ANTICANCER RESEARCH *25*: 1179-1186 (2005)

Mono- or Di-fluorinated Analogues of Flavone-8-acetic Acid: Synthesis and *In Vitro* Biological Activity

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Abstract. *Background: Previously, the antitumour activity of some flavone-8-acetic acid (FAA) derivatives substituted with an acid function in position 2 of the benzene ring was evaluated. The most active compound resulted the one bearing a fluorine atom in position 7 of the flavone nucleus. In this paper, we evaluated new mono- or di-fluorinated FAA derivatives. Materials and Methods: The cytotoxicity towards two human ovarian adenocarcinoma cell lines, the capability to stimulate human mononuclear cells and murine macrophages' lytic properties were evaluated by MTT. Moreover, the potentiation of lipopolysaccharide (LPS) activity was studied by ELISA analysis of TNF-α release. Results: The analogues showed a direct cytotoxicity comparable to that of 5,6-dimethyl-xanthen-9-one-4-acetic acid (DMXAA), at present in clinical trials. None of the tested compounds was able to stimulate human mononuclear cells' lytic properties after either 4- or 24-h treatment, while after 4-h treatment, the derivative 5a was more able to stimulate murine macrophages with respect to DMXAA. Moreover, a significant increase of 5c and 5d activation was obtained with LPS association, reflected by TNF-α production as well. Conclusion: Like FAA, the new fluorinated derivatives 5a, 5c and 5d showed remarkable activity in murine cells, but this was not confirmed in human models.*

Flavone-8-acetic acid (1) (FAA, **1**; Figure 1) is considered a very interesting compound because of its peculiar antitumour profile, in particular its remarkable activity on solid tumours (2). The target of its activity were both the immune and the vascular systems (3-6). Unfortunately, FAA did not confirm in humans the effects found in murine tumour models, not exhibiting any significant activity in clinical trials (7). Extensive SAR studies performed on xanthenone-4-acetic acid (8) (XAA, **2**; Figure 1), closely related to but significantly more potent than FAA, led to 5,6-dimethyl-xanthenone-4-acetic acid (DMXAA, **3**; Figure 1), which resulted the most potent compound synthesized and showed to be active on human models (9). DMXAA was, therefore, selected for further evaluation and it has now completed phase I clinical trials (10).

For some years, our research group has been interested in the synthesis and biological evaluation of FAA analogues (11-13). In a previous work (12), the activity of some FAA derivatives substituted with an acid function in position 2 of the benzene ring was described, preserving the spacial relationship between the acid group and pironic oxygen, even if the acid function is not in position 8 of the flavone ring. As far as the biological activity was concerned, the most interesting compound turned out to be the one carrying a fluorine atom in position 7 of the benzene ring (12).

In this paper, we examined the effects of some more modifications of the lead molecule FAA on antitumour activity. In particular, we synthesized and evaluated derivatives bearing one or two fluorine atoms in the flavone nucleus. The structures of the new compounds are shown in Table I.

Materials and Methods

Chemistry. The studied compounds were synthesized according to Figure 2. Different substituted o-hydroxyphenylketones were heated for 3 h with phthaloyl chloride in pyridine to give an intermediate, which was then cyclized by refluxing it in pyridine in the presence of potassium carbonate. The o-hydroxyphenylketones, that were not commercially available, were prepared by treatment of the substituted phenol with the corresponding acidic chloride and subsequent transposition by heating to 140°C with $AlCl_3$ (Figure 3).

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Key Words: FAA, DMXAA, TNF-α, LPS, murine macrophages.

