

The ability of coumarin-, flavanon- and flavonol-analogues of flavone acetic acid to stimulate human monocytes

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Abstract. Flavone acetic acid (FAA) is a semi-synthetic flavonoid characterised by potent immune-modulatory and antivasular activity on mice but not in humans. Previously, the synthesis and cytotoxic activity on a human adenocarcinoma cell line of coumarin-, flavanon- and flavonol-derivatives of FAA were described. These analogues were able to induce the reduction of lysosomal neutral red uptake at 5×10^{-5} M concentration and some of them were more effective than FAA. Some of these derivatives were selected to investigate their ability to exert immune-modulation on a human model, by using the most potent analogue that has emerged thus far, 5,6-dimethylxanthene-4-acetic acid (DMXAA), as a reference compound. We investigated the cytotoxicity of the selected derivatives on two human ovarian adenocarcinoma cell lines and their ability to activate the immune system by inducing lytic properties, TNF- α and nitric oxide in human monocytes. The immune-modulating activity was assessed by treating a cell line of human monocytes (Mono Mac 6, MM6) with FAA-derivatives alone or in association with lipopolysaccharide (LPS). None of the tested molecules showed any significant ability to directly affect tumor cell proliferation, whereas they were able to induce the lytic properties of MM6 cells. In particular, two coumarin derivatives, *a* and *d*, and the flavonol acetic acid, *l*, showed comparable results to DMXAA. The combination with LPS did not lead to synergistic interactions in the induction of the lytic properties of MM6, but it significantly increased the release of TNF- α , especially after 4 h of treatment. Instead, the maximum release of nitric oxide (NO) was detected after 24 h of treatment and

after exposure to the FAA derivatives alone. Derivative *a* combined with LPS and analogue *d* alone were able to induce a higher TNF- α and NO release, respectively, whereas flavonol acetic acid was characterised by a strictly similar activity to DMXAA.

Introduction

The semi-synthetic molecule flavone-8-acetic acid (FAA, 1; Fig. 1) emerged in a large screening of flavonoids (1,2) for its excellent and peculiar activity against a broad set of transplantable solid murine tumors (such as colon, pancreatic ductal adenocarcinomas, mammary adenocarcinoma, reticulum cell sarcoma and Glasgow's osteosarcoma) normally resistant to many cytotoxic drugs (3). The antitumor profile of FAA involves effects exerted on the immune and vascular systems (4-7). FAA acts on the vascular network not only by altering the blood perfusion, reducing plasma volumes and leading to blood vessel shutdown (8), with a consequent interruption of the tumor blood supply (9), but also by affecting the pro-coagulant activity of endothelial cells (10). The immune-modulation exerted by FAA involves the natural killer cell activity (5) and the release of several cytokines, among which TNF- α plays the main role (4). Unfortunately, FAA did not exhibit any significant activity in clinical trials (2). In order to obtain an effective molecule on human models, extensive SAR studies have been performed on FAA analogues. These investigations first led to the xanthene-4-acetic acid (11) (XAA, 2; Fig. 1), closely related and significantly more potent than FAA, and then to the synthesis of 5,6-dimethyl-xanthene-4-acetic acid (DMXAA, 3; Fig. 1), the most potent derivative, which turned out to be significantly active on human models and selective on tumor tissue (12). Thus, DMXAA was selected for further investigation and, at present, is under phase II clinical trials as a vascular disrupting agent (13). The DMXAA-mediated immune stimulation results in the induction of the nuclear transcription factor NF- κ B, due to the inactivation of its inhibitory factor, I κ B- α . This event likely occurs through the allosteric interaction of DMXAA with IKK, a kinase protein which triggers the destruction of I κ B- α through its phosphorylating activity (14). Furthermore, a very remarkable enhancement of the TNF- α

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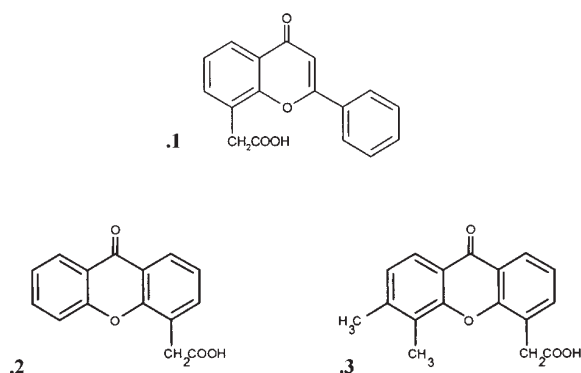


Figure 1. Molecular structures of FAA, XAA and DMXAA.

synthesis in human leukocytes (14) and murine splenocytes (15) was pointed out after exposure to the associations of DMXAA and LPS, suggesting that DMXAA requires prior activation of IKK before acting (16).

For some years, our research group has been interested in the synthesis and biological evaluation of FAA analogues (17-21). In a previous study (17), the synthesis and cytotoxic activity on the human tumor cell lines of some FAA derivatives were described. These coumarin-, flavanon- and flavonol-acetic acids were able to induce a statistically significant reduction of lysosomal neutral red uptake at a 5×10^{-5} M concentration. Some compounds were more active than FAA.

