



Design and synthesis of naphthylchalcones as novel anti-leukaemia agents

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ARTICLE INFO

Keyword:

Chalcones

Flavanones

Claisen-Schmidt condensation, cytotoxicity

Apoptosis

Caspases

ABSTRACT

A series of new hydroxylated chalcone derivatives with different substitution patterns on a phenyl ring A and B, were prepared by Claisen-Schmidt condensation in an aqueous alkaline base. The antiproliferative activity of the studied compounds was evaluated against the human leukaemia cell line U-937. The structure-activity relationship of these naphthylchalcones was investigated by the introduction of one methoxy or two methyl groups on the A ring, the introduction of a methoxy group on the naphthyl ring or by varying the position of the methoxy group on the A ring. The results revealed that the naphthylchalcone containing a methoxy group in position 6 of the A ring was the most cytotoxic compound, with an IC₅₀ value of $4.7 \pm 0.5 \mu\text{M}$ against U-937 cells. This synthetic chalcone induced S and G₂-M cell cycle arrest, a time-dependent increase in sub-G₁ ratio and annexin-V positive cells, caspase activation and poly(ADP-ribose) polymerase cleavage. Apoptosis induction was blocked by a pan-caspase inhibitor and by the selective caspase-3/7 inhibitor and attenuated by the inhibition of c-jun N-terminal kinases / stress-activated protein kinases (JNK/SAPK) and phosphoinositide 3-kinase. The structure-activity relationship of naphthylchalcones against human leukaemia cells reveals that the major determining in cytotoxicity is the presence of a methoxy group in position 6 of the A ring that suggest the potential of this compound or derivatives in the development of new anti-leukaemia drugs.

1. Introduction

Chalcones are an important class of compounds bearing the *trans*-1,3-diphenyl-2-propen-1-ones framework, belonging to an important class of natural products, flavonoids. Chalcones exist as either *E*- (*trans*) or *Z*- (*cis*) isomers (Fig. 1). The *E*-isomer is the thermodynamically most stable form in most cases, so the majority of chalcones are isolated as *E*-isomers. Configuration of *Z* isomer is unstable due to the strong steric effects between the carbonyl group and B-ring.[1]

Chalcones are present in vegetables, fruits, spices, tea and soy based foodstuff.[2] The importance of these compounds is related to the biological activity that have been reported in recent years, such as: anti-inflammatory, antileishmanial, antimalarial, antifungal, anti-diabetic, antimicrobial,[3,4] antibacterial, anticancer, antituberculosis, anti-hyperglycemic, anti-obesity, antidepressant, against cardiovascular diseases, anti-obesity, antioxidant,[4] anticonvulsant,[5] antiviral[6].

They have also been described for application and they may present as well as antitumor activity, that has been observed in different types of cancer, namely, leukaemia, a non-small-cell lung cancer, colon cancer, prostate cancer, and breast cancer.[7,8] It has been found that their biological activities are due to the presence of a reactive α,β -unsaturated keto function in the molecule.[9] The biological activity in these compounds is not new. In fact, prehistoric therapeutic applications of chalcones can be associated with the thousand-year old use of plants and herbs for the treatment of different medical disorders.[10] Chalcone containing plants, such as *Glycyrrhiza*, *Angelica*, *Ruscus* and *Piper* species have been used as medicine in Asia, Africa and South America. Several chalcones were approved for clinical use, such as metochalcone 1 marked as a choleric drug and sofalcone 2 an antiulcer drug, Fig. 2. [11]

The chalcones and their derivatives are important intermediates in organic synthesis because they are resourceful precursors for the

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<https://doi.org/10.1016/j.bioorg.2021.105348>

Received 5 July 2021; Received in revised form 30 July 2021; Accepted 7 September 2021

Available online 21 September 2021

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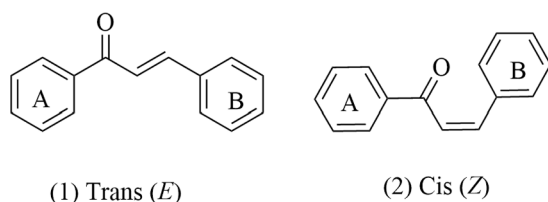


Fig. 1. Structural and numerical representations of chalcone scaffold.

synthesis of heterocyclic compounds (Fig. 2). Chalcones undergo cyclization reactions with different reagents to form diverse classes of heterocyclic compounds ranging from five membered to seven membered rings containing nitrogen, oxygen and sulphur heteroatoms. In the cyclization reactions the highly reactive bielectrophilic ketovinyl chain condenses with a variety of binucleophilic reagents to generate an assortment of heterocyclic systems such as derivatives of pyrazolines, [12,13] phenylpyrazoline and isoxazole (5-membered heterocyclics), [14] derivatives of aminopyrimidines and cyanopyridines (6-membered heterocyclics) [11] and derivatives of 1,5-benzodiazepines, 1,5-benzoxazepines, and 1,5-benzothiazepines (7-membered heterocyclics), [12] Fig. 3.