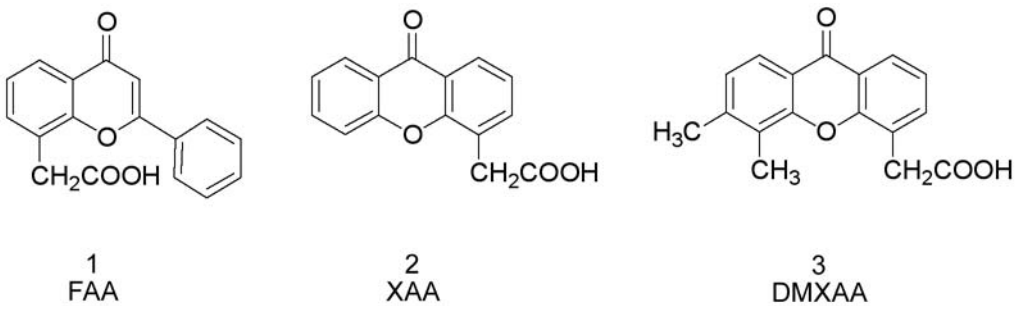


Figure 1. *Reference compounds.*

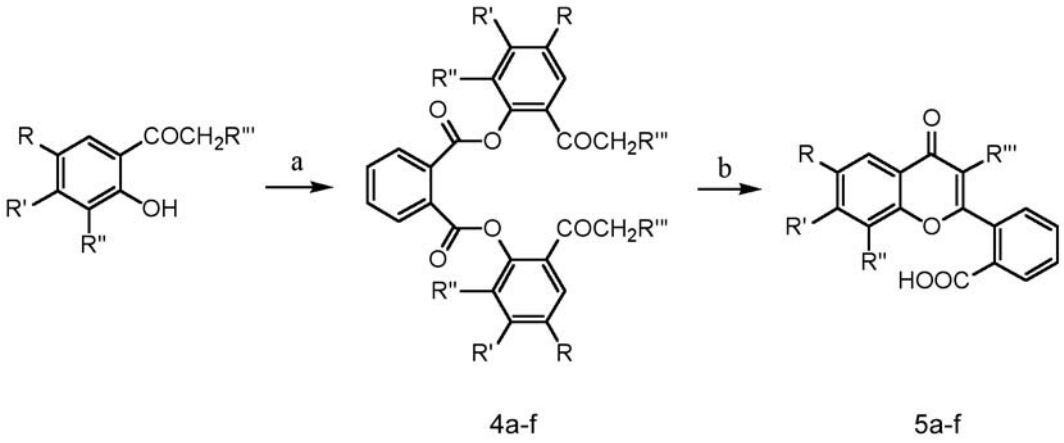


Figure 2. *Reagents: a) Phthaloyl dichloride, pyridine; b) Pyridine, K_2CO_3, reflux 3h.*

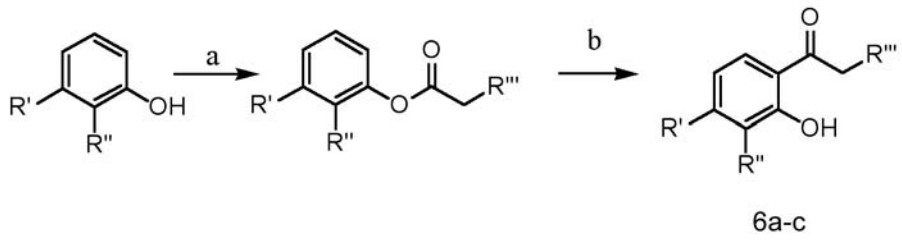


Figure 3. *Reagents: a) selected acidic chloride, reflux 1h; b) $AlCl_3$, 140˚C 6h*

Synthesis. Melting points were determined on a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were obtained for $CDCl_3$ or DMSO-d_6 solutions on a Gemini 300 spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet) or *m* (multiplet). Elemental analyses are within ± 0.4% of the theoretical values. The compounds were named following IUPAC rules as applied by AUTONOM, a PC software for systematic names in organic chemistry, Beilstein-Institut and Springer.

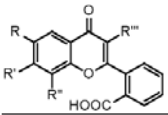
Synthesis of 1-(3,4-difluoro-2-hydroxyphenyl)-ethanone 6c: 2.12 mL (2.35 g, 0.03 mol) of acetyl chloride were added dropwise to a suspension of 2,3-difluorophenol (4 g, 0.03 mol) and potassium carbonate (4.14 g, 0.03 mol) in 10 mL of toluene. The reaction mixture was refluxed for 1h, hot filtered and evaporated to dryness to give 3.4 g (73%) of the corresponding ester. 7.5 g of aluminium chloride (0.056 mol) were then added to 2.5 g of the ester (0.014 mol) and the mixture was heated at 140˚C for 6h. Ice was cautiously added and the solid was collected by filtration (1.3 g, 50%) and crystallized from toluene (mp 49-51˚C). ^{1}H NMR