In this study, we carefully examined the effects on tumor cell proliferation and immune system activation resulting in treatment with some of these previously studied compounds (Fig. 2), using DMXAA as a reference compound. In particular, we investigated the cytotoxic activity on two human tumor cell lines and their ability to activate the immune system, by evaluating the ability of the derivatives to induce, in human monocytes, lytic properties exerted on a tumor cell line and the release of TNF- α and nitric oxide.

Materials and methods

Compounds and sample solutions. DMXAA and FAA derivatives were stored in the dark and at room temperature. The samples for experimental use were prepared from stock solutions (10^{-3} M, in DMSO and distilled water) and diluted with culture medium. The DMSO concentration in sample solutions was lower than 1% v/v. LPS (lipopolysaccharide from *Escherichia coli* serotype 0127; F8, Sigma) was stored at 4 to 8°C in the dark and the sample solutions were prepared by diluting a stock solution (1 mg/ml in distilled water) with culture medium.

Cell lines. Two human ovarian adenocarcinoma cell lines, 2008 and the cis-DDP-resistant cell line C13*, were used. Both were maintained in RPMI-1640 supplemented with 10% heat-inactivated FCS, 1% antibiotics (all products Biochrom KG Seromed) and 200 mM glutamine (Merck). The human monocytic leukaemia cells Mono Mac 6 (MM6), were supplied by the 'Istituto Nazionale per la Ricerca sul Cancro - Genova' and maintained in the same culture medium and then supplemented with 1% sodium pyruvate 100 mM,

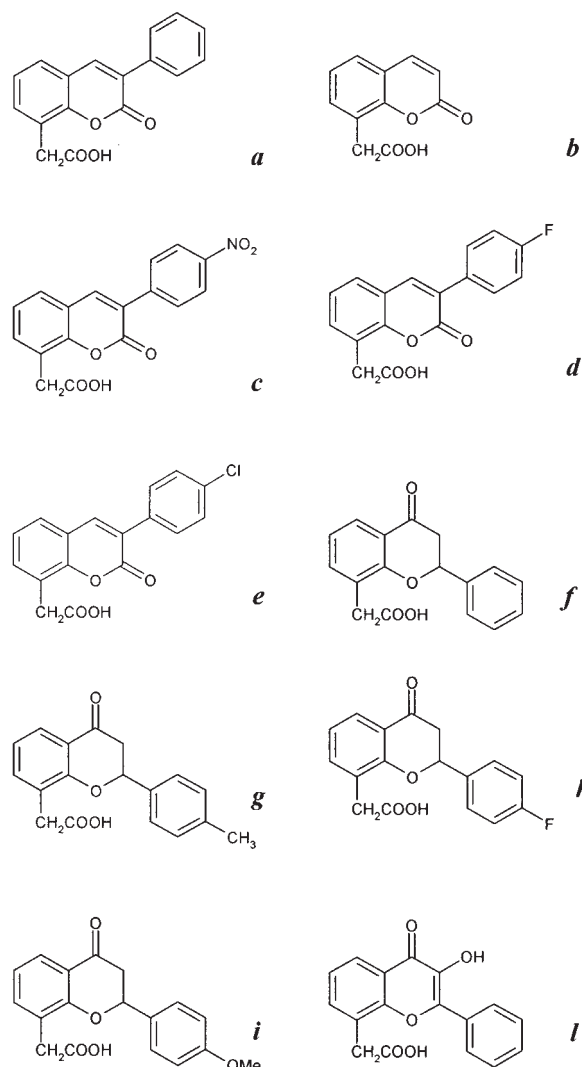


Figure 2. Coumarin (a-e)-, flavanon (f-i)- and flavonol (l)-acetic acid chemical structures.

1% non-essential amino acids and bovine insulin 9 μ g/ml (Biochrom KG Seromed).

Cytotoxicity on 2008 and C13* cells. Cells were seeded (8×10^3 cells/well) in 96-well tissue plates (Falcon) and treated with 100, 250 and 500 μ M DMXAA and FAA derivatives for 24 h. Cell proliferation was evaluated by the tetrazolium salts reduction assay (MTT test).

Cytotoxicity on the Mono Mac 6 cell line. Cells were seeded (2×10^4 cells/well) in 96-well tissue plates and exposed to 25, 50 and 100 μ M DMXAA and FAA derivatives for 24 h. Cell proliferation was assessed by the tetrazolium salts reduction assay (XTT test).

Stimulation of lytic properties of MM6 cells. MM6 cells were seeded and treated as above in the previous assays. After 24 h of treatment the media were drawn and the C13* cells were plated (2×10^3 cells/well) on the wells containing the treated MM6 cells. The optimal MM6/C13* cell ratio was determined

Table I. IC₅₀ μ M and 95% confidence limits, after 24 h of treatment with 100, 250 or 500 μ M DMXAA or FAA-derivatives.

Cell line	DMXAA	<i>a</i>	<i>d</i>	<i>f</i>	<i>l</i>
2008	448.1 (395.9-507.3)	1196.7 (872.9-1641.1)			2514.7 (1586.2-3686.8)
C13*	641.8 (511.1-805.7)		1616.3 (999.6-2613.5)	740.6 (589.7-930.2)	

in previous experiments (results not reported). Cells were co-cultured for 24 h and then the viability of C13* was assessed by the XTT test. As a control, samples containing non-treated MM6 and C13* cells and C13* co-cultured with non-treated MM6 were seeded. The percentages of the specific growth of C13* cells were calculated as follows:

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

The same assay was also performed by treating MM6 with the same doses of DMXAA or the compounds *a*, *d* and *l*, combined with LPS 10 ng/ml.