Chalcones are also considered to be biosynthetic precursors of flavonoids (such as flavones and flavanones) and isoflavonoids. Chalcones and their derivatives have been synthesized through different methods, [4] some of them using quite innovative and very current methodologies. [17,18] Particularly, chalcones may be synthesized by a Claisen Schmidt condensation of aldehyde and ketone, through base or acid catalysis, followed by dehydration to yield chalcones.

Our goal was to synthesize a series of new 2'-hydroxychalcones (naphthylchalcones) and to evaluate their cytotoxicity against the human leukaemia cell line U-937. From these results interesting structure-activity relationships were observed to assess which compound presents more promising results and would justify being further studied on the path of finding new anti-leukaemia drugs.

2. Results and discussion

2.1. Chemistry

Several methods are available for the synthesis of chalcones and some of them can be found in a review published in 2020. [4] However, traditionally, the Claisen-Schmidt condensation is still the best option to prepare chalcones, due to its simplicity and the commercial availability of raw materials. For this reason, the Claisen-Schmidt condensation in basic media was the method used herein to synthesize a series of new 2'-hydroxychalcones (naphthylchalcones) with different substituents on the A and B rings. [19] The proposed mechanism is presented in Scheme 1.

In the first step, the hydroxide deprotonates the proton α of the carbonyl on acetophenone **3**, which creates an alkoxide bonded to an alkene **4-b**. The nucleophilic carbon-carbon double bond in **4-b** attacks the carbonyl carbon in 2-naphthaldehyde **5** to form **6**. The alkoxide **4-a** deprotonates water to give hydroxide, which in turn deprotonates the proton α eliminating water to give the 2'-hydroxychalcones **6**. All chalcone compounds synthesized in this study are *trans* (*E*) isomers (Fig. 1) according to their ^1H NMR spectral analysis in agreement with literature. [4] The flavanone **7** may be obtained by the cyclization of the

2'-hydroxychalcones in basic or acidic media and for this reason is considered the main impurity in the chalcone synthesis.

The mechanism presented may generate some discussion, since the most acidic proton of acetophenone belongs to the hydroxyl group and not that of the methyl group (proton α). Nevertheless, this is the correct mechanism that leads to the product and in a certain way this mechanism was confirmed through the isolation of intermediate **5-b**. However, the existence of other species cannot be ruled out. All naphthylchalcones synthesized are listed in Table 1.

2.2. Biological activity

2.2.1. Screening of synthetic naphthylchalcones reveals that **6-a** is the most potent inhibitor of viability of human leukaemia U-937 cells

The synthesized naphthylchalcones were investigated for their potential antiproliferative activity against the human tumor cell line U-937. This cell line is the most frequently used cell line in biomedical research for the study of neoplasia and therapeutics. The structure-activity relationship of these chalcones was investigated by the introduction of one methoxy or two methyl groups on the A ring, by the introduction of a methoxy group on the naphthyl ring or by varying the position of the methoxy group on the A ring. U-937 cells were treated with increasing concentrations of the synthesized compounds (**6-a**, **6-b**, **6-c**, **6-d**, **6-e**, **6-f**, **6-g**, and **7-a**) and the IC_{50} values (the concentration that induces a 50% inhibition of cell growth) were determined with an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Table 2).

The results indicate that in the case of naphthylchalcones with a methoxy group on the A ring, the position of the methoxy group determines the cytotoxicity. The introduction of a methoxy group at position 6 on the A ring (compound **6-a**) led to a significant improvement in cytotoxic activity in comparison with the compound containing the methoxy group at position 5 on the A ring [$\text{IC}_{50} = 4.7 \pm 0.5 \mu\text{M}$ for chalcone **6-a** vs. $\text{IC}_{50} = 14.5 \pm 2.6 \mu\text{M}$ for chalcone **6-d**]; 3-fold increase in cytotoxicity] and with the compound containing the methoxy group at position 4 on the A ring [$\text{IC}_{50} = 4.7 \pm 0.5 \mu\text{M}$ for chalcone **6-a** vs. $\text{IC}_{50} = 22.8 \pm 4.0 \mu\text{M}$ for chalcone **6-c**]; 4.8-fold increase in cytotoxicity].