($CDCl_3$): δ 2.60 (s, 3H, CO*CH*$_3$), 6.70-7.30 (m, 2H, Ar*H*). *Synthesis of 1-(4-fluoro-2-hydroxyphenyl)-propan-1-one 6e:* Using the previous procedures and starting from 2.63 mL (2.77 g, 0.03 mol) of propionyl chloride and 3-fluorophenol (3.36 g, 0.03 mol), 6e (3.6 g, 72%) was obtained as an oil. ^{1}H NMR ($CDCl_3$): δ 1.20 (t, 3H, $COCH_2CH_3$), 3.0 (q, 2H, $COCH_2CH_3$), 6.60-7.85 (m, 3H, Ar*H*). *Synthesis of 1-(4-fluoro-2-hydroxyphenyl)-2-phenylethanone 6f:* Using the previous procedures and starting from 3.96 mL (4.63 g, 0.03 mol) of phenylacetyl chloride and 3-fluorophenol (3.36 g, 0.03 mol), 6f (5.2g, 73%) was obtained as an oil. ^{1}H NMR ($CDCl_3$): δ 4.20 (s, 2H, $COCH_2$Phe), 6.60-7.95 (m, 8H, Ar*H*).

General procedure for the preparation of phthalates 4a-f: A solution of phthaloyl chloride (0.005 mol) and the selected o-hydroxyphenylketone (0.01 mol) in pyridine (20 mL) was heated in a steam bath for 30 min. After cooling, the reaction mixture was acidified with diluted H_2SO_4. The separated solid was collected by filtration, dried and crystallized from the appropriate solvent: *Phthalic acid bis-(2-acetyl-4-fluorophenyl) ester 4a:* Yield 45%, mp 116-118°C (methanol). ^{1}H NMR ($CDCl_3$): δ 2.55 (s, 6H, CO*CH*$_3$), 7.2-8.15 (m, 10H, Ar*H*). *Phthalic acid bis-(2-acetyl-4,5-difluorophenyl) ester 4b:* Yield 50%, oily compound. ^{1}H NMR ($CDCl_3$): δ 2.50 (s, 6H, CO*CH*$_3$), 7.2-8.15 (m, 8H, Ar*H*). *Phthalic acid bis-(6-acetyl-2,3-difluorophenyl) ester 4c:* Yield 30%, mp 94-96°C (methanol). ^{1}H NMR ($CDCl_3$): δ 2.60 (s, 6H, CO*CH*$_3$), 7.15-8.2 (m, 8H, Ar*H*). *Phthalic acid bis-(2-acetyl-4,6-difluorophenyl) ester 4d:* Yield 50%, oily compound. ^{1}H NMR ($CDCl_3$): δ 2.55 (s, 6H, CO*CH*$_3$), 7.2-8.15 (m, 8H, Ar*H*). *Phthalic acid bis-(5-fluoro-2-propionylphenyl) ester 4e:* Yield 40%, mp 90-92°C (ligroin). ^{1}H NMR ($CDCl_3$): δ 1.55 (t, 6H, $COCH_2CH_3$), 2.95 (q, 4H, $COCH_2CH_3$) 7.2-8.15 (m, 10H, Ar*H*). *Phthalic acid bis-(5-fluoro-2-phenylacetylphenyl) ester 4f:* Yield 45%, oily compound. ^{1}H NMR ($CDCl_3$): δ 4.15 (s, 4H, $COCH_2$Phe), 6.8-8.05 (m, 20H, Ar*H*).

