.2	2.1[b]
4f	248.5 (169.1−367.8)	1.1	127.1 (89.1−181.2)	1	1.9[b]
4g	182.3 (132.5−250.7)	1.6[b]	*a*		
4h	*a*		192.8 (140.6−264.3)	0.7	
4i	293.4 (199.9−434.5)	0.9	105.1 (73.8−149.6)	1.3	2.8[b]
4j	283.8 (189.9−424.2)	1.0	*a*		
4k	430.6 (241.3−768.4)	0.7	*a*		
4l	224.9 (156.0−324.2)	1.3	*a*		
4m	*a*		18.3 (12.7−26.4)	7.3[b]	
4n	238.6 (166.6−339.4)	1.2	36.8 (25.5−51.0)	3.7[b]	6.5[b]
4o	*a*		212.7 (145.0−227.1)	0.6[c]	

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

show any significant activity on human cells while compounds **4c**, **4h, 4m**, and **4o** proved to be only active on human monocytes. Thus, most of the compounds showed important activity on human monocytes, which was not detected before with any flavone derivative, and a certain degree of species specificity could also be noted.

By comparison of the responses of human monocytes to DMXAA and to the new derivatives, it should be pointed out that two of the compounds (**4m** and **4n**) showed activity 7.3 and 3.7 times higher than the reference compound, respectively. It is interesting to note that compound **4m** showed higher activity than DMXAA in the human model, while it proved to be inactive on murine macrophages.

Thus, considering the effect of alkoxy groups in position 3 of the flavone ring, it should be noted that activity was lost with the introduction of a methoxy group. However, compounds **4k** and **4l**, bearing larger groups in that position, still showed activity on murine macrophages while the same substitutions seemed to be detrimental for the activity on human monocytes. Taking into account the effect of the substitution on the phenyl ring in position 2, it appears that no clear structure−activity relationships can be established. Focusing on the activity on human monocytes, the introduction of a substituent seemed to improve the activity, compound **4a**, without any substituent, being the only one totally inactive, but neither the position nor the properties of substituents seemed to be crucial for activity. However, the best results were obtained by introducing electron-withdrawing groups in the 3′ position, as in compounds **4m** and **4n**, respectively 7.3 and

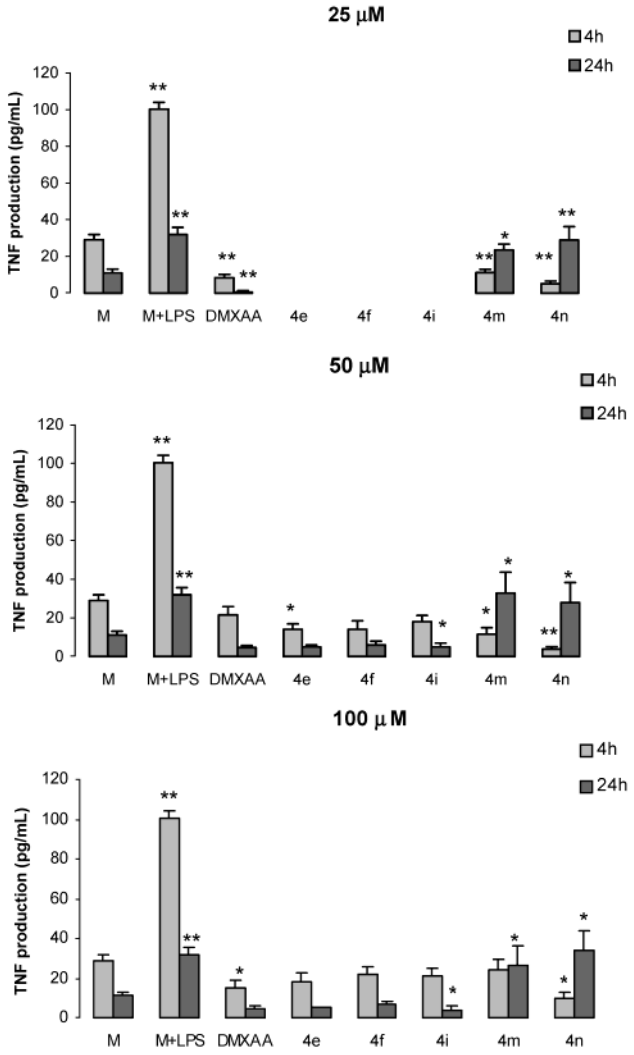


Figure 1. Effect of DMXAA and analogues tested at different concentrations on TNF-α production by human monocytes. The first two bars represent medium from unstimulated cells. A paired *t*-test was used to compare TNF-α production of stimulated and control (unstimulated) cells. ∗ or ∗∗ represent significant TNF-α production with $p < 0.05$ or $p < 0.01$, respectively.