The introduction of an additional methoxy group in position 6 of the 2-naphthyl group did not amplify the potency against cell growth inhibition [$\text{IC}_{50} = 14.5 \pm 2.6 \mu\text{M}$ for chalcone **6-d** vs. $\text{IC}_{50} = 23.8 \pm 2.5 \mu\text{M}$ for chalcone **6-e**]; 1.6-fold decrease in cytotoxicity; and [$\text{IC}_{50} = 4.7 \pm 0.5 \mu\text{M}$ for chalcone **6-a** vs. $\text{IC}_{50} = 9.0 \pm 1.8 \mu\text{M}$ for chalcone **6-b**]; 1.9-fold decrease in cytotoxicity].

The introduction of one additional methoxy group in position 4 on the A ring significantly decreased cytotoxicity ($\text{IC}_{50} > 30 \mu\text{M}$ for chalcone **6-f** vs. $\text{IC}_{50} = 9.0 \pm 1.8 \mu\text{M}$ for chalcone **6-b**).

The cytotoxicity of compound **6-h** was not tested because it was used to prepare the flavanone **7-a**, however, considering structure-activity the demethylation of the methoxy group in position 5 on the A ring of the chalcone **6-e** followed by cyclization to the flavanone **7-a** did not change the cytotoxicity of the chalcone ($\text{IC}_{50} = 23.8 \pm 2.5 \mu\text{M}$ for chalcone **6-e** vs. $\text{IC}_{50} = 22.0 \pm 2.4 \mu\text{M}$ for flavanone **7-a**).

The substitution of a methoxy group by a methyl group in position 5 and the introduction of a methyl group in position 3 on the A ring seems to be irrelevant in determining cytotoxicity ($\text{IC}_{50} = 21.8 \pm 2.7 \mu\text{M}$ for chalcone **6-g** vs. $\text{IC}_{50} = 23.8 \pm 2.5 \mu\text{M}$ for chalcone **6-e**). In these experiments, the standard antitumor agent etoposide was included as a positive control.

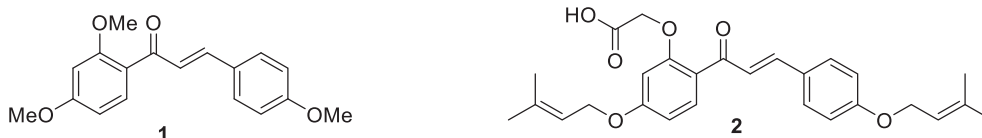


Fig. 2. Chalcones approved for clinical use.