MTT test. MTT (Sigma) solution (20 μ l of which 5 mg/ml was in PBS) were added to each well and the plates were incubated for 4 h at 37°C. Then the media were discarded and the dark blue crystals were dissolved in DMSO (150 μ l/well). The absorbance was measured on a micro-culture plate reader (Titertek Multiscan) using 570 and 630 nm as a test and reference wavelength, respectively.

XTT test. A commercially available kit (Cell proliferation kit II-XTT, Roche) was used to obtain an XTT solution, according to the manufacturer's instructions. XTT (75 μ l) was added to each well and the plates were incubated at 37°C. After 2 h, absorbance was directly measured on a micro-culture plate reader using 450 and 630 nm as a test and reference wavelength, respectively.

Stimulation of TNF- α release. MM6 cells were treated with DMXAA or the compounds *a*, *d* and *l*, with or without LPS at the same concentration used for the MM6-mediated cytotoxicity assays. After 4 or 24 h incubation, culture media were collected and stored at -80°C. A commercially available ELISA kit was used to measure the concentration of TNF- α (Human TNF- α Instant ELISA, Biosource) according to the manufacturer's instructions. The results were normalised according to the protein content of each sample.

Nitric oxide release by 'Griess' reaction. The nitrite/nitrate concentration, a reliable indicator of nitroxide production and release, was evaluated by the photometric 'Griess' reaction, in the same culture media tested for the TNF- α release assay. The samples were previously treated as described by Miranda *et al* (22), in order to reduce all the nitrate ions to nitrite. Then, 500 μ l of each sample was mixed with 250 μ l of Griess A (1% sulphanilamide in 5% phosphoric acid) and Griess B (0.1% naphthylethylenediamine dihydrochloride in distilled

water) reagents (all from Sigma) and the absorbance was measured at 543 nm. The results were normalised according to the protein content of each sample.

Bradford protein assay. The evaluation of the content of proteins in the samples treated for the TNF- α and nitrite assays was carried out according to the Bradford method (23). After treatment of the TNF- α and nitrite assays, the MM6 cells were washed and centrifuged 3 times with ice-cold PBS and then incubated on ice for 30 min with 0.5 ml lysis buffer (50 mM Tris HCl, 1 mM DTT, 1 mM Na₂ EDTA and 1% v/v Triton X-100). The lysates were then centrifuged for 15 min at 4°C and 15,000 rpm and the supernatants were stored at -80°C. The lysates were then diluted 15-fold and mixed with the Bio-Rad protein assay dye (Bio-Rad) according to the manufacturer's instructions. After 10-15 min of incubation in the dark absorbance at 595 nm wavelength was measured using the 15-fold diluted lysis buffer as a background.

Statistical analysis. At least three different experiments were performed in triplicate for each drug dose. The results were statistically analysed by the Student's t-test. The IC₅₀s, the confidence limits (95%) and the potency ratios between DMXAA and each analogue were estimated using the Litchfield and Wilcoxon method.

Results

Cytotoxicity on 2008, C13* and Mono Mac 6 cell lines. On both of the ovarian adenocarcinoma cell lines, 2008 and C13*, DMXAA showed a statistically significant cytotoxic activity only for the highest dose used (500 μ M). None of the FAA analogues were able to induce inhibition of cell growth comparable to that obtained with the reference compound (Fig. 3). Derivatives *a* and *l*, on 2008 cells and analogues *d* and *f*, on C13*, were the most effective, even though their IC₅₀ was significantly higher than those obtained with DMXAA [2008: IC₅₀=448.1 (395.9-507.3) μ M, C13*: IC₅₀=641.8 (511.1-805.7) μ M] (Table I).

Of the tested doses, neither DMXAA nor any FAA analogue was significantly cytotoxic towards the Mono Mac 6 (MM6) cells (data not shown).

Stimulation of the lytic properties of MM6 cells. The host-mediated antitumor effects induced *in vitro* by DMXAA and FAA analogues were investigated by measuring their ability to stimulate MM6 cells into exerting a cytotoxic action on C13* tumor cells.