3.7 times more active than DMXAA, which is considered a very promising compound and is now undergoing clinical trials. Unfortunately, in our hands, the analogues bearing a nitro group on the benzene ring could not be obtained in acceptable yields and purity for pharmacological testing.

It is interesting to note that the results obtained with this series of 3-alkoxy analogues of FAA seem to differ from those reported in a paper by Aitken et al.,[24] which showed how, in a series of flavone derivatives, electron-donating substituents seemed to be preferred for in vitro activity.

Tumor Necrosis Factor α (TNF-α) Production. The effects of selected compounds (**4e**, **4f**, **4i**, **4m**, and **4n**), tested at three different concentrations, on TNF-α production by HPBMC are shown in Figure 1. As expected from Philpott's results,[25] no significant cytokine production was stimulated by DMXAA alone at any of the concentrations tested. The responses of HPBMC to the new derivatives after 4 h of incubation were also significantly lower than the response to LPS, while after

24 h of incubation the levels of TNF-α obtained in response to compounds **4m** and **4n** were, at all tested doses, similar to those obtained with LPS and significantly larger than the level of the negative control (Figure 1). LPS is known to form complexes with LPS binding proteins (LBP), which interact with the CD14 receptor, a membrane-bound glycoprotein[26] linked to the TNF and IL-1 kinase cascades by the TLR2 protein on the plasma membrane,[27,28] inducing NF-κB activity.[27] DMXAA induces TNF-α by a pathway that is not identical to the one used by LPS.[22] The action of DMXAA converges on the NF-κB activation pathway through the phosphorylation and proteolysis of the IκB inhibitory subunit, which is carried out by an enzyme complex called IκB kinase (IKK).[25] Since TNF is synthesized by human peripheral blood mononuclear cells in response to the tested compounds as well as to DMXAA, it could be possible that the activation pathways are the same. Since recent results demonstrated that DMXAA stimulates TNF synthesis in cultured human leucocytes by amplifying a signal induced by another agent, it would be interesting to study the possible synergic effect between LPS and compounds **4m** and **4n**.

Conclusions

New derivatives of flavone-8-acetic acid were prepared in which different alkoxy groups in position 3 and different substituents on the phenyl ring in position 2 were introduced to further investigate the structural requirements of these parts of the molecule. The modifications performed led to a decrease in direct cytotoxicity with respect to the parent compound FAA, but almost all the compounds proved to be able to enhance the lytic potential of immune cells, both murine or human, in some cases being more active than FAA and DMXAA and supporting the hypothesis of species specificity. While insertion of an alkoxy group in position 3 alone seemed to be detrimental for activity, especially on human monocytes (compounds **4a**, **4k**, and **4l**), the simultaneous introduction of a substituent on the benzene ring in position 2 of the benzopyrane nucleus proved to be more interesting. As a result, most of the synthesized compounds showed significant activity on human monocytes and could be considered as the first group of flavone derivatives endowed with such activity. Particularly promising seemed to be compounds **4m** and **4n**, bearing electron-withdrawing substituents in the 3′ position, which proved to be 7.3- and 3.7-fold more active than DMXAA on human monocytes and were 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 $CDCl_3$ (unless otherwise indicated) 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.

General Method for the Preparation of 2′-Hydroxy-3′-alkylchalcones (5a−j,o). A 50% KOH solution (10 mL) was added dropwise to a mixture of 2′-hydroxy-3′-allylacetophenone or 2′-hydroxy-3′-methylacetophenone (compounds **5c**, **5d**, and **5o**) (28 mmol) and the selected benzaldehyde (28 mmol) in EtOH (50 mL), and the mixture was stirred at room temperature overnight. The reaction mixture was then poured into ice and acidified with HCl. The precipitate was filtered (or the mixture was extracted with CH_2Cl_2) and crystallized from EtOH.