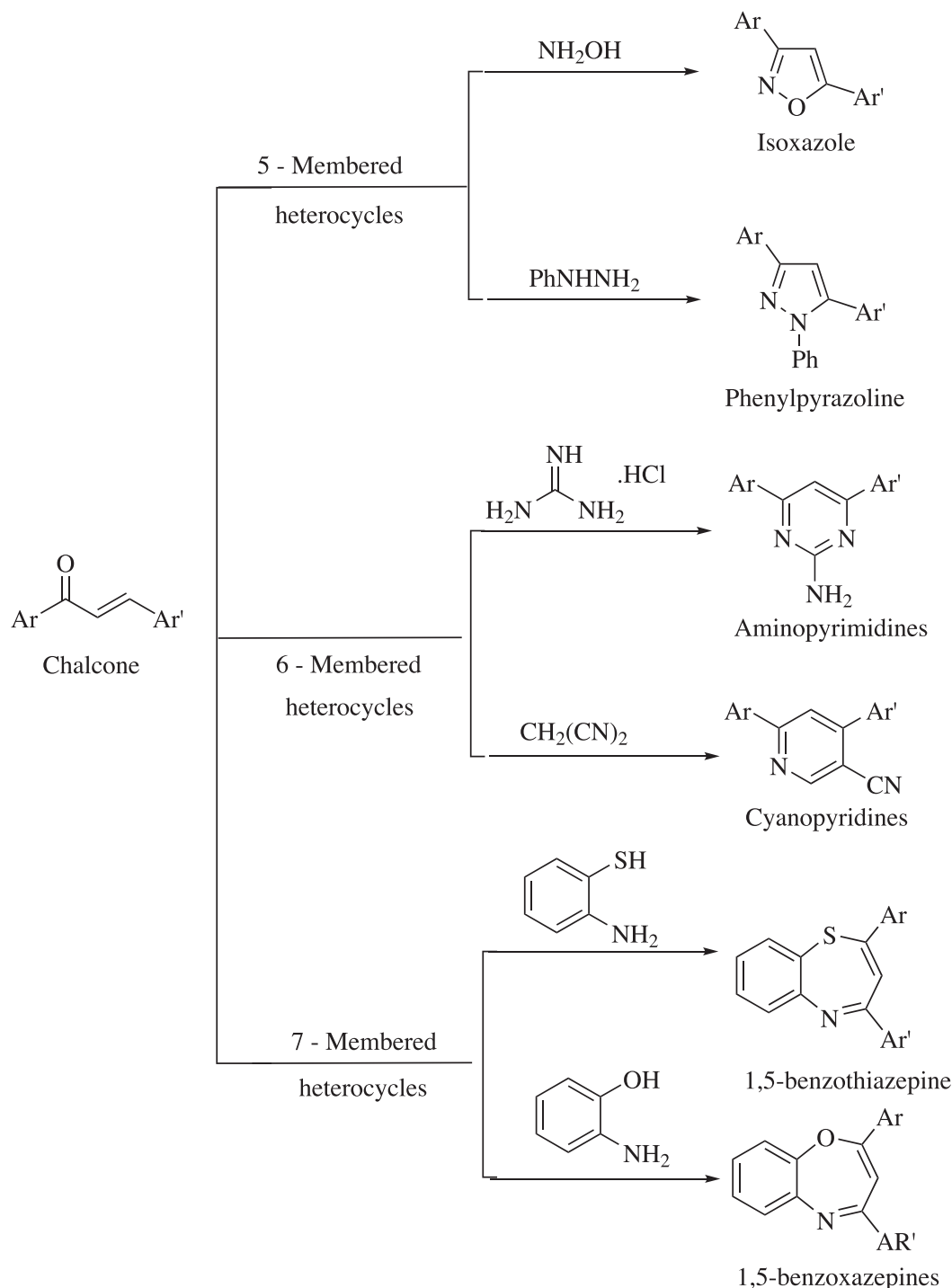


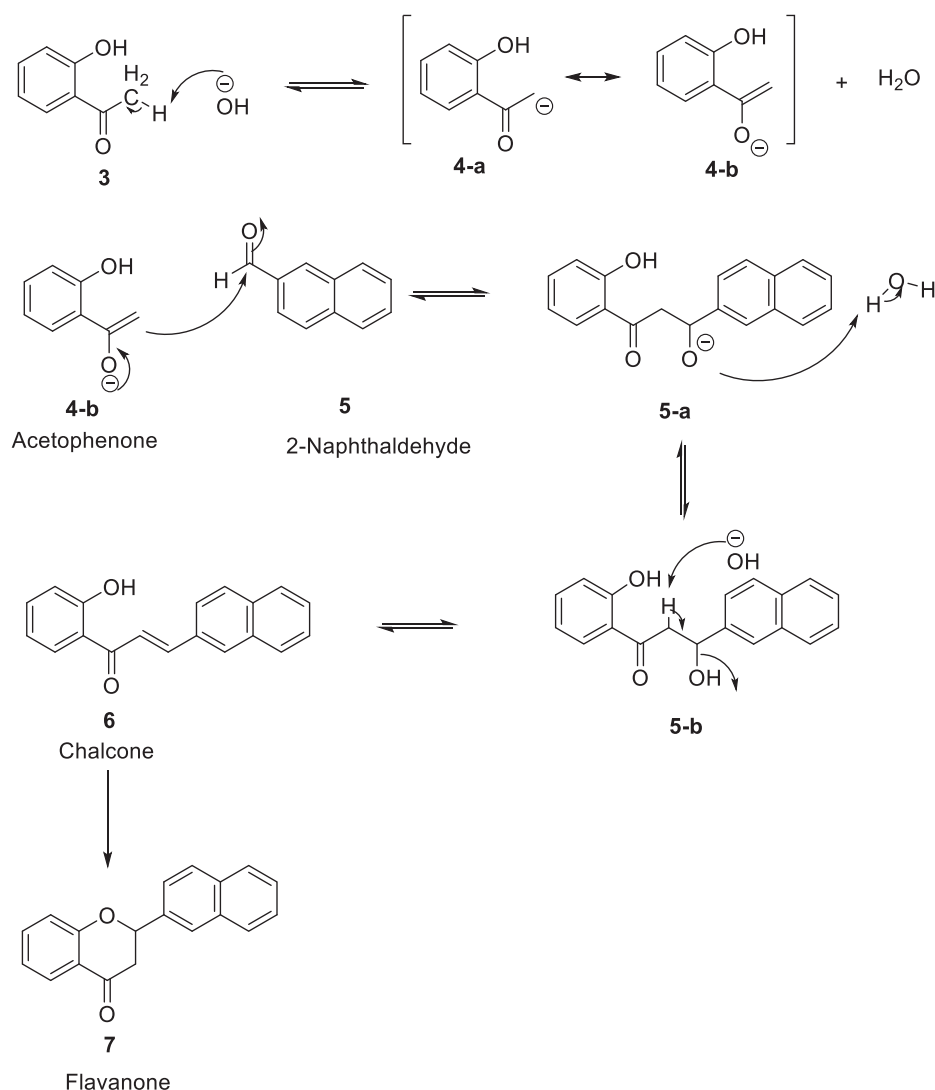
Fig. 3. Synthetic potential of chalcone derivatives for the synthesis of heterocyclic analogues.