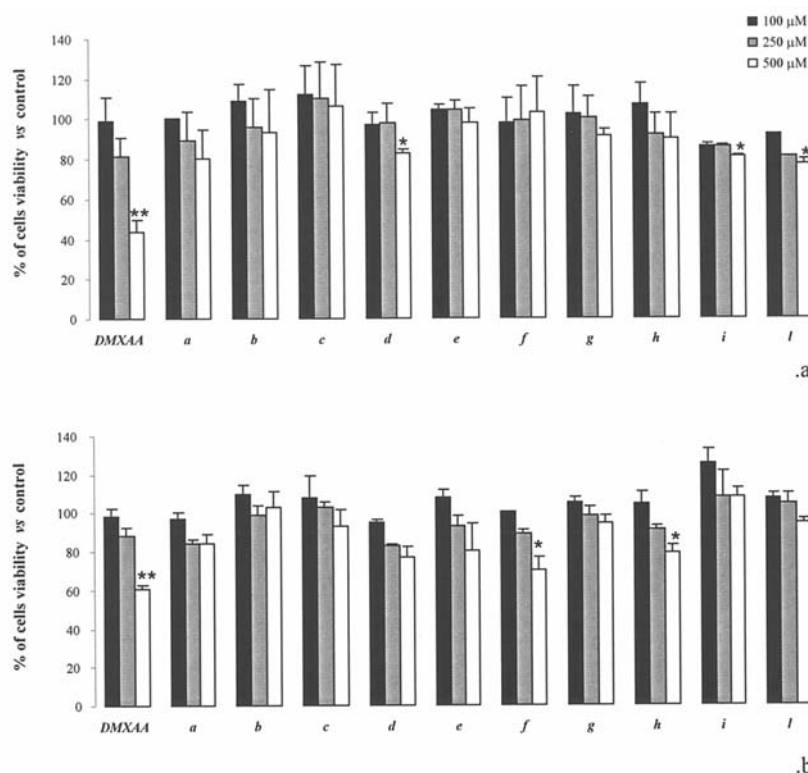


Figure 3. Percentage with respect to the control of the tetrazolium salts reduction (MTT) as an index of the viability of the 2008 (a) and C13* (b) cells, after 24 h of treatment with 100, 250 or 500 μ M DMXAA or FAA derivatives; * p <0.05, ** p <0.01.

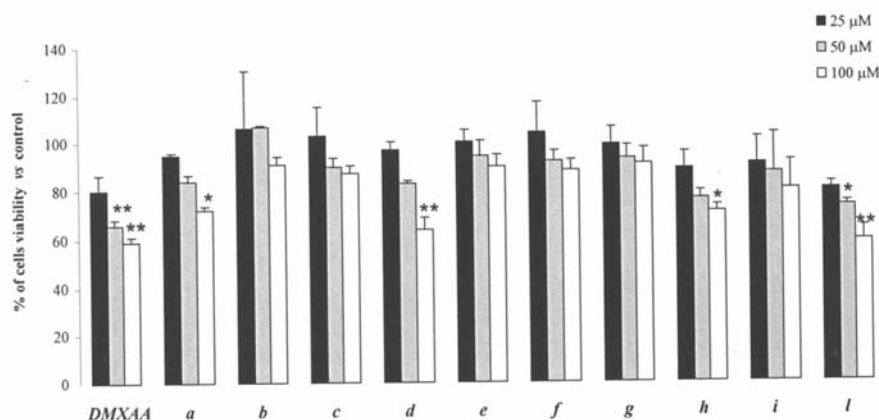


Figure 4. Percentage with respect to the control of the tetrazolium salts reduction (MTT) as an index of the viability of the C13* cells, after 24 h of co-culture with MM6 cells pre-treated for 24 h with 100, 250 or 500 μ M DMXAA or FAA derivatives; * p <0.05, ** p <0.01.

DMXAA showed a remarkable ability to stimulate lytic properties of MM6 (Fig. 4) reducing C13* proliferation in a dose-dependent manner, with an IC_{50} value <4 times than that obtained in cytotoxic assays [IC_{50} =152.3 (106.4-210.0) μ M versus IC_{50} =641.8 (511.1-805.7) μ M] (Table II). FAA-derivatives also generally increased their ability to inhibit C13* growth in comparison to the previous evaluation. In particular, analogues *a*, *d*, *h* and *l* exerted a statistically significant stimulation of MM6 cell antitumor activity at the highest dose used (100 μ M, Fig. 4). The most effective compounds, *a*, *d* and *l*, showed similar activities and IC_{50} values to the reference compound: 203.0 (141.7-290.7),

131.7 (102.5-169.3) and 177.1 (123.7-253.5) μ M, respectively (Table II).

When the same protocol was performed by treating the cells with the LPS associations, the results obtained showed not only that DMXAA and the three tested derivatives did not improve their ability to stimulate MM6 lytic properties, but also that they were less effective than when they were used alone (Fig. 5). Notably, the association with LPS led to IC_{50} values 1.5- (DMXAA) and 2-fold (*a*, *d* and *l*) higher than those obtained in the previous experiment (Table IV). Compound *d* showed an IC_{50} value totally comparable to that obtained with DMXAA (Table III).

Table II. IC₅₀ μ M, 95% confidence limits and power ratios versus DMXAA and the direct cytotoxicity assays, after 24 h of co-culture of the C13* cells with MM6 cells pre-treated with DMXAA or FAA derivatives (100, 250 or 500 μ M).

	DMXAA	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>h</i>	<i>i</i>	<i>l</i>
IC ₅₀	152.3 (106.4-210.0)	203.0 (141.7-290.7)	-	-	131.7 (102.5-169.3)	-	-	-	275.1 (184.2-410.7)	1067.5 (497.0-2293)	177.1 (123.7-253.5)
P.R. vs DMXAA	1.0	0.7	-	-	1.2	-	-	-	0.6	0.1	0.9
P.R. vs Cytotoxicity	4.2	-	-	-	12.3	-	-	-	-	-	-

Table III. IC₅₀ μ M, 95% confidence limits and power ratios versus DMXAA after 24 h of co-culture of the C13* cells with MM6 cells pre-treated with DMXAA or FAA derivatives (100, 250 or 500 μ M) associated with 10 ng/ml LPS.

