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Synthesis and Biological Activity of Some Rigid Analogues of Flavone-8-acetic Acid

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Abstract—Some rigid analogues of flavone-8-acetic acid are described. Direct in vitro toxicity of the synthesised compounds was evaluated towards four tumoral cell lines and the ability of these compounds to stimulate mouse peritoneal macrophages in culture to become tumoricidal (indirect toxicity) was also studied. All compounds were able to induce direct cytotoxicity only at very high concentrations but showed a remarkable indirect activity. In particular compound **4d** was able to significantly increase macrophage lytic properties and has been selected for further investigations.

Introduction

Flavone-8-acetic acid (FAA, **1**) is a flavonoid synthesised by Atassi et al.[1] with a unique pattern of preclinical antitumour activity. A peculiar feature of FAA is its low activity against fast-growing tumours, like leukemia, and its striking, broad activity against slow-growing solid tumours (colon, pancreatic and mammary adenocarcinomas, Glasgow osteogenic sarcoma) that are usually insensitive to most cytotoxic drugs.[1–3] Its toxicological profile is also different from conventional chemotherapeutic agents, since it does not cause myelosuppression.[4] Anyway, its potency is low[5] and high doses and long exposure times are required for a direct cytotoxic effect. Unfortunately, the activity of the drug on murine tumours was not confirmed by the subsequent clinical trials, which led to disappointing results showing no activity on human tumours.[6,7]

The activity of FAA seems to be due to indirect effects more than to direct cytotoxicity. Factors involved are its ability to induce haemorrhagic necrosis of subcutaneous tumours,[8] that may be attributed to vascular collapse,[9–11] its ability to induce production of tumour necrosis factor-α (TNF-α),[12] increase of natural killer (NK) cells[13,14] through the production of interferons,[12] activation of macrophage tumouricidal activity[15] and the production of nitric oxide.[16] Therefore, the action of FAA involves also the immune system, since it acts as a biological response modifier stimulating natural killer cells' activity[13,14] and enhancing the lytic potential of macrophages.[15] Moreover, there is evidence[16] for FAA induced increase in the expression of some cytokine genes in mice, such as TNF gene, for which Mahadevan et al.[17] postulated a role in FAA induced tumour vascular shutdown. A paper from Futami et al.[18] demonstrated that FAA can directly stimulate cytokine gene expression in mouse but not in human leukocytes; this difference in sensitivity to FAA between human and mouse immune systems may account for the lack of activity seen in human tumours.

Extensive studies have been carried out to elucidate the mechanism of action of FAA at molecular level.[19–22] Despite these studies, it has not been fully elucidated yet and synthesis and evaluation of novel analogues may provide useful information, since the biological receptor for FAA is presently unknown. Furthermore, the structure–activity relationships for FAA have not been thoroughly investigated. Recent papers[23–25] reported some novel modifications of the parent structure, but these did not lead to major improvements in potency. Remarkable activity and higer potency was shown by a series of analogues of xanthenone-4-acetic acid (XAA, **2**), where the xanthenone moiety can be regarded as a 'fused' flavone, expecially if substituted in positions 5

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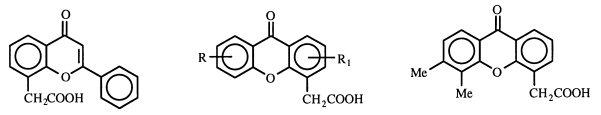
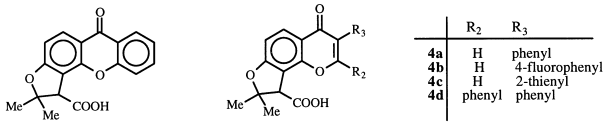
and/or 6: the 5,6-dimethylxanthenone-4-acetic acid (**3**) proved to be the most potent compound synthesised so far, and was selected for clinical evaluation.[26–30]



1 **2** **3**

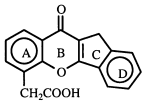
In continuing our studies on the structure–activity relationships of FAA,[31,32] in this paper we report the synthesis and in vitro study of some furochromene and furoxanthene carboxylic acids of general formulas **4** and **5** in which the acetic chain of FAA and XAA is locked in a rigid structure. This introduces a significant change in the α position to the carboxyl group, a change shown not to greatly influence activity in the xanthenone derivatives.[30]



	R_2	R_3
4a	H	phenyl
4b	H	4-fluorophenyl
4c	H	2-thienyl
4d	phenyl	phenyl

5 **4**

A rigid analogue of FAA (i.e. 10-oxo-10,11-dihydro-indeno-[1,2-*b*]-chromen-6-yl-acetic acid (**6**)) was also prepared in which a five membered ring is introduced in order to fix the position of the benzene ring in position 2 on the flavone moiety, closely resembling the structure of xanthenone-4-acetic acid (**2**).