5a: yield 50%; mp 103−105 °C; ^{1}H NMR δ 3.48 (d, J = 6 Hz, 2H, CH_2Ar), 5.14 (m, 2H, CH_2=CH−), 6.08 (m, 1H, CH_2=CH−CH_2−), 6.85−8.0 (m, 10H, olefinic and aromatic). Anal. ($C_{18}H_{16}O_2$) C, H.

5b: yield 20%; mp 67−70 °C; ^{1}H NMR δ 2.40 (s, 3H, CH_3), 3.48 (d, J = 6 Hz, 2H, CH_2−Ar), 5.12 (m, 2H, CH_2=CH−), 6.05 (m, 1H, CH_2=CH−CH_2−), 6.86−7.96 (m, 9H, olefinic and aromatic). Anal. ($C_{19}H_{18}O_2$) C, H.

5c: yield 75%; mp 104−107 °C; ^{1}H NMR δ 2.3 (s, 3H, CH_3), 6.8−7.9 (m, 9H, olefinic and aromatic). Anal. ($C_{16}H_{13}FO_2$) C, H.

5d: yield 70%; mp 104−107 °C; ^{1}H NMR δ 2.3 (s, 3H, CH_3), 3.9 (s, 3H, OCH_3), 6.8−7.95 (m, 9H, olefinic and aromatic). Anal. ($C_{17}H_{16}O_3$) C, H.

5e: yield 45%; mp 54−55 °C; ^{1}H NMR δ 2.4 (s, 3H, CH_3), 3.5 (m, 2H, CH_2Ar), 5.15 (m, 2H, CH_2=CH−), 6.1 (m, 1H, CH_2=CH−CH_2−), 6.95−7.9 (m, 9H, olefinic and aromatic), 13.2 (s, 1H, OH). Anal. ($C_{19}H_{18}O_2$) C, H.

5f: yield 35%; mp 48−49 °C; ^{1}H NMR δ 2.5 (s, 3H, CH_3), 3.5 (m, 2H, CH_2Ar), 5.15 (m, 2H, CH_2=CH−), 6.1 (m, 1H, CH_2=CH−CH_2−), 6.9−8.3 (m, 9H, olefinic and aromatic). Anal. ($C_{19}H_{18}O_2$) C, H.

5g: yield 28%; mp 52−53 °C; ^{1}H NMR δ 3.5 (m, 2H, CH_2-Ar), 3.9 (s, 3H, OCH_3), 5.1 (m, 2H, CH_2=CH−), 6.05 (m, 1H, CH_2=CH−CH_2−), 6.9−7.9 (m, 9H, olefinic and aromatic). Anal. ($C_{19}H_{18}O_3$) C, H.

5h: yield 43%; mp 72−73 °C; ^{1}H NMR δ 3.45 (m, 2H, CH_2-Ar), 3.95 (s, 3H, OCH_3), 5.1 (m, 2H, CH_2=CH−), 6.05 (m, 1H, CH_2=CH−CH_2−), 6.9−8.25 (m, 9H, olefinic and aromatic). Anal. ($C_{19}H_{18}O_3$) C, H.

5i: yield 23%; mp 97−100 °C; ^{1}H NMR δ 3.5 (m, 2H, CH_2-Ar), 5.15 (m, 2H, CH_2=CH−), 6.05 (m, 1H, CH_2=CH−CH_2−), 6.9−7.9 (m, 9H, olefinic and aromatic), 13.15 (s, 1H, OH). Anal. ($C_{18}H_{15}ClO_2$) C, H.

5j: yield 96%; mp 54−55 °C; ^{1}H NMR δ 3.5 (m, 2H, CH_2Ar), 5.15 (m, 2H, CH_2=CH−), 6.05 (m, 1H, CH_2=CH−CH_2−), 6.9−8.35 (m, 9H, olefinic and aromatic), 13.1 (s, 1H, OH). Anal. ($C_{18}H_{15}ClO_2$) C, H.

5o: yield 60%; mp 64−66 °C; ^{1}H NMR δ 2.4 (s, 3H, CH_3), 6.85−8.2 (m, 8H, olefinic and aromatic). Anal. ($C_{14}H_{12}O_2S$) C, H.

General Method for the Preparation of 8-Alkylflavonols (6a−j,o). A 30% H_2O_2 (7 mL) solution was added dropwise to a mixture of the appropriate chalcone **5** (0.01 mol) and NaOH (5 g) in 50% EtOH (50 mL). The mixture was stirred at room temperature overnight and then poured into ice and acidified with HCl. The precipitate was filtered and crystallized from MeOH.