In summary, these results revealed that the major determinant of cytotoxicity in this series of naphthylchalcones is the presence of only one methoxy group in position 6 the A ring and the most cytotoxic compound was **6-a**. Since this compound was the most potent in the MTT assays, it was selected for further experiments. The impact of the synthesized compounds (**6-a**, **6-b**, **6-c**, **6-d**, **6-e**, **6-f**, **6-g**, and **7-a**) on the growth of human U-937 leukaemia cells are summarized on Table 2.

2.2.2. Naphthylchalcone **6-a** induces S and G₂-M phases cell cycle arrest and apoptosis in human leukaemia U-937 cells

To determine whether the effects of **6-a** on cell growth inhibition

were mediated by alterations in cell cycle progression, cells were treated with 10 μM **6-a** for different durations (6–24 h), stained with propidium iodide and analyzed by flow cytometry. As shown (Table 3), this chalcone induced a significant S and G₂-M arrest at expense of G₁ phase at 6–12 h which was accompanied with an increase in the percentage of hypodiploid (sub-G₁) cells (i.e., apoptotic cells). The percentage of cells in S and G₂-M phases increased from ~ 24% and ~ 19% in control cells to ~ 34% after treatment with **6-a** for 12 h. The quantification of apoptosis obtained by measurement of the hypodiploid cells revealed that the percentage of apoptotic cells increased in a time-dependent manner (Fig. 3.A and B). Approximately 17-fold increase of apoptotic



Scheme 1. Claisen-Schmidt condensation of benzaldehyde and 2-hydroxyacetophenone, followed by a cyclization of 2'-hydroxychalcone to flavanone.

cells with respect to control was observed at 24 h of treatment (Table 3, Fig. 4.A and B). Estimates of apoptosis obtained from the number of hypodiploid cells and from Annexin V-FITC staining were similar (results not shown). In sum, these results indicate that the synthetic naphthylchalcone **6-a** induces cell cycle arrest in the S and G₂-M phases of the cell cycle and apoptosis on U-937 cells.

We performed additional experiments to explore whether compound **6-a** directly targets tubulin since many cytotoxic drugs induce G₂-M arrest by targeting microtubules and some flavonoids have been shown to perturb microtubules polymerization via tubulin binding.[20–23]

To confirm this hypothesis, it was performed an *in vitro* tubulin polymerization assay in the presence of compound **6-a** and observed that this naphthylchalcone led to an inhibition of tubulin assembly. Colchicine was used as positive control of inhibition of tubulin polymerization (Fig. 4C).

2.2.3. Naphthylchalcone **6-a** induces caspase activation, poly(ADP-ribose) polymerase cleavage and cytochrome *c* release in human leukaemia U-937 cells

To determine whether the mechanism of cell death triggered by the naphthylchalcone was associated with caspase activation, U-937 cells were treated with 10 μM **6-a** and cell lysates were assayed for cleavage of specific tetrapeptide substrates DEVD-pNA, IETD-pNA and LEHD-pNA for caspase-3/7, caspase-8 and caspase-9, respectively. As shown, a 4-

fold increase in caspase-3/7 and caspase-9 activities and a 2-fold increase in caspase-8 activity was observed in treated cells with respect to control (Fig. 5A). Caspases processing was also analyzed by Western blot using specific antibodies. Treatment with the naphthylchalcone induced processing of the initiator caspases, caspase-8 and -9 which was detected as a decrease of the proenzyme, while in the case of caspase-3 the fragments of 18–20 kDa resulting of the proenzyme cleavage were detected (Fig. 5B). In accordance with caspase-3 activation, the DNA repair enzyme poly(ADP-ribose)polymerase was cleaved after treatment with the naphthylchalcone, generating the 85 kDa fragment (Fig. 5C). In addition, it was analyzed the mitochondrial cytochrome *c* release since this is a key point in apoptotic signalling. To this end, cells were treated with **6-a** for 24 h and cytosolic fractions were prepared and analyzed by Western blot. The results showed a significant increase of cytochrome *c* in the cytosol (Fig. 5C). In these experiments etoposide was included as a positive control.