6

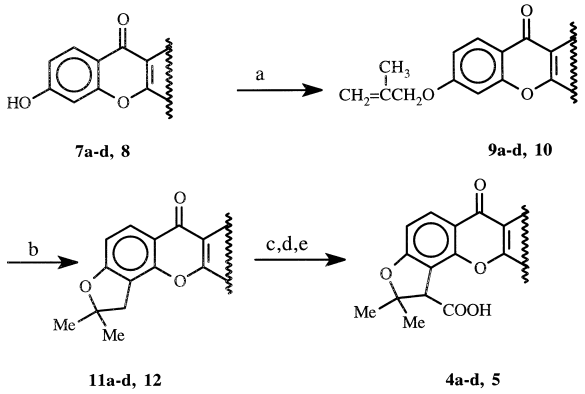
It resembles particularly 5,6-dimethylxanthenone-4-acetic acid (**3**), as the A, B, and C rings can mimic the xanthenone moiety and the D ring can mimic the substituents in positions 5 and 6 included in a cyclic structure.

Direct in vitro toxicity of the synthesised compounds (tested as racemates) was evaluated towards four tumoral cell lines and the ability of these compounds to stimulate mouse peritoneal macrophages in culture to become tumoricidal (indirect toxicity) was also studied.

Results and Discussion

Chemistry

The studied compounds **4a–d** and **5** were prepared according to Scheme 1. The substituted 7-hydroxy-



7a-d, 8 **9a-d, 10**

11a-d, 12 **4a-d, 5**

Scheme 1. Reagents and conditions: (a) methylallylbromide, K_2CO_3 reflux; (b) 2,6-dimethylphenol 230–240 °C, 8 h; (c) NBS; (d) CuCN; (e) AcOH, H_2SO_4, H_2O reflux, 2 h.

chromones (**7a,b,d**) were prepared via Baker–Venkataraman rearrangement of resacetophenone dibenzoates, which were prepared from resacetophenone and benzoyl chlorides, to the corresponding diketones followed by cyclisation.[33–35] The same procedure was used to prepare the 7-hydroxy-2-thenoylchromone (**7c**). The substituted 7-hydroxychromones and 3-hydroxyxanthenone[36] (**8**) were heated with methylallylbromide in presence of K_2CO_3 to afford, respectively, **9a–d** and **10**.[28] These compounds were cyclised to **11a–d** and **12**[37] by heating at 230–240 °C in the presence of 2,6-dimethylphenol. Subsequent bromination with *N*-bromosuccinimide (compounds **13a-d** and **14**[37]), treatment with CuCN (not isolable compounds) and hydrolysis with AcOH, H_2SO_4, H_2O afforded the required compounds **4a–d** and **5**.

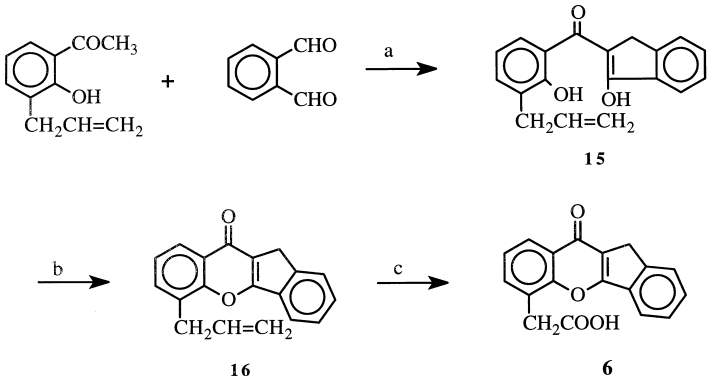
Compound **6** was prepared following Scheme 2. The 2-hydroxy-3-allylacetophenone was condensed with *o*-phthalaldehyde using 50% KOH solution at room temperature to afford **15**, which was cyclised to **16** by refluxing with MeOH–HCl (g). Oxidation of **16** with $KMnO_4$ afforded **6**.

Biological evaluation

We have studied direct in vitro toxicity of FAA and XAA analogues towards tumoral cell lines and also the ability of these compounds to stimulate mouse peritoneal macrophages in culture to become tumoricidal (indirect toxicity).