6a: yield 70%; mp 176−179 °C; ^{1}H NMR δ 3.8 (d, J = 6 Hz, 2H, CH_2−Ar), 5.20 (m, 2H, CH_2=CH−), 6.12 (m, 1H, CH_2=CH−CH_2−), 7.1−8.22 (m, 8H, aromatic). Anal. ($C_{18}H_{14}O_3$) C, H.

6b: yield 50%; mp 186−190 °C; ^{1}H NMR δ 2.48 (s, 3H, CH_3), 3.80 (d, J = 6 Hz, 2H, CH_2−Ar), 5.20 (m, 2H, CH_2=CH−), 6.12 (m, 1H, CH_2=CH−CH_2), 6.96−8.25 (m, 7H, aromatic). Anal. ($C_{19}H_{16}O_3$) C, H.

6c: yield 50%; mp 200−204 °C; ^{1}H NMR δ 2.65 (s, 3H, CH_3), 7.1 (s, 1H, OH), 7.2−8.3 (m, 7H, aromatic). Anal. ($C_{16}H_{11}FO_3$) C, H.

6d: yield 40%; mp 211−214 °C; ^{1}H NMR δ 2.65 (s, 3H, CH_3), 3.9 (s, 3H, OCH_3), 7.0 (s, 1H, OH), 7.05−8.3 (m, 7H, aromatic). Anal. ($C_{17}H_{14}O_4$) C, H.

6e: yield 19%; mp 119−120 °C; ^{1}H NMR δ 2.5 (s, 3H, CH_3), 3.8 (m, 2H, CH_2Ar), 5.2 (m, 2H, CH_2=CH−), 6.1 (m, 1H, CH_2=

CH−CH_2−), 7.05 (broad, 1H, OH), 7.3−8.2 (m, 7H, aromatic). Anal. ($C_{19}H_{16}O_3$) C, H.

6f: yield 21%; mp 128−129 °C; ^{1}H NMR δ 2.4 (s, 3H, CH_3), 3.65 (m, 2H, *CH_2*Ar), 5.1 (m, 2H, *CH_2*=CH−), 6.0 (m, 1H, CH_2=*CH*−CH_2−), 6.5 (broad, 1H, OH), 7.3−8.2 (m, 7H, aromatic). Anal. ($C_{19}H_{16}O_3$) C, H.

6g: yield 10%; mp 103−105 °C; ^{1}H NMR δ 3.8 (m, 2H, *CH_2*-Ar), 3.9 (s, 3H, OCH_3), 5.15 (m, 2H, *CH_2*=CH−), 6.1 (m, 1H, CH_2=*CH*−CH_2−), 7.0−8.15 (m, 8H, OH and aromatic). Anal. ($C_{19}H_{16}O_4$) C, H.

6h: yield 31%; mp 183−184 °C; ^{1}H NMR δ 3.65 (m, 2H, *CH_2*-Ar), 3.9 (s, 3H, OCH_3), 5.1 (m, 2H, *CH_2*=CH−), 6.0 (m, 1H, CH_2=*CH*−CH_2−), 6.5 (s, 1H, OH), 7.1−8.2 (m, 7H, aromatic). Anal. ($C_{19}H_{16}O_4$) C, H.

6i: yield 10%; mp 195−198 °C; ^{1}H NMR δ 3.8 (m, 2H, *CH_2*-Ar), 5.15 (m, 2H, *CH_2*=CH−), 6.1 (m, 1H, CH_2=*CH*−CH_2−), 7.05 (broad, 1H, OH), 7.35−8.25 (m, 7H, aromatic). Anal. ($C_{18}H_{13}ClO_3$) C, H.

6j: yield 15%; mp 120−122 °C; ^{1}H NMR δ 3.7 (m, 2H, *CH_2*-Ar), 5.1 (m, 2H, *CH_2*=CH−), 6.05 (m, 1H, CH_2=*CH*−CH_2−), 6.5 (broad, 1H, OH), 7.35−8.2 (m, 7H, aromatic). Anal. ($C_{18}H_{13}ClO_3$) C, H.

6o: yield 40%; mp 206−208 °C; ^{1}H NMR δ 2.6 (s, 3H, CH_3), 7.25−8.1 (m, 6H, aromatic). Anal. ($C_{14}H_{10}O_3S$) C, H.