To confirm that the apoptosis triggered by the naphthylchalcone was dependent on caspase activation, we used the pan caspase inhibitor z-VAD-fmk. Pretreatment with this general caspase inhibitor mostly blocked apoptosis, indicating that cell death is dependent on caspase activation (Fig. 6A). To determine which specific caspase was determining in the mechanism of cell death we used selective permeable caspase inhibitors, including z-DEVD-fmk, z-IETD-fmk and z-LEHD-fmk as caspase-3/7, -8 and -9 inhibitors, respectively. As shown in Fig. 6B,

Table 1

Products produced via the mechanism presented in Scheme 1

Chalcone - 6

Flavanone - 7

Entry	Product	R1	R2	R3	R4	R5	Conditions	pH ^c	Yield %
6-a	OMe	H	H	H	H	H	Aldehyde ^a (1.0 eq), Ca(OH) ₂ (1.0 eq.), KOH (1.0 eq.), MeOH 50 (v/w), 6 h at 64 °C	2	66
6-b	OMe	H	H	H	H	OMe	Aldehyde (1.65 eq), NaOH (3.0 eq), EtOH 50 (v/w), 16 h at 20–25 °C	3	65
6-c	H	H	OMe	H	H	H	Aldehyde (1.1 eq), KOH (2.5 eq.), EtOH (50 v/w), 1 h at –5/–10 °C plus 16 h at –5/–10 °C	2	41
6-d	H	OMe	H	H	H	H	Aldehyde (1.1 eq), KOH (2.5 eq.) / H ₂ O (2 v/w) EtOH 50 (v/w), 1 h at –5/–10 °C plus 16 h at 20–25 °C	3	34
6-e	H	OMe	H	H	OMe	OMe	Aldehyde (1.5 eq), KOH (1.0 eq.) PEG-200 (25 v/w), 1 h at 50–60 °C	2	64
6-f	OMe	H	OMe	H	OMe	OMe	Aldehyde (1.5 eq), NaOH (2.5 eq.) H ₂ O (2 v/w) EtOH 50 (v/w), 16 h at 20–25 °C	8	30
6-g	H	CH ₃	H	CH ₃	OMe	OMe	Aldehyde (1.65 eq), NaOH (3.0 eq.), EtOH (50 v/w), 16 h at 20–25 °C	2	30
6-h	H	OH	H	H	OMe	OMe	Aldehyde (1.1 eq), KOH (2.5 eq.), H ₂ O (2 v/w), EtOH (50 v/w), 16 h at 20–25 °C	2	49
7-a ^b	H	OH	H	H	OMe	OMe	Glacial AcOH, 72 h at reflux temperature	–	34

^a Number of aldehyde equivalents.^b This flavanone was prepared from the naphthyl-chalcone **6-h**.^c pH obtained during the workup and after the adjustment with concentrated HCl.

Table 2

Effects of Synthetic Compounds on the Growth of Human U-937 Leukaemia Cells.

IC ₅₀ (μM)								
6-a	6-b	6-c	6-d	6-e	6-f	6-g	7-a	Etoposide
4.7 ± 0.5	9.0 ± 1.8	22.8 ± 4.0	14.5 ± 2.6	23.8 ± 2.5	>30	21.8 ± 2.7	22.0 ± 2.4	2.1 ± 0.4

Cells were cultured for 72 h in presence of specified compounds and the IC₅₀ (50% inhibition of cell growth) values were calculated from dose-response curves using a colorimetric MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay. Etoposide was included as a positive control. The data shown represent means ± SE of three independent experiments with three determinations in each.

Table 3

Effect of different durations of treatment with naphthylchalcone **6-a** on cell cycle phase distribution of U-937 cells.

		% SubG ₁	% G ₁	% S	% G _{2-M}
6 h	Control	0.7 ± 0.1	52.4 ± 1.7	28.5 ± 0.2	17.2 ± 1.5
	6-a	14.3 ± 2.1*	18.3 ± 0.2*	35.8 ± 0.4*	30.2 ± 0.1*
12 h	Control	2.4 ± 0.3	52.9 ± 3.0	24.4 ± 0.3	19.1 ± 3.0
	6-a	18.8 ± 0.4*	12.2 ± 1.5*	33.9 ± 0.7*	34.1 ± 1.5*
24 h	Control	2.0 ± 0.2	56.5 ± 2.3	25.1 ± 0.4	14.9 ± 1.3
	6-a	34.1 ± 2.2*	25.4 ± 1.2*	25.0 ± 2.5	14.8 ± 0.9

Cells were cultured with 10 μM **6-a** for the indicated period of times and the cell cycle phase distribution was determined by flow cytometry. The values are means ± S.E. of two independent experiments with two determinations in each. Asterisks indicate a significant difference ($P < 0.05$) compared with the corresponding controls.

these specific inhibitors blocked significantly apoptosis induction, being the selective caspase-3/7 inhibitor the most potent in blocking cell death.