The direct effect of the tested compounds was determined on four human cell lines: two of these, LoVo S and LoVo R arise from colon adenocarcinoma, while 2008 and C13* from ovarian adenocarcinoma. 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 show reduced cell membrane permeability to passive diffusion[38] and mitocondrial membrane functionality.[39,40]

Direct toxicity. Data obtained for the tested compounds are summarised in Table 1.



Scheme 2. Reagents: (a) KOH 50%, room temperature, 12 h; (b) HCl(g), MeOH reflux 2 h; (c) $KMnO_4$, 0–5 °C, 6 h.

On LoVo S cells XAA showed toxic effect similar to FAA, in fact at the highest doses tested (250 and 500 μM) XAA was able to induce 28 and 44% reduction of cell growth, against 24 and 42% obtained with FAA. On this cell line all the synthesised compounds appeared more active than the two references, showing a significant cytotoxic effect, about 30%, at the lowest concentration tested (100 μM). Nevertheless, it should be stressed that their direct toxicity on LoVo S cells at higher concentrations was not dose-dependent. Considering the IC_{50} values and the potency ratio (P.R.) versus the two references, the new analogues appeared 1.4–2.4 times more potent than FAA and 1.8–3 times more potent than XAA. In particular, **5** and **4c** were the two most toxic compounds.

On LoVo R cells, both FAA and XAA proved to be more cytotoxic than on the parental cell line, whereas the analogues showed toxic effect similar to the reference compounds (except for **6**, more active) but lower than their effect on LoVo S cells.

Fully different were the results obtained on the two ovarian cell lines, especially on 2008 cells. In fact, not only was FAA the most active compound on this line, but among the new derivatives only **4b** and **6** showed the same cytotoxic activity. The other compounds were quite inactive and a significant cell growth reduction (about 35%) was observed, only with the 500 μM dose.

Generally, the cell line bearing resistance (C13*) was more sensitive to cytotoxic effect of the new derivatives than the parental one, even if the best results were obtained at the highest concentration tested (500 μM). In these experiments FAA was the most active compound on both cell lines and was more cytotoxic on resistant C13* cells. XAA showed toxic effect similar to FAA on 2008 cells, while it was 2.7 times less potent than FAA on C13* cells. Among the analogues, only **6** showed toxic effect similar to FAA both on 2008 and C13* cells, whereas the other compounds were inactive or less potent.

In conclusion, these analogues were able to induce direct cytotoxicity only at very high concentrations. The four cell lines used showed a different response to FAA: generally, ovarian cells were more sensitive than colon cells. On the contrary, the new derivatives appeared to be more active on colon cells with respect to ovarian cells. In particular, all analogues were significantly more potent than FAA itself on LoVo S.

Indirect toxicity. The immune system plays an essential role in the physiological defence against tumoural cell proliferation and several lymphocyte subpopulations are involved in immunosurveillance against tumour cells.[41,42] Natural killer (NK) cells are a subpopulation of large granular lymphocytes that display spontaneous cytotoxic activity against tumour cells without restriction by molecule of the major histocompatibility complex.[43] The lytic activity of NK lymphocytes can be regulated by different cytokines, such as interferon α, interferon β and interleukin-2.[44–47]

Flavone-8-acetic acid seems to act by an indirect mechanism, involving the stimulation of NK cell activity,[13,14] as well as the increase of macrophage-mediated cytotoxicity.[48] Furthermore, FAA induces interferon α, β, γ and TNF.[49] In order to evaluate the true biological meaning of the changes in the chemical structure of FAA or XAA it is interesting to consider the ability of the new derivatives to stimulate mouse peritoneal macrophages in culture to become tumoricidal, evaluated by measuring the cytotoxicity induced on C13* cells co-cultivated with macrophages pre-treated with the reference compounds or one of the new analogues. The results were collected in Table 2.

FAA seems unable to stimulate lytic activity of macrophages. Comparing the IC_{50} values obtained on C13* cells for direct effects, no appreciable difference was seen (Table 2). On the contrary, XAA was the most active compound, in fact its indirect toxicity was three times more than its direct effect. Comparing the indirect effect of the new derivatives with their direct activity, a general increase of toxicity was observed, that for **4a**, **4c** and **4d** was about 1.7–2.6 times. Interesting were also the results obtained with compound **5**, which was inactive on C13* cells, but was able, like XAA, to stimulate lytic activity of macrophages.