	IC ₅₀ μ M	P.R. vs DMXAA
DMXAA	220.7 (144.3-337.6)	1.0
<i>a</i>	350.0 (218.6-563.4)	0.6
<i>d</i>	211.9 (154.0-291.7)	1.0
<i>l</i>	398.8 (225.6-704.9)	0.6

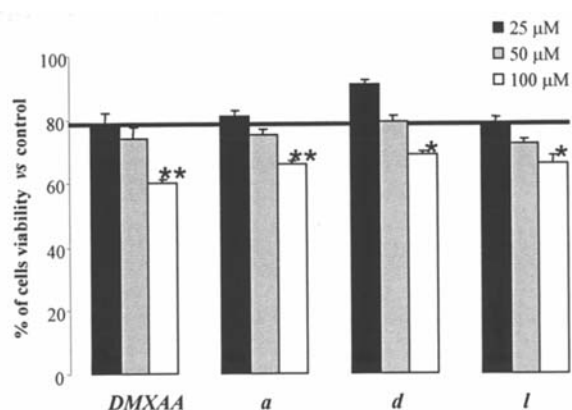


Figure 5. Percentage with respect to the control of the tetrazolium salts reduction (MTT) as an index of the viability of the C13* cells, after 24 h of co-culture with MM6 cells pre-treated for 24 h with 100, 250 or 500 μ M DMXAA or FAA derivatives associated with 10 ng/ml LPS. Black line: C13* co-cultured with MM6 treated only with 10 ng/ml LPS; * p <0.05, ** p <0.01.

Stimulation of TNF- α release. The release of TNF- α by MM6 was measured after a 4- or 24-h exposure to DMXAA or the compounds *a*, *d* and *l*, with or without LPS.

When used alone, the exposure time was the main factor affecting the release of the cytokine. After 4 h of treatment,

derivatives *d* and *l* stimulated a remarkable TNF- α release. In particular, the cells exposed to compound *d* released TNF- α levels significantly higher not only in comparison to the control cells, but also to that exposed to the DMXAA (Fig. 6a). A remarkable TNF- α release was also obtained by treating cells with derivative *l* at 25 μ M. When the exposure time was extended to 24 h, the DMXAA and FAA analogues completely lost their stimulating ability. Notably, the measured levels of TNF- α were comparable or decreased in compared to the control (Fig. 6b).

When MM6 cells were treated with LPS associations the time reliance of the TNF- α release was confirmed and then increased. In general, after 4 h of exposure all the tested associations were able to strongly induce TNF- α production and the cytokine levels were significantly higher than those obtained with either the DMXAA or FAA derivatives alone (Fig. 6c). The result obtained after 4 h of exposure to LPS association with 25 and 50 μ M *a* was noteworthy, as it showed a significant synergistic interaction with the bacterial endotoxin. After 24 h of treatment, none of the associations led to significant results in comparison to those obtained for the shorter exposure time. Under these experimental conditions, the production of TNF- α was in any case significantly lower than that obtained with LPS alone, suggesting antagonistic interactions (Fig. 6d).

Nitric oxide release by 'Griess' reaction. The nitric oxide release was evaluated in the same medium as the TNF- α assay, by using the nitrite 'Griess reaction' as a reliable indicator of radical release.

Moreover, the time of exposure affected the obtained results but in an inverse manner in compared to the TNF- α release assays. When the MM6 cells were treated with compounds alone, a considerable nitric oxide induction was obtained only after 24 h of exposure. After 4 h of treatment not only none of the tested compounds was able to stimulate nitric oxide production, but they also seemed to inhibit its production if compared to the control (Fig. 7a). Instead, following the 24 h of exposure all the tested compounds powerfully stimulated nitric oxide production (Fig. 7b). In particular, the most remarkable results were obtained with the derivative *d*, which led to a significant nitric oxide release that, in comparison to the control, was about 800% at 25 μ M and 500% at both 50 and 100 μ M. Under these experimental conditions, DMXAA increased the radical release only at 25 μ M.

Table IV. Summary of the IC_{50} , 95% confidence limits and power ratios of the IC_{50} s after 24 h of co-culture of the C13* - MM6 cells pre-treated with DMXAA or FAA derivatives (100, 250 or 500 μ M) associated with LPS (10 ng/ml) versus previous experiments.

Compounds	IC_{50} μ M Cytotoxicity on C13*	IC_{50} μ M Mediated by Mono Mac 6	IC_{50} μ M Association LPS	P.R. associations LPS versus Cytotoxicity on C13*	P.R. associations LPS versus Mediated by MM6
DMXAA	641.8 (511.1-805.7)	152.3 (106.4-210.0)	220.7 (144.3-337.6)	2.9	0.7
<i>a</i>	- -	203.0 (141.7-290.7)	350.0 (218.6-563.4)	-	0.6
<i>d</i>	1616.3 (999.6-2613.5)	131.7 (102.5-169.3)	211.9 (154.0-291.7)	7.6	0.6
<i>l</i>	- -	177.1 (123.7-253.5)	398.8 (225.6-704.9)	-	0.4