To determine whether the mitogen-activated protein kinase and the phosphoinositide 3-kinase pathways are involved in the mechanism of cell death triggered by this specific naphthylchalcone, the effects of specific inhibitors were evaluated (Fig. 6C). Inhibition of phosphoinositide 3-kinase using LY294002 was found to suppress partially the increase in the percentage of hypodiploid cells induced by **6-a**. The blockage of phosphoinositide 3-kinase pathway with LY294002 (20 μM) decreased the percentage of apoptotic cells from 31.7 ± 4.0% in **6-a**-treated cells to 21.2 ± 1.0% in the combination group (LY294002 + **6-a**).

Inhibition of p38^{MAPK} with the inhibitor SB 203,580 (2 μM) or the

inhibition of MEK1/2 using U0126 or PD98059 did not affect the rate of naphthylchalcone-induced apoptosis. These results suggest that activation of p38^{MAPK} or the ERK1/2 is not involved in chalcone-induced cell death (Fig. 6C). However, inhibition of *c-jun* N-terminal kinases / stress-activated protein kinases (JNK/SAPK) with SP600125 was found to suppress in great part the increase in the percentage of hypodiploid cells induced by the naphthylchalcone. The percentage of apoptotic cells decreased from 31.7 ± 4.0% in **6-a**-treated cells to 11.0 ± 2.0% in the combination group (SP600125 + **6-a**) (Fig. 6C). It was also explored the effects of the naphthylchalcone **6-a** on the activation of JNK/SAPK. Incubation of U-937 cells with **6-a** leads to phosphorylation of JNK/SAPK (Fig. 6D).

These results suggest that activation of phosphoinositide 3-kinase and JNK/SAPK is involved in chalcone-induced apoptosis.

3. Conclusion

We optimise the conditions to prepare pure 2'-hydroxychalcones (naphthylchalcones) with methoxy and methyl substitution in phenyl ring A, without the use of chromatography. Compound **6-h**, which has a hydroxyl group at 5' position gave rise to secondary reactions, being necessary to purify it by column chromatography. In this case, the reaction should be carried out starting from an acetophenone with the hydroxyl group, at 5' position, protected.

During the preparation of the naphthylchalcones presented, it was always observed a small formation of the corresponding naphthylflavanone. We observed that the amount of naphthylflavanone formed, was temperature and reaction time dependent. High temperatures and long reaction times favoured the cyclization of naphthylchalcone in naphthylflavanone. However, the work-up developed purged the

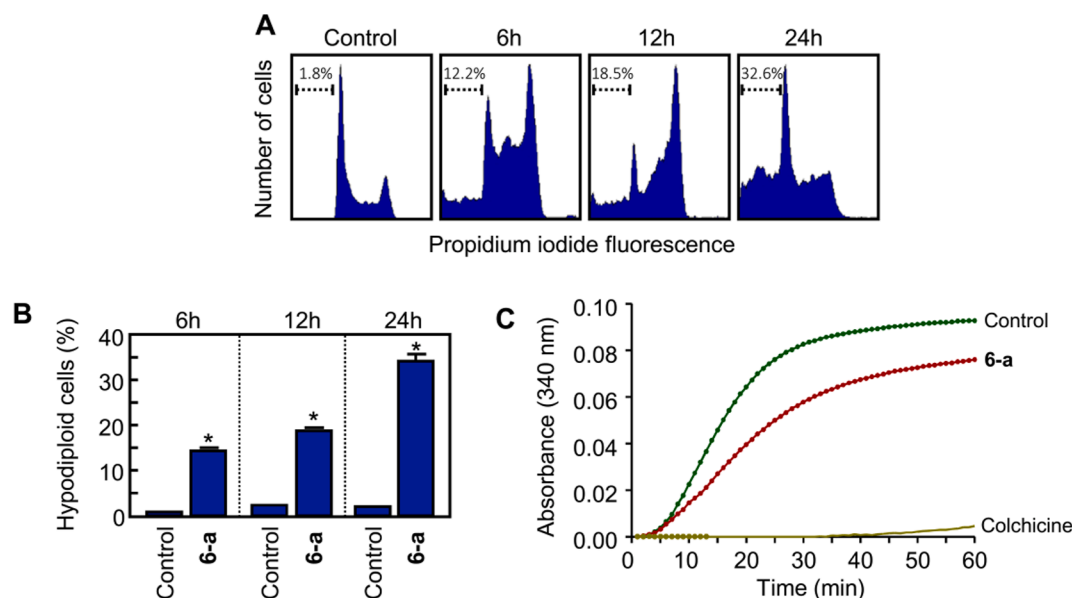


Fig. 4. (A) Cell cycle phase distribution determined by flow cytometry after propidium iodide staining. (B) U-937 cells were treated with 10 μ M 6-a and the percentage of hypodiploid cells was determined as in (A). * $P < 0.05$, significantly different from control. (C) Naphthylchalcone 6-a blocks the tubulin polymerization *in vitro*. Purified tubulin protein in a reaction buffer was incubated at 37 °C in the absence (control) or in the presence of colchicine (5 μ M) or the naphthylchalcone 6-a (30 μ M) and the absorbance at 340 nm was measured in a microplate reader.