Table 1. IC_{50}, 95% confidence limits and potency ratio (PR) versus FAA and versus XAA for each derivative and for each cell line

Compd	IC_{50} μM LoVo S	PR versus FAA	PR versus XAA	IC_{50} μM LoVo R	PR versus FAA	PR versus XAA	IC_{50} μM 2008	PR versus FAA	PR versus XAA	IC_{50} μM C13*	PR versus FAA	PR versus XAA
FAA	564 (465–684)	1	—	383 (314–469)	1	—	460 (419–471)	1	—	233 (183–297)	1	1
XAA	711 (502–979)	—	1	510 (417–628)	—	1	448 (396–508)	—	1	642 (511–806)	—	1
4a	242 (134–437)	2.3[a] (1.5–4.3)	2.9[a] (2.2–3.6)	562 (494–640)	0.7[b] (0.5–0.8)	0.9 (0.5–1.3)	—	—	—	500 (424–667)	0.4[b] (0.3–0.6)	1.2 (0.7–2.3)
4b	271 (146–402)	2.1[a] (1.1–3.9)	2.6[a] (2.0–3.2)	548 (483–623)	0.7[b] (0.5–0.7)	0.9 (0.5–2.0)	568 (374–785)	0.8 (0.5–1.2)	0.8 (0.5–1.3)	496 (425–578)	0.5[b] (0.3–0.7)	1.3 (0.9–1.9)
4c	236 (152–366)	2.4[a] (1.5–3.9)	3.0[a] (2.5–3.5)	498 (437–567)	0.8 (0.6–1.0)	1.0 (0.6–1.6)	—	—	—	548 (460–652)	0.4[b] (0.3–0.5)	1.1 (0.6–2.0)
4d	264 (206–338)	2.1[a] (1.4–3.0)	2.7[a] (1.9–3.5)	391 (199–482)	0.9 (0.5–2.0)	1.3 (1.0–1.8)	—	—	—	613 (483–777)	0.3[b] (0.1–1.5)	1.0 (0.7–1.7)
5	238 (189–299)	2.4[a] (1.7–3.3)	3.0[a] (2.0–4.0)	453 (226–563)	0.8 (0.2–1.3)	1.1 (0.9–1.5)	—	—	—	—	—	—
6	378 (311–461)	1.4[a] (1.1–1.9)	1.8[a] (1.2–2.7)	391 (329–465)	1.1 (0.8–1.5)	1.5[a] (1.2–2.0)	371 (329–418)	1.2 (0.5–1.9)	1.2 (1.0–1.4)	294 (243–356)	0.7 (0.5–0.9)	2.2[a] (1.6–2.9)

[a]More potent.
[b]Less potent.

Table 2. IC_{50}, 95% confidence limits and potency ratio (PR) versus FAA and versus XAA for each derivative on co-cultured macrophages + C13* cells and PR for IC_{50} of each compound obtained in co-culture macrophages + C13* versus its direct toxicity on C13* cells

Compd	IC_{50} μM	PR versus FAA	PR versus XAA	PR versus C13*
FAA	285 (211–386)	1	—	0.8 (0.5–1.2)
XAA	219 (164–293)	—	1	3.0 (2.2–4.2)[a]
4a	288 (201–414)	1.0 (0.6–1.6)	0.8 (0.2–1.3)	1.7 (1.3–2.0)[a]
4b	481 (317–730)	0.6 (0.3–1.0)[b]	0.4 (0.3–0.6)[b]	1.2 (0.7–2.1)
4c	275 (209–362)	1.0 (0.7–1.5)	0.7 (0.5–0.9)	2.0 (1.4–2.7)[a]
4d	240 (179–322)	1.2 (0.8–1.8)	0.9 (0.5–2.0)	2.6 (1.7–3.7)[a]
5	304 (211–439)	0.9 (0.6–1.5)	0.7 (0.4–1.1)	—
6	293 (232–373)	1.0 (0.7–1.4)	0.7 (0.5–1.0)	1.0 (0.7–1.3)

[a]More potent.
[b]Less potent.

Conclusion

Some furochromene and furoxanthene carboxylic acids (**4a**–**d** and **5**) in which the acetic chain of FAA and XAA is locked in a rigid structure and a rigid analogue of FAA (compound **6**) were synthesized. Direct cytotoxic activity, evaluated on four human cell lines, was obtained only at very high concentrations. Generally, FAA was more cytotoxic than XAA and ovarian cells were more sensitive than colon cells to FAA activity. On the contrary, the new derivatives appeared to be more active on colon cells with respect to ovarian cells. In particular, all analogues were significantly more potent than FAA itself on LoVo S.