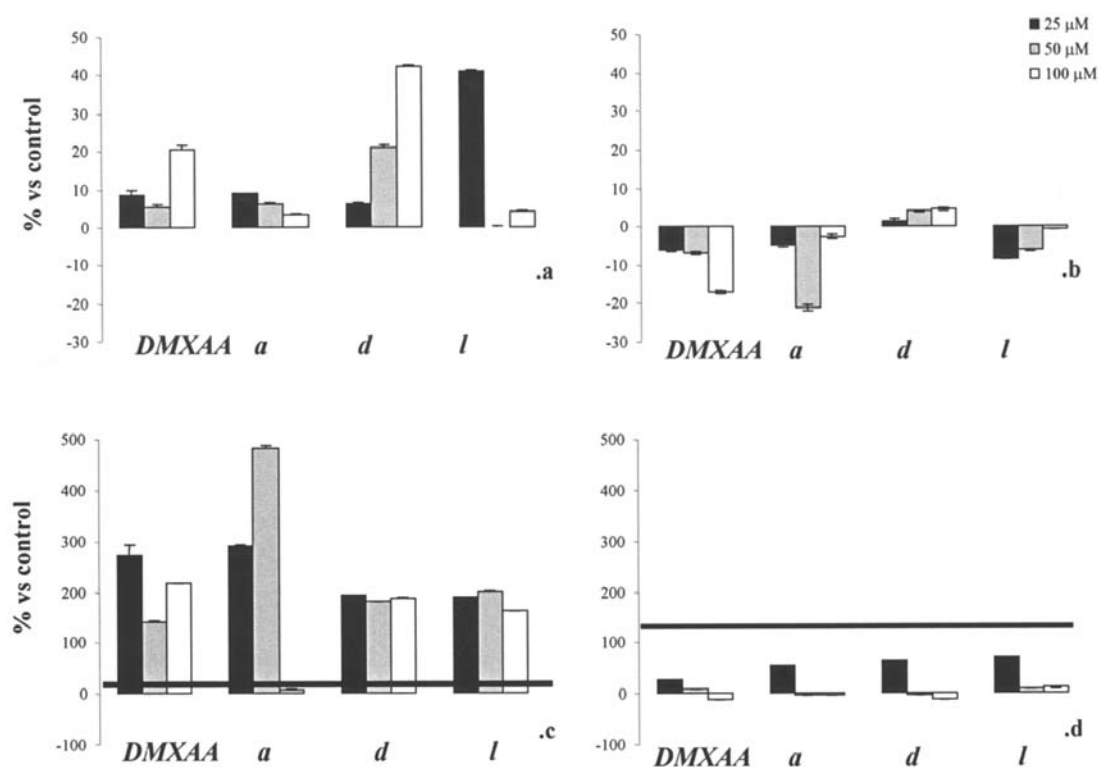


Figure 6. TNF- α (% vs control) in MM6 supernatants after 5 (a) or 24 h (b) treatment with 25, 50 or 100 μ M DMXAA or derivatives *a*, *d* or *l* alone, or associated with 10 ng/ml LPS for 4 (c) or 24 h (d). Black lines: TNF- α in MM6 cells treated with 10 ng/ml LPS alone.

Similar results were obtained when the nitric oxide release was assessed on the MM6 cell media after 4 h of exposure to the derivatives combined with LPS. Neither the DMXAA nor any FAA analogue were able to synergise with the bacterial endotoxin to stimulate the nitric oxide release. Furthermore, all the associations led to a significant decrease both in comparison to the control and to that obtained with the LPS alone (Fig. 7c).

When exposure time was extended to 24 h, the LPS completely lost any ability to stimulate the nitric oxide release, which was quite similar to that obtained with the non-treated cells, while the association with 50 μ M compound *l* led to a significant increase of the nitric oxide production both in compared to the derivative *l* alone and to the LPS alone (Fig. 7d). A strongly antagonistic interaction was obtained with the association LPS-compound *d*, which completely lost