General Method for the Preparation of 3-Alkoxy-8-alkylflavones (7a−l,o). A mixture of the appropriate 8-alkylflavonol **6** (1.8 mmol), dry K_2CO_3 (1.8 mmol), and dimethyl sulfate (1.8 mmol) or the selected alkyl halide (3.6 mmol) in acetone (50 mL) was refluxed for 7−9 h and hot-filtered. The solvent was evaporated, and the residue was crystallized from EtOH.

7a: yield 95%; mp 99−100 °C; ^{1}H NMR δ 3.75 (m, 2H, *CH_2*-Ar), 3.92 (s, 3H, OCH_3), 5.15 (m, 2H, *CH_2*=CH), 6.1 (m, 1H, CH_2=*CH*−CH_2−), 7.35−8.15 (m, 8H, aromatic). Anal. ($C_{19}H_{16}O_3$) C, H.

7b: yield 83%; mp 128−129 °C; ^{1}H NMR δ 2.4 (s, 3H, CH_3), 3.7 (m, 2H, *CH_2*Ar), 3.9 (s, 3H, OCH_3), 5.1 (m, 2H, *CH_2*=CH−), 6.05 (m, 1H, CH_2=*CH*−CH_2−), 7.3−8.1 (m, 7H, aromatic). Anal. ($C_{20}H_{18}O_3$) C, H.

7c: yield 90%; mp 169−171 °C; ^{1}H NMR δ 2.5 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 7.15−8.2 (m, 7H, aromatic). Anal. ($C_{17}H_{13}FO_3$) C, H.

7d: quantitative yield; mp 147−150 °C; ^{1}H NMR δ 2.5 (s, 3H, CH_3), 3.85 (s, 6H, OCH_3), 7.0−8.2 (m, 7H, aromatic). Anal. ($C_{18}H_{16}O_4$) C, H.

7e: yield 83%; mp 95−96 °C; ^{1}H NMR δ 2.5 (s, 3H, CH_3), 3.75 (m, 2H, *CH_2*Ar), 3.9 (s, 3H, OCH_3), 5.2 (m, 2H, *CH_2*=CH−), 6.05 (m, 1H, CH_2=*CH*−CH_2−), 7.35−8.2 (m, 7H, aromatic). Anal. ($C_{20}H_{18}O_3$) C, H.

7f: yield 50%; mp 82−85 °C; ^{1}H NMR δ 2.4 (s, 3H, CH_3), 3.6 (m, 2H, *CH_2*Ar), 3.72 (s, 3H, OCH_3), 5.1 (m, 2H, *CH_2*=CH−), 6.0 (m, 1H, CH_2=*CH*−CH_2), 7.35−8.2 (m, 7H, aromatic). Anal. ($C_{20}H_{18}O_3$) C, H.

7g: quantitative yield; mp 76−78 °C; ^{1}H NMR δ 3.7 (m, 2H, *CH_2*Ar), 3.9 (s, 6H, 2 × OCH_3), 5.1 (m, 2H, *CH_2*=CH−), 6.1 (m, 1H, CH_2=*CH*−CH_2−), 7.05−8.15 (m, 7H, aromatic). Anal. ($C_{20}H_{18}O_4$) C, H.

7h: quantitative yield; mp 104−105 °C; ^{1}H NMR δ 3.6 (m, 2H, *CH_2*Ar), 3.85 (s, 3H, OCH_3), 3.9 (s, 3H, OCH_3), 5.1 (m, 2H, *CH_2*=CH−), 6.0 (m, 1H, CH_2=*CH*−CH_2−), 7.1−8.2 (m, 7H, aromatic). Anal. ($C_{20}H_{18}O_4$) C, H.

7i: yield 25%; mp 132−134 °C; ^{1}H NMR δ 3.72 (m, 2H, *CH_2*-Ar), 3.9 (s, 3H, OCH_3), 5.15 (m, 2H, *CH_2*=CH−), 6.1 (m, 1H, CH_2=*CH*−CH_2−), 7.35−8.2 (m, 7H, aromatic). Anal. ($C_{19}H_{15}ClO_3$) C, H.

7j: yield 71%; mp 96−98 °C; ^{1}H NMR δ 3.6 (m, 2H, *CH_2*Ar), 3.85 (s, 3H, OCH_3), 5.1 (m, 2H, *CH_2*=CH−), 6.0 (m, 1H, CH_2=*CH*−CH_2−), 7.35−8.2 (m, 7H, aromatic). Anal. ($C_{19}H_{15}ClO_3$) C, H.