All compounds showed an interesting indirect mechanism of antitumour action. In particular, compound **4d** was able to significantly increase the macrophage lytic properties and has been selected for further investigations.

Experimental

Chemistry

A Büchi apparatus and open glass capillaries were used to determine all melting points (mp), which are not corrected. Wherever analyses are only indicated with element symbols, analytical results obtained for those elements are within 0.4% of the theoretical values. ^{1}H NMR were obtained on Gemini 300 spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS). Mass spectra were recorded on a V.G. 7070 E spectrometer.

7-Hydroxy-2-thenoylchromone (7c). To a solution of resacetophenone (15.2 g, 0.1 mol), pyridine (23.7 g, 0.3 mol), and anhydrous ether (100 mL) at 0 °C (ice bath), 2-thiophenecarbonyl chloride (43.8 g, 0.3 mol) was added, dropwise, over 1 h. After the addition, the mixture was stirred at 0 °C for 1 h and then at room temperature for 1 h. The solid was collected by filtration and washed with ether (200 mL). The filtrate was washed with water, 10% aq HCl, 10% aq $NaHCO_3$, and saturated brine solution and dried (Na_2SO_4). The

filtered solution was evaporated to give 26 g (70%) of product mp 120–123 °C (EtOH).

A suspension of this product and K_2CO_3 (28 g, 0.2 mol) in acetone (200 mL) was stirred and heated under reflux in presence of nitrogen for 2 days. After cooling the yellow solid was collected by filtration and washed with toluene and then with water. The solid was stirred with 10% aq HCl and collected by filtration. The desired product, 20.8 g (80%), mp 144–147 °C was obtained.

A yellow suspension of this compound, glacial acetic acid (200 mL) and anhydrous sodium acetate (50 g) was refluxed under nitrogen for 16 h. The reaction mixture was cooled and water was added until no further white precipitate was formed. The solid was collected by filtration to yield 10.2 g (60%) of product mp 290 °C (dec.) (EtOH). Anal. ($C_{13}H_8O_3S$): C, H. ^{1}H NMR ($CDCl_3$) (6.7 δs, 1H, CH-3), 7.5–8.4 (m, 6H, aromatic).

7-β-Methylallylflavone (9a). A mixture of 7-hydroxyflavone (9.52 g, 0.04 mol), K_2CO_3 (7 g, 0.05 mol) and methylallylchloride (7.2 g, 0.08 mol) in acetone (100 mL) was refluxed, with stirring, for 24 h and hot filtered. The solvent was evaporated and the residue was taken up in CH_2Cl_2. The organic layer was washed with 5% NaOH solution, H_2O and dried (Na_2SO_4). Evaporation of the solvent gave a residue which was crystallised from EtOH to give 8.2 g (70%) of product mp 118–120 °C. Anal. ($C_{19}H_{16}O_3$): C, H. ^{1}H NMR ($CDCl_3$) δ 1.9 (s, 3H, CH_3), 4.55 (s, 2H, CH_2), 5.05 (s, 1H, CH_2), 5.15 (s, 1H, CH_2), 6.7 (s, 1H, CH), 6.95–8.15 (m, 8H, aromatic).

The same procedure was used to prepare the following compounds.

7-β-Methylallyl-4′-fluoroflavone (9b). This compound was obtained in 80% yield, mp 136–137 °C (EtOH). Anal. ($C_{19}H_{15}FO_3$): C, H. ^{1}H NMR ($CDCl_3$) δ 1.9 (s, 3H, CH_3), 4.55 (s, 2H, CH_2), 5.05 (s, 1H, CH_2), 5.15 (s, 1H, CH_2), 6.6 (s, 1H, CH), 6.95–8.15 (m, 7H, aromatic).

7-β-Methylallyl-2-thienylchromone (9c). This compound was obtained in 65% yield, mp 139–141 °C (EtOH). Anal. ($C_{17}H_{14}O_3S$): C, H. ^{1}H NMR ($CDCl_3$) δ 1.9 (s, 3H, CH_3), 4.55 (s, 2H, CH_2), 5.05 (s, 1H, CH_2), 5.15 (s, 1H, CH_2), 6.6 (s, 1H, CH), 6.95–8.2 (m, 6H, aromatic).

7-β-Methylallyl-2,3-diphenylchromone (9d). This compound was obtained in 75% yield, mp 106–108 °C (EtOH). Anal. ($C_{25}H_{20}O_3$): C, H. ^{1}H NMR ($CDCl_3$) δ 1.9 (s, 3H, CH_3), 4.55 (s, 2H, CH_2), 5.1 (d, 2H, CH_2), 6.95–8.2 (m, 13H, aromatic).