General synthesis of substituted flavone-2-carboxylic acids 5a-f: To a solution of the selected phthalate (0.01 mol) in pyridine (100 mL), K_2CO_3 (0.02 mol) was added and the reaction mixture was refluxed with stirring for 3 h. After cooling, the solution obtained was acidified with diluted HCl. The separated solid was collected by filtration, dried and crystallized from the appropriate solvent: *2-(6-Fluoro-4-oxo-4H-chromen-2-yl)-benzoic acid 5a:* Yield 45%, mp 230-232°C (methanol). ^{1}H NMR (DMSO-d_6): δ 6.55 (s, 1H, C*H*-3), 7.65-7.95 (m, 7H, Ar*H*). MS *m/z* (rel.abund.): 284 (M^+, 34,35), 240 (38,64), 138 (100). *2-(6,7-Difluoro-4-oxo-4H-chromen-2-yl)-benzoic acid 5b:* Yield 40%, mp 136-138°C (methanol). ^{1}H NMR (DMSO-d_6): δ 6.50 (s, 1H, C*H*-3), 7.45-7.90 (m, 6H, Ar*H*). MS *m/z* (rel.abund.): 302 (M^+, 6,30), 207 (21,07), 44 (100). *2-(7,8-Difluoro-4-oxo-4H-chromen-2-yl)-benzoic acid 5c:* Yield 42%, mp 198-201°C (methanol). ^{1}H NMR (DMSO-d_6): δ 6.7 (s, 1H, C*H*-3), 7.50-7.90 (m, 6H, Ar*H*). MS *m/z* (rel.abund.): 302 (M^+, 34,29), 258 (83,70), 156 (100). *2-(6,8-Difluoro-4-oxo-4H-chromen-2-yl)-benzoic acid 5d:* Yield 38%, mp 234-236°C (methanol). ^{1}H NMR (DMSO-d_6): δ 6.65 (s, 1H, C*H*-3), 7.60-8.05 (m, 6H, Ar*H*). MS *m/z* (rel.abund.): 302 (M^+, 39,65), 258 (23,68), 156 (100). *2-(7-Fluoro-3-methyl-4-oxo-4H-chromen-2-yl)-benzoic acid 5e:* Yield 40%, mp 143-145°C (toluene). ^{1}H NMR (DMSO-d_6): δ 2.80 (s, 3H, *CH*$_3$), 7.40-8.25 (m, 7H, Ar*H*). MS *m/z* (rel.abund.): 297 (M^+, 36,80), 254 (20,46), 253 (100). *2-(7-Fluoro-4-oxo-3-phenyl-4H-chromen-2-yl)-benzoic acid 5f:* Yield 38%, mp 132-134°C (toluene).^{1}H NMR (DMSO-d_6): δ 7.05-8.30 (m, 12H, Ar*H*). MS *m/z* (rel.abund.): 360 (M^+, 80,38), 314 (100), 222 (26,99).

Table I. *Structure of synthesized compounds.*



Compound	R	R'	R''	R'''
5a	F	H	H	H
5b	F	F	H	H
5c	H	F	F	H
5d	F	H	F	H
5e	H	F	H	CH_3
5f	H	F	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 ovarian adenocarcinoma cell line 2008 and the *cis*-DDP-resistant sub-line C13*, kindly supplied by Prof. G. Marverti (Department of Biomedical Sciences, University of Modena, Italy), were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1% antibiotics (all products of Biochrom KG Seromed), and 200 mM l-glutamine (Merck).

Growth inhibition. Cells were seeded in 96-well tissue plates (Falcon) and treated 24 h later with each agent at different concentrations (100, 250 and 500 μM). Cell growth was determined by tetrazolium salts reduction assay (MTT) after 24-h incubation (14).

Murine macrophages-mediated toxicity. The ability of the new derivatives to stimulate murine macrophages in culture to become tumoricidal was evaluated using resident peritoneal macrophages isolated from male CD-1 mouse (Charles River) by two injections of 5 mL of PBS containing 10 U/mL heparin into the peritoneal cavity (15). The cavity was gently massaged for 2 min, and the cells were removed by drawing fluid out with a syringe (16).

The recovered cell suspension was centrifuged, and the pellet was washed twice in sterile PBS. The cells were suspended in RPMI 1640 with 5% FCS, viability was assessed by trypan blue dye exclusion (always more than 95%), seeded in 96-well plates (Falcon) at a density of 13.3 x 10^3 cells/well and incubated for 2 h. Then the plates were washed in order to discard non-adherent cells, and the adhering macrophages were treated with the new FAA derivatives or DMXAA at 25, 50 and 100 μM in triplicate wells. After 4- or 24-h exposure, the medium was removed, the C13* cells were plated above (2 x 10^3 cells/well) and co-cultured for 24 h. Furthermore, the same assay was performed treating macrophages with new fluorinated derivatives or DMXAA in association with LPS 10 ng/ml (lipopolysaccharide from *E. coli* serotype 0127; F8, Sigma). The optimal macrophages/C13* cells ratio had been determined in preliminary experiments (results not reported). Lysis of C13* cells was assessed by MTT test and the percentages of specific cytotoxicity were calculated as follows (17):

Table II. *Cytotoxicity on C13* cells and toxicity mediated by murine macrophages of new compounds (PR = Potency Ratio).*