7k: starting from **6a** and ethyl bromide, yield 36%; mp 88−89 °C; ^{1}H NMR δ 1.3 (t, J = 7 Hz, 3H, CH_3), 3.7 (m, 2H, *CH_2*-Ar), 4.15 (q, J = 7 Hz, 2H, O*CH_2*CH_3), 5.1 (m, 2H, *CH_2*=CH−), 6.05 (m, 1H, CH_2=*CH*−CH_2−), 7.3−8.15 (m, 8H, aromatic). Anal. ($C_{20}H_{18}O_3$) C, H.

7l: starting from **6a** and isopropyl bromide, yield 37%; mp 66−67 °C; ^{1}H NMR δ 1.2 (d, J = 6 Hz, 6H, 2 × CH_3), 3.6 (m, 2H, *CH_2*Ar), 4.65 (m, 1H, CH), 5.1 (m, 2H, *CH_2*=CH−), 6.0 (m, 1H, CH_2=*CH*−CH_2), 7.35−8.25 (m, 8H, aromatic). Anal. ($C_{21}H_{20}O_3$) C, H.

7o: yield 80%; mp 133−134 °C; ^{1}H NMR δ 2.6 (s, 3H, CH_3), 4.05 (s, 3H, OCH_3), 6.65−8.1 (m, 6H, aromatic). Anal. ($C_{15}H_{12}O_3S$) C, H.

2′-Hydroxy-3′-allyl-2-methoxyacetophenone (12). A mixture of **11**[18] (6.6 g, 0.04 mol), dry K_2CO_3 (5.5 g, 0.04 mol), and allyl bromide (6.3 g, 0.05 mol) in acetone (40 mL) was refluxed for 15 h and hot-filtered. The solution was washed with 2 N NaOH and then with H_2O, and the solvent was evaporated to give 2′-allyloxy-2-methoxyacetophenone, which was heated to 250 °C for 4 h. The residue was then resuspended in ether and extracted with 2 N NaOH. The aqueous layer was acidified with HCl and extracted with ether, which was then evaporated to dryness to give **12** as an oil (33%): ^{1}H NMR δ 3.45 (m, 2H, *CH_2*Ar), 3.50 (s, 3H, OCH_3), 3.70 (s, 2H, CO*CH_2*), 5.1 (m, 2H, *CH_2*=CH−), 6.0 (m, 1H, CH_2=*CH*−CH_2−), 6.80−7.60 (m, 3H, aromatic), 12.10 (s, 1H, OH). Anal. ($C_{12}H_{14}O_3$) C, H.

3-Methoxy-8-allyl-3′-trifluoromethylflavone (7m). A mixture of **12** (2.75 g, 13.4 mmol), 3-trifluoromethylbenzoyl chloride (6 g, 26.8 mmol, prepared by refluxing 6 g of the corresponding acid with 60 mL of $SOCl_2$ for 8 h and evaporating to dryness), and the sodium salt of 3-trifluoromethylbenzoic acid (8.5 g, 40.1 mmol, prepared by stirring at room temperature 10 g of the corresponding acid and 1.21 g of Na in EtOH) was heated to 180−190 °C for 7 h. The solid was then resuspended in H_2O and extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried, and evaporated. The residue was purified by column chromatography (petroleum ether/ethyl acetate 9:1) to give 0.5 g (10%) of **7m**: mp 76−77 °C; ^{1}H NMR δ 3.70 (m, 2H, *CH_2*Ar), 3.90 (s, 3H, OCH_3), 5.05 (m, 2H, *CH_2*=CH−), 6.0 (m, 1H, CH_2=*CH*−CH_2−), 7.25−8.45 (m, 7H, aromatic). Anal. ($C_{20}H_{15}F_3O_3$) C, H.

3-Methoxy-8-allyl-3′-chloroflavone (7n). By use of the same procedure and starting from **12** (1.6 g, 7.76 mmol), 3-chlorobenzoyl chloride (2.8 g, 16 mmol), and the sodium salt of 3-chlorobenzoic acid (4.25 g, 23.8 mmol, prepared by stirring at room temperature 5 g of the corresponding acid and 0.74 g of Na in EtOH), 1.7 g (67%) of **7n** was obtained: mp 109−112 °C; ^{1}H NMR δ 3.70 (m, 2H, *CH_2*Ar), 3.90 (s, 3H, OCH_3), 5.20 (m, 2H, *CH_2*=CH−), 6.10 (m, 1H, CH_2=*CH*−CH_2−), 7.25−8.20 (m, 7H, aromatic). Anal. ($C_{19}H_{15}ClO_3$) C, H.