8,8-Dimethyl-2-phenyl-8,9-dihydrofuro-[2,3-*h*]-chromen-4-one (11a). Compound **9a** (1.46 g, 0.005 mol) was heated at 230–240 °C with 1.5 g of 2,6-dimethylphenol for 8 h. After cooling the reaction mixture was taken up in diethyl ether. The organic layer was washed with 10% NaOH solution, H_2O, dried (Na_2SO_4) and evaporated to dryness. The residue on crystallizing from ligroin afforded 1.0 g (70%) of product mp 184–186 °C. Anal. ($C_{19}H_{16}O_3$); C, H. ^{1}H NMR ($CDCl_3$) (1.6 (s, 6H, CH_3), 3.3 (s, 2H, CH_2), 6.75 (s, 1H, CH), 6.85–8.1 (m, 7H, aromatic).

The same procedure was used to prepare the following compounds.

8,8-Dimethyl-2-(4′-fluoro)phenyl-8,9-dihydrofuro-[2,3-*h*]-chromen-4-one (11b). This compound was obtained in 65% yield, mp 204–206 °C (toluene). Anal. ($C_{19}H_{15}FO_3$): C, H. ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 6H, CH_3), 3.25 (s, 2H, CH_2), 6.7 (s, 1H, CH), 6.8–8.1 (m, 6H, aromatic).

8,8-Dimethyl-2-thienyl-8,9-dihydrofuro-[2,3-*h*]-chromen-4-one (11c). This compound was obtained in 55% yield, mp 219–221 °C (toluene). Anal. ($C_{17}H_{14}O_3S$): C, H. ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 6H, CH_3), 3.25 (s, 2H, CH_2), 6.6 (s, 1H, CH), 6.8–8.1 (m, 5H, aromatic).

8,8-Dimethyl-2,3-diphenyl-8,9-dihydrofuro-[2,3-*h*]chromen-4-one (11d). This compound was obtained in 55% yield, mp 245–247 °C (toluene). Anal. ($C_{25}H_{20}O_3$): C, H. ^{1}H NMR ($CDCl_3$) δ 1.6 (s, 6H, CH_3), 3.25 (s, 2H, CH_2), 6.8–8.1 (m, 12H, aromatic).

9-Bromo-8,8-dimethyl-8,9-dihydro-2-phenylfuro-[2,3-*h*]-chromen-4-one (13a). A mixture of **11a** (2.92 g, 0.01 mol), *N*-bromosuccinimide (1.78 g, 0.01 mol) and a catalytic amount of benzoyl peroxide in CCl_4 (50 mL) was refluxed for 3 h and then hot filtered. The filtrate was evaporated to dryness and the residue on crystallising from toluene gave 1.8 g (50%) of product mp 218–220 °C. Anal. ($C_{19}H_{15}BrO_3$): C, H. ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 3H, CH_3), 1.85 (s, 3H, CH_3), 5.7 (s, 1H, CHBr), 6.8 (s, 1H, CH), 6.9–8.2 (m, 7H, aromatic).

The same procedure was used to prepare the following compounds.

9-Bromo-8,8-dimethyl-8,9-dihydro-2-(4-fluoro)phenylfuro-[2,3-*h*]-chromen-4-one (13b). This compound was obtained in 60% yield, mp 242–246 °C (toluene). Anal. ($C_{19}H_{14}BrFO_3$): C, H. ^{1}H NMR (DMSO-d_6) δ 1.5 (s, 3H, CH_3), 1.7 (s, 3H, CH_3), 5.2 (s, 1H, CHBr), 6.35 (s, 1H, CH), 7.0–8.3 (m, 6H aromatic).

9-Bromo-8,8-dimethyl-8,9-dihydro-2-thienylfuro-[2,3-*h*]-chromen-4-one (13c). This compound was obtained in 50% yield, mp 170–176 °C (toluene). Anal. ($C_{17}H_{13}BrO_3S$): C, H. ^{1}H NMR (DMSO-d_6) (1.3 (s, 3H, CH_3), 1.45 (s, 3H, CH_3), 5.15 (s, 1H, CHBr), 6.8 (s, 1H, CH), 6.9–8.05 (m, 5H aromatic).