Compound	C*13 cells IC_{50} (μM)	PR *vs* DMXAA	Murine macrophages IC_{50} (μM)	PR *vs* DMXAA	PR *vs* C13*	Association with LPS 4h IC_{50} (μM)	PR *vs* murine macrophages
DMXAA	641.8 (511.1-805.7)	1.0	176.0 (106.8-290.0)	1.0	3.6	a	-
5a	538.7 (457.4-634.3)	1.2[b]	137.8 (96.2-197.3)	1.3[b]	3.9	103.9 (65.2-165.4)	1.3
5b	1037.4 (782.1-1376.0)	0.6[c]	360.1 (143.7-901.7)	0.5[c]	2.9	a	-
5c	491.9 (405.8-596.2)	1.3[b]	272.2 (150.1-511.9)	0.6[c]	1.8	77.7 (49.2-122.7)	3.5
5d	536.8 (452.9-636.4)	1.2[b]	a	-	-	83.8 (64.8-108.5)	-
5e	634.7 (507.6-793.6)	1.0	a	-	-	a	-
5f	544.8 (458.3-647.8)	1.2[b]	a	-	-	a	-

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

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

Tumour necrosis factor a (TNF-a) production. Murine macrophages, isolated as described above, were treated with DMXAA or the selected compounds at concentrations of 25, 50 and 100 μM in the presence of LPS (10 ng/ml). After 4-h incubation, the medium was carefully collected and stored at –70˚C until assayed. A commercially available enzymes-linked immunosorbent assay (ELISA) kit was used to determine the concentration of TNF-α (Biotrak ELISA System, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Human mononuclear cells-mediated toxicity. Human peripheral blood mononuclear cells (HPBMC) were isolated from heparinized whole blood by centrifugation over Ficoll-Paque (Pharmacia), seeded (10^4 cells/well) in 96-well plates and allowed to adhere at 37˚C. Viability of the cells was assessed by trypan blue dye exclusion and it was always more than 95%. After 2 h, the medium and the non-adherent cells were discarded, the plates were vigorously washed three times with RPMI 1640 medium and further incubated by the same protocols treatment reported above for murine macrophages.

Statistical analysis. For each assay, three different experiments were performed in triplicate. The results were statistically evaluated by the Student's *t*-test (18). 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 (18).

Results

Growth inhibition. On both 2008 and C13* cells, DMXAA and FAA derivatives revealed a dose-dependent cytotoxic activity. All compounds inhibited cell growth only at high concentrations (500 μM) and IC_{50} values were superimposable to that of the reference compound DMXAA [2008: IC_{50} = 448.1(395.9-507.3) μM; C13*: IC_{50} = 641.8 (511.1-805.7) μM]. The two cell lines showed different responses to DMXAA and to some FAA derivatives, DMXAA and compound *5e* being more potent on 2008 cells than on C13* cells [*5e*: IC_{50} = 468.4 (413.2-530.9) μM *versus* IC_{50} = 634.7 (507.6-793.6) μM] and compound *5b* completely inactive on the resistant line, while the derivative *5d* resulted more active on C13* cells [IC_{50} = 754.7 (583.5-976.2) μM *versus* IC_{50} = 536.8 (452.9-636.4) μM]. Instead, the analogues *5a*, *5c* and *5f* maintained the same cytotoxicity passing from the sensitive to resistant line. The IC_{50} values obtained for the C13* cell line are listed in Table II, where DMXAA was taken as reference.

Murine macrophages-mediated toxicity. The host-mediated antitumour effect was measured as cytotoxicity induced on C13* cells co-cultured with murine macrophages pre-treated with the new compounds alone or in association with LPS. The obtained IC_{50} values are shown in Table II.

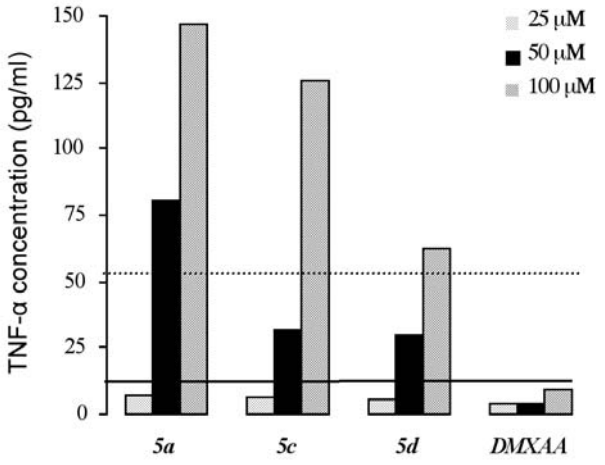


Figure 4. *Effect on TNF-α production by murine macrophages treated for 4 h with different concentrations of DMXAA or selected FAA-derivatives tested in association with LPS.*
-------- LPS 10 ng/ml
——— control