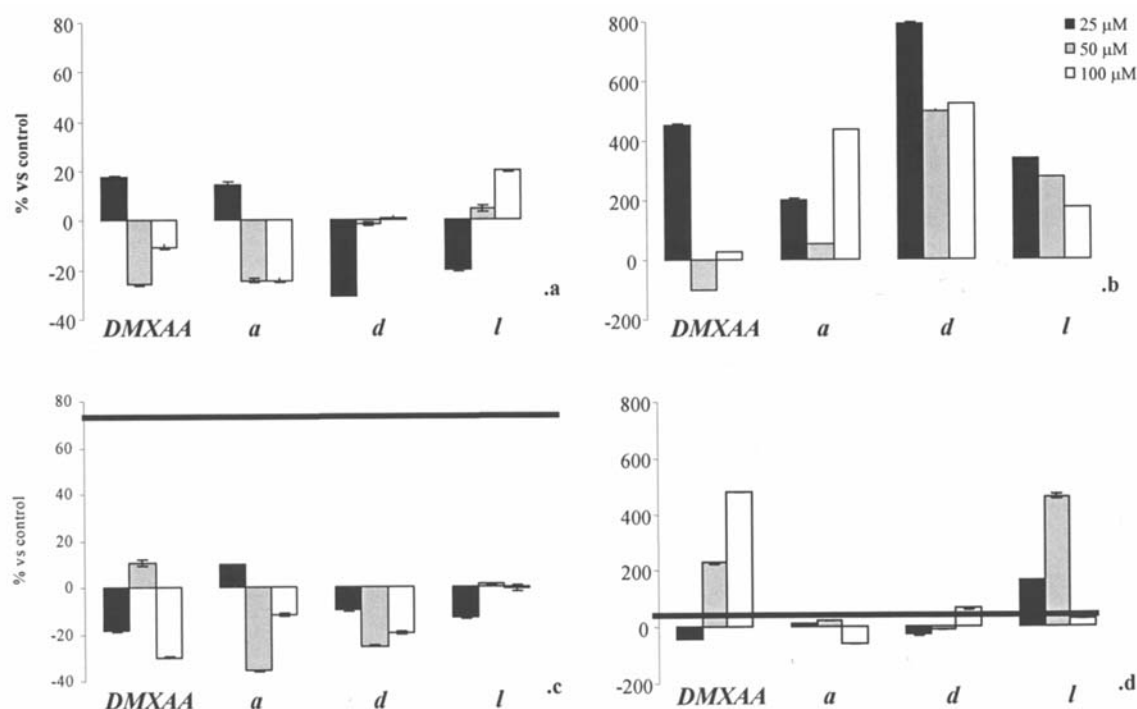


Figure 7. Nitrites (% vs control) in MM6 supernatants after 5 (a) or 24 h (b) treatment with 25, 50 or 100 μ M DMXAA or derivatives *a*, *d* or *l* alone, or associated with 10 ng/ml LPS for 4 (c) or 24 h (d). Black lines: nitrite in the MM6 cells treated with 10 ng/ml LPS alone.

its ability to stimulate the release of nitric oxide in comparison to when it was used alone. A synergistic increase of radical production was obtained with the association LPS-DMXAA, which was about 230 and 500% at 25 and 100 μ M, respectively (Fig. 7d).

Discussion

The interest in studies concerning FAA-derivatives, their synthesis and their biological action is based on the evidence that these molecules are able to exert peculiar antitumor effects involving the immune system and the tumor vascular network. Although FAA failed the clinical trials (24), the potential effectiveness of this approach was confirmed by DMXAA (3,25-27), the most potent FAA and XAA derivative obtained up to now, currently in phase II clinical trials.

The significant activity on human models showed by DMXAA and its selectivity on tumor tissue, resulted first in *in vitro* (28-30) and then *in vivo* (16,25,31,32) studies, making this molecule one of the most noteworthy vascular disrupting agents currently in clinical trials. Although DMXAA acts as a powerful antivascular agent in both mice and human tumors, the only evidence of moderate antitumor activity has been obtained in clinical trials, in comparison to the pre-clinical studies (13,33). For that reason further investigation on FAA and XAA derivatives may lead to the detection of more notable molecules.

The cytotoxic activity of some coumarin-, flavanon- and flavonol-FAA derivatives on a colon cancer cell line was the subject of our previous studies (17). In this study, by using DMXAA as a reference, we carefully examined the ability of some of these previously-studied derivatives to exert, *in vitro*, an immune-modulating activity leading to an indirect

antiproliferative action on human tumor cells by the induction of physiological factors involved in the immune response.

All the compounds were tested for cytotoxicity towards the two tumor cell lines, 2008 and C13*, arising from a human ovarian adenocarcinoma. The C13* cells showed a reduced cell membrane permeability to passive diffusion (34) and mitochondrial membrane functionality (35,36). On both cell lines, FAA derivatives revealed a very low ability to reduce cell proliferation. In fact, cytotoxic effects were obtained only with few derivatives and only at the maximum-tested dose. Few IC_{50} values could be calculated (for analogues *a* and *l* on 2008 and for analogues *d* and *f* on C13*) and they were even higher than that of DMXAA, taken as a reference for all the evaluations. One of the interesting characteristics first shown by FAA and then by DMXAA was their marginal ability to directly affect the cell proliferation and the results obtained suggest and confirm that these FAA derivatives are not characterised by any remarkable cytotoxic property.

The antitumor effects shown by both FAA and DMXAA seem to involve the immune system by different pathways, including an increase in macrophage-mediated cytotoxicity (4), stimulation of the activity of NK cells (5) and the induction of different cytokines (37,38). Thus, the main key to clarifying the biological meaning of the structural changes brought in by the FAA molecule was the evaluation of host-mediated effects of the synthesized compounds, in terms of their ability to exert, *in vitro*, an immune-modulating activity leading to an indirect antiproliferative action on human tumor cells by induction of physiological factors involved in the immune response. For this reason we investigated the immune system activation by evaluating the ability of these derivatives to induce, in human monocytes (MM6), lytic properties exerted on a tumor cell line, and TNF- α and nitric oxide release. The

ability to induce lytic properties in MM6 cells was evaluated as cytotoxicity, induced on C13⁺ cells co-cultivated with MM6 and pre-treated with the new compounds. MM6, a human monoblastic leukaemia, is a mature macrophage-like cell line that expresses phenotypic and functional properties of mature monocytes (39).

A remarkable increase in antiproliferative activity, compared to the direct cytotoxicity, was observed for both DMXAA and the ten FAA derivatives. The reference compound showed an IC₅₀ <4 times than that obtained in the cytotoxicity assay. Among the coumarin-derivatives, *a* and *d* showed a significantly mediated cytotoxicity. In particular, a 12-fold enhancement was obtained with the analogue *d*. The substitution of the flavonic core with a flavanonic one did not lead to the molecules being able to stimulate MM6 more than both DMXAA and the other analogues. The two compounds *h* and *i* were able to stimulate MM6, but their IC₅₀ values were significantly higher than that obtained with the reference compound, while the flavonol acetic acid (*l*), which bears a hydroxyl group in position 3 of the FAA core, showed an ability to stimulate MM6 lytic properties similar to that of DMXAA.