9-Bromo-8,8-dimethyl-8,9-dihydro-2,3-diphenylfuro-[2,3-*h*]-chromen-4-one (13d). This compound was obtained in 55% yield, mp 197–200 °C (toluene). Anal. ($C_{25}H_{19}BrO_3$): C, H. ^{1}H NMR ($CDCl_3$) (1.55 (s, 3H, CH_3), 1.9 (s, 3H, CH3), 5.65 (s, 1H, CHBr), 6.9–8.3 (m, 12H aromatic).

2,2-Dimethyl-1,2-dihydro-6*H*-furo-[2,3-*c*]-xanthen-6-on-1-carboxylic acid (5). A solution of 1-bromo-2,2-dimethyl-1,2-dihydro-6*H*-furo-[2,3-*c*]-xanthen-6-one[37] (**14**, 1.72 g, 0.005 mol) and CuCN (0.9 g, 0.01 mol) in DMF (20 mL) was refluxed for 20 h under N_2 atmosphere.

After cooling, the reaction mixture was poured into H_2O. The separated solid was collected by filtration, washed with H_2O and dried. This solid was refluxed for 2 h with a mixture of AcOH (5 mL), H_2SO_4 (5 mL) and H_2O (5 mL). After cooling the reaction mixture was poured into H_2O. The separated solid was collected by filtration, washed with H_2O and suspended in a saturated $NaHCO_3$ solution. After filtration the solution was acidified with dil HCl: the separated solid was collected and crystallised from EtOH to give 0.31 g (20%) of product mp 268–270 °C. Anal. ($C_{18}H_{14}O_5$): C, H. ^{1}H NMR (DMSO-d_6) δ 1.5 (s, 3H, CH_3), 1.6 (s, 3H, CH_3), 4.3 (s, 1H, CH), 6.95–8.2 (m, 6H, aromatic). MS: *m*/*z* (relative abundance): 310 (M^+, 37), 265 (100), 31 (29).

The same procedure was used to prepare the following compounds.

8,8-Dimethyl-8,9-dihydro-2-phenylfuro-[2,3-*h*]-chromen-4-on-9-carboxylic acid (4a). This compound was obtained in 30% yield, mp 198–202 °C (EtOH/H_2O). Anal. ($C_{20}H_{15}O_5$): C, H. ^{1}H NMR (DMSO-d_6) δ 1.5 (s, 3H, CH_3), 1.6 (s, 3H, CH_3), 4.4 (s, 1H, C*H*COOH), 6.95 (s, 1H, CH), 7.0–8.05 (m, 7H, aromatic). MS: *m*/*z* (relative abundance): 336 (M^+, 56), 291 (100), 292 (34).

8,8-Dimethyl-8,9-dihydro-2-(4′-fluoro)phenylfuro-[2,3-*h*]-chromen-4-on-9-carboxylic acid (4b). This compound was obtained in 30% yield, mp 260–262 °C (EtOH). Anal. ($C_{20}H_{14}FO_5$): C, H. ^{1}H NMR (DMSO-d_6) δ 1.5 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 4.45 (s, 1H, C*H*COOH), 6.95 (s, 1H, CH), 7.0–8.1 (m, 6H, aromatic). MS: *m*/*z* (relative abundance): 354 (M^+, 76), 309 (100), 296 (72).

8,8-Dimethyl-8,9-dihydro-2-thienylfuro-[2,3-*h*]-chromen-4-on-9-carboxylic acid (4c). This compound was obtained in 25% yield, mp 151–153 °C (EtOH). Anal. ($C_{18}H_{14}O_5S$): C, H. ^{1}H NMR (DMSO-d_6) δ 1.5 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 4.35 (s, 1H, C*H*COOH), 6.85 (s, 1H, CH), 7.0–8.0 (m, 5H, aromatic). MS: *m*/*z* (relative abundance): 342 (M^+, 38), 297 (64), 58 (100).

8,8-Dimethyl-8,9-dihydro-2,3-diphenylfuro-[2,3-*h*]-chromen-4-on-9-carboxylic acid (4d). This compound was obtained in 40% yield, mp 292–294 °C (EtOH). Anal. ($C_{26}H_{20}O_5$): C, H. ^{1}H NMR (DMSO-d_6) δ 1.5 (s, 3H, CH_3), 1.6 (s, 3H, CH_3), 4.4 (s, 1H, C*H*COOH), 7.0–8.0 (m, 12H, aromatic). MS: spectrum not registered.