General Method for the Preparation of Substituted 3-Alkoxyflavone-8-acetic Acids (4a,b,e−n). Solid $KMnO_4$ (0.1 mol) was added portionwise over 6 h to an ice-cold solution of the allyl derivative (0.02 mol) in a mixture of acetic acid (45 mL), acetone (90 mL), and water (35 mL). The mixture was stirred at room temperature for another 1 h and poured into water. The aqueous solution was then decolorated with H_2O_2, extracted with CH_2Cl_2, and evaporated to dryness (or the precipitate was filtered). The crude products were crystallized from EtOH (see Table 1).

3,4′-Dimethoxyflavone-8-acetic Acid (4d). A mixture of **7d** (3 g, 10 mmol), *N*-bromosuccinimide (7.6 g, 0.04 mol), and a catalytic amount of benzoyl peroxide in CCl_4 (300 mL) was refluxed for 5 h and hot-filtered. The precipitate formed was filtered and crystallized from EtOH to give the bromo derivative (2.6 g, 70%): mp 189−191 °C. To a solution of this compound (1.8 g, 5 mmol) in EtOH (400 mL), KCN (0.4 g, 5.9 mmol) in water (10 mL) was added and the mixture was refluxed for 20 h. Then the solvent was evaporated, and the residue was resuspended in H_2O and extracted with CH_2Cl_2. The organic layer was dried and evaporated, and the residue was crystallized from EtOH/H_2O to give the corresponding nitrile (1.1 g, 75%): mp 148−153 °C. The resulting compound (1.1 g, 3 mmol) was then dissolved in a mixture of acetic acid (4 mL), H_2O (4 mL), and concentrated H_2SO_4 (4 mL), and the mixture was refluxed for 2 h. H_2O was then added, and a precipitate was formed, which was filtered and resuspended in aqueous $NaHCO_3$. The solution was filtered and acidified

with HCl, and the precipitate formed was filtered, dried, and crystallized from EtOH to give **4d** (see Table 1).

3-Methoxy-4′-fluoroflavone-8-acetic Acid (4c). Starting from **7c** and by use of the same procedure described above, the following compounds were prepared: bromo derivative (75%, ethyl acetate), mp 160−163 °C; nitrile (60%, EtOH/H_2O), mp 165−166 °C; **4c** (see Table 1).

2-Tienyl-3-methoxychromone-8-acetic Acid (4o). Starting from **7o** and by use of the same procedure described above, the following compounds were prepared: bromo derivative (60%, ethyl acetate), mp 126−128 °C; nitrile (40%, EtOH/H_2O), mp 165−168 °C; **4o** (see Table 1).

^{1}H NMR and Mass Spectrum Data for Compounds 4a−o. 4a: ^{1}H NMR δ 3.9 (s, 3H, OCH_3), 4.0 (s, 2H, CH_2COOH), 7.4−8.25 (m, 8H, aromatic). MS *m*/*z* (rel abundance): 310 (M^+, 69.66), 309 (100), 77 (47.22).

4b: ^{1}H NMR δ 2.4 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 4.0 (s, 2H, CH_2COOH), 7.3−8.25 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 324 (M^+, 72.75), 323 (100), 309 (48.04).

4c: ^{1}H NMR (DMSO-d_6) δ 3.8 (s, 3H, OCH_3), 3.95 (s, 2H, CH_2COOH), 7.35−8.1 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 328 (M^+, 78.57), 327 (100), 133 (32.27).

4d: ^{1}H NMR (DMSO-d_6) δ 3.8 (s, 3H, OCH_3), 3.9 (s, 3H, OCH_3), 4.0 (s, 2H, CH_2COOH), 7.1−8.1 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 340 (M^+, 76.52), 339 (100), 133 (24.47).

4e: ^{1}H NMR δ 2.4 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 4.0 (s, 2H, CH_2COOH), 7.2−8.25 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 324 (M^+, 88.03), 323 (100), 309 (60.43).