After 4-h treatment, the new compounds *5d, 5e* and *5f* resulted inactive, while an increase in antitumour activity with respect to direct toxicity was observed for DMXAA [IC_{50}=176.0 (106.8-290.0) μM *versus* IC_{50}=641.8 (511.1-805.7) μM] and for derivatives 5a, 5b and 5c, the IC_{50} of which diminished 4, 3 and 2 times, respectively.

Regarding the anticancer activity of the new compounds in association with LPS, after 4-h co-treatment the derivatives *5e* and *5f* continued to be inactive while compound *5b*, like DMXAA, lost its activity completely. Instead, considering their activity without LPS, *5a* resulted equally able to stimulate macrophages [IC_{50}=103.9 (65.2-165.4) μM *versus* IC_{50}=137.8 (96.2-197.3) μM], the compound *5c* improved 3.5 times its indirect toxicity [IC_{50}=77.7 (49.2-122.7) μM *versus* IC_{50}=272.2 (150.1-511.9) μM] and the analogue *5d*, which was inactive when used alone, was activated by the association with LPS [IC_{50}= 83.8 (64.8-108.5) μM].

When the murine macrophages were pre-treated for 24 h, no FAA derivative displayed any significant antitumour activity, while DMXAA considerably reduced C13* viability [IC_{50}= 211.0 (155.4-287.6) μM]. In association with LPS, neither DMXAA nor the new derivatives induced a host-mediated cytotoxicity higher than that of LPS alone, and the activity of FAA analogues themselves was also reduced (data not shown).

TNF-a production. The macrophages' TNF production and release after 4-h exposure to DMXAA and to compounds *5a*, *5c* and *5d*, with or without LPS, was measured.

When used alone, neither DMXAA nor FAA-derivatives were able to stimulate TNF-α production (data not shown). Figure 4 shows the dose-dependent TNF release induced by compounds *5a*, *5c* and *5d* in association with LPS. The analogue *5a*, already at 50 μM, was able to induce a TNF-α production greater than that obtained by both DMXAA and LPS used alone. Compounds *5c* and *5d*, when tested at 50 μM, resulted more potent with respect to DMXAA, while at 100 μM they induced a release higher than that due to LPS (Figure 4).

Human mononuclear cells-mediated toxicity. None of the tested compounds was able to stimulate human mononuclear cells' lytic properties either after 4- or 24-h treatment. When new fluorinated derivatives were associated with LPS, the same results were obtained.

Discussion

One of the peculiar features of FAA is its remarkable preferential activity on murine slow growing solid tumours (2), despite its low potency (19). Therefore, all the synthesized compounds were tested for cytotoxicity against two tumour cell lines, 2008 and C13*, arising from human ovarian adenocarcinoma. C13* cells appeared to be 10-fold more resistant to cisplatin than the original 2008 line and, furthermore, they showed reduced cell membrane permeability to passive diffusion (20) and mitochondrial membrane functionality (21,22).

On both cell lines, FAA derivatives revealed a dose-dependent cytotoxic activity, but significant toxicity was seen only at the maximum tested dose. The IC_{50} values obtained with the analogues were similar to that of DMXAA, which was taken as reference because of its potency on human models (9). The comparison between the activity of new FAA analogues on the two cell lines showed that the analogues *5a*, *5c* and *5f* maintained the same cytotoxicity passing from 2008 to C13* lines, compound *5b* resulted completely inactive on the resistant line, compound *5e,* as well as DMXAA, was less potent on C13* than on 2008 cells, while the derivative *5d* proved to be more active on C13* cells.

The antitumour effects shown by both FAA and XAA seem to involve the immune system by different pathways, including increase in macrophage-mediated cytotoxicity (3), stimulation of the activity of NK cells (4) and induction of different cytokines (23, 24). 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 co-cultured with murine macrophages or human mononuclear cells pre-treated with the new compounds was, therefore, measured to test them for their ability to improve the lytic properties of these immune cells.