(3-Allyl-2-hydroxyphenyl)-(3-hydroxy-1*H*-inden-2-yl)-methanone (15). To a mixture of 2-hydroxy-3-allylacetophenone (4.27 g, 24 mmol) and *o*-phthalaldehyde (3.22 g, 24 mmol) in EtOH (45 mL) 50% KOH solution (10 mL) was added dropwise. The reaction mixture was stirred for 12 h, poured into ice and acidified with dil HCl. The separated solid was collected by filtration and crystallised from EtOH to give 2.12 g (30%) of **15**: mp 134–136 °C. Anal. ($C_{19}H_{16}O_3$): C, H. ^{1}H NMR ($CDCl_3$) δ 3.5 (m, 2H, CH_2CH=CH_2), 4.05 (s, 2H, CH_2), 5.15 (m, 2H, CH_2=CH-), 6.05 (m, 1H, CH_2=*CH*-), 6.95–7.9 (m, 7H, Ar).

6-Allyl-11*H*-indeno-[1,2-*b*]-chromen-10-one (16). A solution of **15** (2 g, 7 mmol) in MeOH saturated with HCl (g) was refluxed for 2 h and then evaporated to dryness. The residue was crystallised from EtOH to give 1.8 g (95%) of **16**: mp 155–158 °C. Anal. ($C_{19}H_{14}O_2$): C, H. ^{1}H NMR ($CDCl_3$) (3.8 (m, 2H, *C*H_2CH=CH_2), 3.9 (s, 2H, CH_2), 5.2 (m, 2H, *C*H_2=CH-), 6.1 (m, 1H, CH_2=*CH*-), 7.4–8.25 (m, 7H, Ar).

(10-Oxo-10,11-dihydro-indeno-[1,2-*b*]-chromen-6-yl)acetic acid (6). To a cold solution of **16** (1.37 g, 5 mmol) in AcOH (20 mL), acetone (20 mL) and H_2O (10 mL) solid $KMnO_4$ (1.58 g, 10 mmol) was added in portions in 6 h. The reaction mixture was stirred for 1 h at room temp and poured into $Na_2S_2O_8$ solution. The separated solid was collected by filtration and crystallised from EtOH to give 1.02 g (70%) of **6**: mp 250 °C (dec). Anal. ($C_{18}H_{12}O_4$): C, H. ^{1}H NMR (DMSO-d_6) δ 3.8 (s, 2H, *C*H_2COOH), 4.0 (m, 2H, *C*H_2), 7.45–8.05 (m, 7H, Ar) MS: *m*/*z* (relative abundance): 292 (M^+, 100), 278 (20), 247 (18), 189 (19).

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 by 100 ng/mL of doxorubicin.

The human ovarian adenocarcinoma cell line 2008 and the *cis*-DDP-resistant subline C13*, kindly supplied by Professor 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

Tetrazolium salts assay (MTT). 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 MTT reduction assay after 24 h of incubation.[50]

Twenty microlitres of MTT solution (5 mg/mL in PBS) were added to each well, and plates were incubated for 4 h at 37 °C. DMSO (150 μL) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. The absorbance was measured on a micro-culture plate reader (Titertek Multiscan) using a test wavelength of 570 nm and a reference wavelength of 630 nm.

Indirect cytotoxicity

The ability of the new derivatives to stimulate mouse macrophages in culture to become tumoricidal was evaluated using resident peritoneal macrophages.[51]

Resident peritoneal cells were isolated by two injections of 5 mL PBS containing 10 U/mL of eparin into the

peritoneal cavity. The cavity was gently massaged for 2 min and the cells removed by drawing fluid out with a syringe.[52] The recovered cell suspension was centrifuged and the pellet was washed twice in sterile PBS. The cells were then re-suspended in RPMI 1640 plus 5% FCS and plated in a culture flask left to adhere at 37 °C. After 2 h medium and non-adherent cells were discarded, the flask washed with sterile PBS, cells adhering (macrophages) were resuspended, centrifuged, counted using 0.5% Trypan blue, re-suspended in complete medium and then plated in 96-well plates (Falcon) at a concentration of 1×10^4 cells/well in presence of different concentrations of FAA 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 co-cultivated for 24 h. Lysis of C13* cells was assessed by MTT test[53] and the percentages of specific cytotoxicity were calculated as follows:

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

Statistical analysis. For each assay three different experiments were performed in triplicate. The results were statistically evaluated by Student's *t*-test.[54] The IC_{50}, 95% confidence limits and the potency ratio between FAA and each analogue (IC_{50}FAA/IC_{50}derivative) were estimated using the Litchfield and Wilcoxon method.[54]

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