4f: ^{1}H NMR δ 2.4 (s, 3H, CH_3), 3.8 (s, 3H, OCH_3), 3.9 (s, 2H, CH_2COOH), 7.15−8.15 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 324 (M^+, 27.43), 309 (100), 77 (72.61).

4g: ^{1}H NMR δ 3.85 (s, 3H, OCH_3), 3.9 (s, 3H, OCH_3), 4.0 (s, 2H, CH_2COOH), 7.05−8.25 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 340 (M^+, 21.41), 310 (100), 129 (50.94).

4h: ^{1}H NMR δ 3.75 (s, 3H, OCH_3), 3.8 (s, 2H, CH_2COOH), 3.85 (s, 3H, OCH_3), 7.0−8.15 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 340 (M^+, 40.11), 309 (59.36), 32 (100).

4i: ^{1}H NMR δ 3.9 (s, 3H, OCH_3), 4.0 (s, 2H, CH_2COOH), 7.4−8.25 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 344 (M^+, 79.15), 343 (100), 77 (79.99).

4j: ^{1}H NMR δ 3.8 (s, 3H, OCH_3), 3.95 (s, 2H, CH_2COOH), 7.35−8.3 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 344 (M^+, 21.50), 309 (100), 129 (64.21).

4k: ^{1}H NMR δ 1.3 (t, J = 7 Hz, 3H, CH_3), 4.0 (s, 2H, CH_2-COOH), 4.15 (q, J = 7 Hz, 2H, OCH_2CH_3), 7.35−8.25 (m, 8H, aromatic). MS *m*/*z* (rel abundance): 324 (M^+, 100), 105 (62.35), 77 (62.79).

4l: ^{1}H NMR δ 1.2 (d, J = 6 Hz, 6H, 2 × CH_3), 4.0 (s, 2H, CH_2COOH), 4.65 (m, 1H, CH), 7.35−8.25 (m, 8H, aromatic). MS *m*/*z* (rel abundance): 338 (M^+, 16.43), 296 (100), 77 (26.67).

4m: ^{1}H NMR (DMSO-d_6) δ 3.85 (s, 3H, OCH_3), 3.92 (s, 2H, CH_2COOH), 7.35−8.4 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 378 (M^+, 31.19), 173 (99.04), 44 (100).

4n: ^{1}H NMR (DMSO-d_6) δ 3.90 (s, 3H, OCH_3), 4.0 (s, 2H, CH_2COOH), 7.35−8.15 (m, 7H, aromatic). MS *m*/*z* (rel abundance): 344 (M^+, 88.99), 343 (100), 133 (84.51).

4o: ^{1}H NMR (DMSO-d_6) δ 3.95 (s, 3H, OCH_3), 4.0 (s, 2H, CH_2COOH), 7.35−8.0 (m, 6H, aromatic). MS *m*/*z* (rel abundance): 316 (M^+, 100), 245 (29.03), 133 (58.68).

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.[29]

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 Macrophages. The ability of the new derivatives to stimulate mouse macrophages in culture to become tumoricidal was evaluated using resident peritoneal macrophages.[30] 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.[31]

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 FAA, DMXAA, and analogues, using triplicate wells per drug dose. After 24 h, the medium was discarded and the C13* (2×10^3 cells/well) cells were plated as above. The optimal macrophages/C13* cells ratio has been determined in preliminary experiments (results not reported). The cells were cocultured for 24 h. Lysis of C13* cells was assessed by the MTT test,[32] and the percentages of specific cytotoxicity were calculated as follows:

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

Cultures of macrophages in the presence of the same drug concentrations were included in all experiments, but the levels of direct drug toxicity were always not significant, less than 5%.

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 FAA, 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.

Cultures of monocytes in the presence of the same drug concentrations were included in all experiments, but the levels of direct drug toxicity were always not significant, less than 5%.

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; F8, 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. Viability

of the cells was assessed by Trypan blue dye exclusion and was always more than 95%.

Commercially available enzymes-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.[33] The IC_{50}, 95% confidence limits, and the potency ratio of FAA or DMXAA to each analogue (IC_{50}(FAA)/IC_{50}(derivative) or IC_{50}(DMXAA)/IC_{50}(derivative)) were estimated using the Litchfield and Wilcoxon method.[33]

The effect of DMXAA and analogues on TNF-α production by human monocytes was compared using a paired *t*-test.[33]

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