Bacterial endotoxin lipopolysaccharide (LPS) is able to induce a powerfully immune stimulation (40) mainly by activating the NF- κ B pathway through the activation of the membrane receptor CD14 and the induction of NIK (NF- κ B inducing kinase), which activates IKK, another kinase, which causes phosphorylation and disruption of I κ B (NF- κ B inhibitor factor) (14). Through this pathway, LPS was able to stimulate the synthesis of many mediators such as TNF- α , other cytokines and iNOS. In human cultured leukocytes, DMXAA showed its ability to amplify the immune stimulation effects of LPS with a synergistic interaction (14). Circumstantial evidence points to IKK as a biochemical target for DMXAA provided by the observation that the action of DMXAA on human peripheral blood leukocytes is inhibited by salicylate and the observation that the kinetics of inhibition suggests competition for the same site. Salicylate binds to the β -subunit of IKK (41). Endotoxin, interleukin-1 and other agents are required to activate IKK and it is likely that DMXAA requires IKK pre-activation before acting. This would explain why DMXAA acts as a co-stimulator rather than an inducer of the TNF- α synthesis and why it can have selective effects on tumor tissue (16). For this reason the hypothesis of the ability of LPS to increase the host-mediated cytotoxicity of DMXAA and selected derivatives, inducing TNF- α and nitric oxide synthesis, was considered. Then, the same protocol reported above was performed in association with LPS.

LPS was able to modify the ability of the FAA derivatives to stimulate the MM6 lytic action, but this influence was negative. The results obtained showed that not only did DMXAA and the three tested derivatives not improve their ability to stimulate MM6 lytic properties, but also that they were less effective in comparison to when they were used alone. In particular, the association with LPS led to IC₅₀ values 1.5- (DMXAA) and 2-fold (*a*, *d* and *l*) higher than those obtained in the previous experiment.

The evaluation of TNF- α released by MM6 pre-treated with DMXAA and selected derivatives, alone or associated with LPS, could be a useful indicator of the activity of this

class of agents (42). When DMXAA and the selected derivatives were used alone, the exposure time seemed to play an important role. After 4 h of treatment with DMXAA, 100 μ M compound *d* and 25 μ M *l*, a remarkable release of TNF- α was obtained. A different trend was shown when the exposure time was extended to 24 h. In this case, a remarkable loss of ability to affect the release of TNF- α , in comparison either to the result obtained with 4 h of exposure or to the control, was detected.

Moreover, when the MM6 cells were treated with DMXAA or selected FAA derivatives associated with LPS, the exposure time played a main role in affecting the cytokine release. When the cells were treated for the shortest time a synergistic effect was obtained, but this ability was completely lost when the exposure time was extended to 24 h.

The most significant MM6 stimulation to TNF- α release was obtained using short time exposure and LPS association.

The release of nitric oxide may contribute to the antivasular action of DMXAA. Nitric oxide, although potentially protective against antivasular effects by relaxing arterioles, also increases vascular permeability in tumors by directly affecting endothelial cells (16). Experiments using tumors grown *in vitro* demonstrated that the pharmacological inhibition of the nitric oxide synthesis reduced the antitumor activity of DMXAA which strongly implicates nitric oxide synthesis in the antitumor action of DMXAA (16).

As with the TNF- α assay, time played a significant role in the release of nitric oxide, but in this case the most interesting results were obtained after 24 h of treatment. When the cells were treated to the shortest time point none of the tested compounds was able to induce a powerfully nitric oxide release, whereas when the exposure time was extended to 24 h each compound was able to induce a powerful release compared to the control. In particular, when used alone, compound *d* was able to induce a powerfully nitric oxide release, 790, 500 and 525% higher to that induced by the control at 25, 50 and 100 μ M, respectively. Under these experimental conditions, DMXAA substantially modified radical production only at 25 μ M.

The ability of DMXAA and the new FAA derivatives to stimulate MM6 nitric oxide production was not affected by the combinations with LPS. Of note is that after 24 h of exposure, compound *d* completely lost its activity, whereas compound *l*, 25 and 50 μ M and DMXAA, 50 and 100 μ M, led to a significant increase of the radical production both in comparison to the compounds alone and to the LPS alone. Thus the nitric oxide release was delayed in compared to the TNF- α , probably because the production of the radical is not directly affected by NF- κ B activation, but is a result of the iNOS activation that can be enhanced by previously synthesised TNF- α (43).

In conclusion, the modifications made on the FAA structure led to completely different results. The substitution of the flavonic core with a coumarinic structure resulted in the synthesis of two derivatives, *a* and *d*, which showed an equal ability to stimulate the MM6 lytic properties in comparison to DMXAA. Compound *a*, when associated with LPS and *d* when used alone, were able to induce a higher TNF- α and nitric oxide release, respectively. The good activity profile of *a* compared to the inactive compound *b*

pointed out the importance of the phenyl nucleus in position 3 of the coumarin core, as well as the positive influence of a fluorine atom on that phenyl compared to other substituents that led to inactive compounds (*c* and *e*). The substitution of flavonic core with a flavanonic structure (compounds *f*, *g*, *h* and *i*) did not lead to any significant improvement of the FAA activity, suggesting a possible main role played by the unsaturated bond between the carbon atoms in positions 2 and 3. The introduction of a hydroxyl group in the molecule of FAA (compound *l*) led to the flavonol acetic acid, characterised by a biological profile strictly similar to the reference compound DMXAA.

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