After 4-h treatment, the new compounds *5d*, *5e* and *5f* resulted inactive on murine macrophages, while a remarkable increase in antitumour activity with respect to direct toxicity was observed for DMXAA and derivatives *5a*, *5b* and *5c*, even though only the analogue *5a* proved to be able to stimulate murine macrophages at least as much as DMXAA. Since, after 24-h treatment, none of the new derivatives showed any significant antitumour activity, as seen also for the parent compound, it can be inferred that the introduction of the fluorine atoms in the flavone ring did not determine an increase of biological activity.

As regards structure-activity relationships, it has to be considered that this series of compounds was synthesized as derivatives of a previously selected one, 2-(7-Fluoro-4-oxo-4H-chromen-2-yl)-benzoic acid (12). The latter has been recently tested for its macrophages' mediated toxicity (IC_{50} = 95 (70-128) μM, data not published) and, comparing its activity with the results obtained with the new derivatives, it can be seen, in the first place, that the introduction of a substituent in position 3 led to loss of activity, showing compounds *5e* and *5f* had no effects on murine macrophages. On moving the fluorine atom from position 7 to position 6, the activity was substantially maintained, and going to di-fluorinated compounds a slight loss in activity could be seen, but only when position 7 was substituted, as in *5b* and *5c*. Interestingly enough, compound *5d* showed some activity on macrophages only when associated with LPS.

The induction of cytokines, particularly TNF-α, appears to be critical for the action of FAA (24) and DMXAA (25). Furthermore, it is well known that LPS is able to induce monocytic-line cells to produce and release various cytotoxic factors by different pathways, *i.e.* NF-Î B translocation and NOS II induction (26). For these reasons, it was interesting to examine the hypothesis of a potentiation of murine macrophages' activity, resulting from the association of these compounds with LPS. After 4-h treatment, all analogues increased macrophages' stimulation exerted by LPS, but only derivatives *5a*, *5c* and *5d* exhibited a significant indirect toxicity. Regarding their activity without LPS, *5a* resulted equally able to stimulate macrophages, DMXAA and derivative *5b* lost their activities completely, while compound *5c* improved 3.5 times its indirect toxicity. The association with LPS significantly activated the analogue *5d*, which was inactive when used alone. Instead, after 24-h exposure, not only none of the associations tested was able to give a host-mediated cytotoxicity higher than that of LPS alone, but also the combination with LPS further reduced the toxic effect of the single FAA derivatives.

In order to explain the disappearance of the effect measured after 4 h, it should be taken into account that LPS forms complexes with LPS binding protein (LBP), which interact with the CD14 receptor, a membrane-bound glycoprotein (27) linked to the TNF and IL-1 kinase cascades by the plasma membrane protein TLR2 (28,29), inducing NF-Î B activity (28). Therefore, the swiftness of the effect could find justification in the rapid turnover of CD14 on the membrane, of about 3 h, or in its rapid internalisation, as recently shown for other substances (30).

Since the *in vitro* analysis of TNF-α production as a result of monocytic-line cells' stimulation could be a useful indicator of the activity of this class of agents (31), the effects on murine macrophages' TNF-α production as a consequence of the stimulation with DMXAA and selected compounds (*5a*, *5c* and *5d*), used alone or in association with LPS, were investigated. The release of this cytokine in the medium of the macrophages exposed for 4 h to DMXAA and to compounds *5a*, *5c* and *5d*, used alone or in association with LPS, confirmed the results obtained before: when used alone, neither DMXAA nor FAA derivatives were able to stimulate TNF-α release, while in association with LPS (Figure 4) *5a, 5c* and *5d* induced a significant dose-dependent cytokine production, greater than that obtained by both DMXAA and LPS. Unfortunately, none of the tested compounds was able to stimulate human mononuclear cells' lytic properties either after 4- or 24-h treatment. In particular, when tested on human mononuclear cells models, neither the promising compounds *5a*, *5c* and *5d*, which, in association with LPS, exhibited significant indirect toxicity and induction of TNF-α release, nor the other FAA derivatives, confirmed the remarkable activity found in murine cells.

In conclusion, the introduction of one or two fluorine atoms in the flavone nucleus of FAA led to a significant potentiation of murine macrophages' activity, which unfortunately disappeared in human models, a profile similar to the parent compound FAA.

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Received August 6, 2004
Revised February 1, 2005
Accepted February 16, 2005