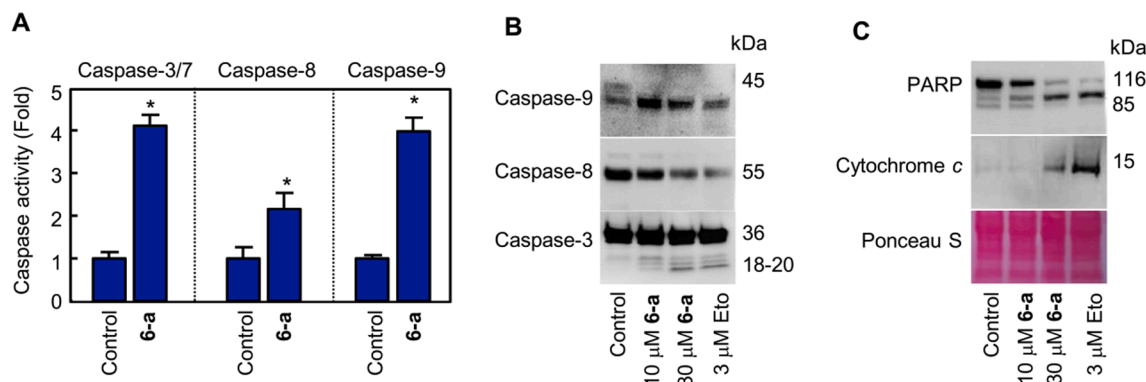


Fig. 5. Involvement of caspases in the mechanism of cell death triggered by 6-a in U-937 cells. (A) Cells were treated with 6-a for 24 h and cell lysates were assayed for caspase-3/7, -8 and -9 activities using colorimetric substrates. Results are expressed as *n*-fold increases in activity compared with control. Bars represent the means $\pm$ SE of two independent experiments each performed in triplicate. * $P < 0.05$, significantly different from control. (B) Immunoblotting for the cleavage of caspases. (C) Western blot of PARP [poly(ADP-ribose)polymerase] cleavage and cytochrome *c* release after 24 h of treatment with the specified compounds. Whole cell lysates or cytosolic fractions - in the case of cytochrome *c* - were probed with antibodies raised against the indicated proteins. Equal protein loading in the cytosolic fraction was controlled by staining the membrane with Ponceau S (a representative section of the stained membrane is shown). Etoposide (Eto) was included as a positive control.

naphthylflavanones formed and produced high quality chalcones.

In conclusion, the structure-activity relationship of naphthylchalcones against human leukaemia cells reveals that the major determining in cytotoxicity is the presence of only one methoxy group in position 6 of the A ring. The most cytotoxic compound induces cell death by activation and processing of caspases and apoptosis was mostly blocked by a pan-caspase inhibitor as well as the selective caspase-3/7 inhibitor. In addition, inhibition studies show that cell death triggered by this specific naphthylchalcone is modulated by the mitogen activated protein kinase and the phosphoinositide 3-kinase pathways. These studies suggest the potential of this compound or derivatives in the development of new anti-leukaemia drugs.

4. Experimental section

The acetophenones were synthesized but can be commercially

obtained (e.g. Alfa-Aesar). The aldehydes (2-naphthaldehyde and 6-methoxy-2-naphthaldehyde) were commercially obtained from Sigma-Aldrich (Saint Louis, MO, USA). All the naphthylchalcones were synthesized by base catalyzed Claisen-Schmidt condensation, from selected naphthaldehydes and acetophenones (Scheme 1). Melting points of newly compounds were determined in a Buchi Melting Point B-540 apparatus. The purity of all compounds was confirmed by NMR and HRMS analyses. ^1H NMR spectra were obtained at 400 MHz in CDCl_3 or $\text{DMSO}-d_6$ with chemical shift values (δ) in ppm downfield from tetramethylsilane and ^{13}C NMR spectra were obtained at 100.61 MHz. Assignments are supported by 2D correlation NMR studies. The reactions were monitored by Waters High Performance Liquid Chromatographer (HPLC) model 600, equipped with auto sampler w717 plus and Photo Diode Array (PDA) detector W996 and by analytical thin layer chromatography (TLC) on silica gel plates (aluminium-backed Silica Gel Merck 60 F254) using as eluent a mixture of hexane/ ethyl acetate. The

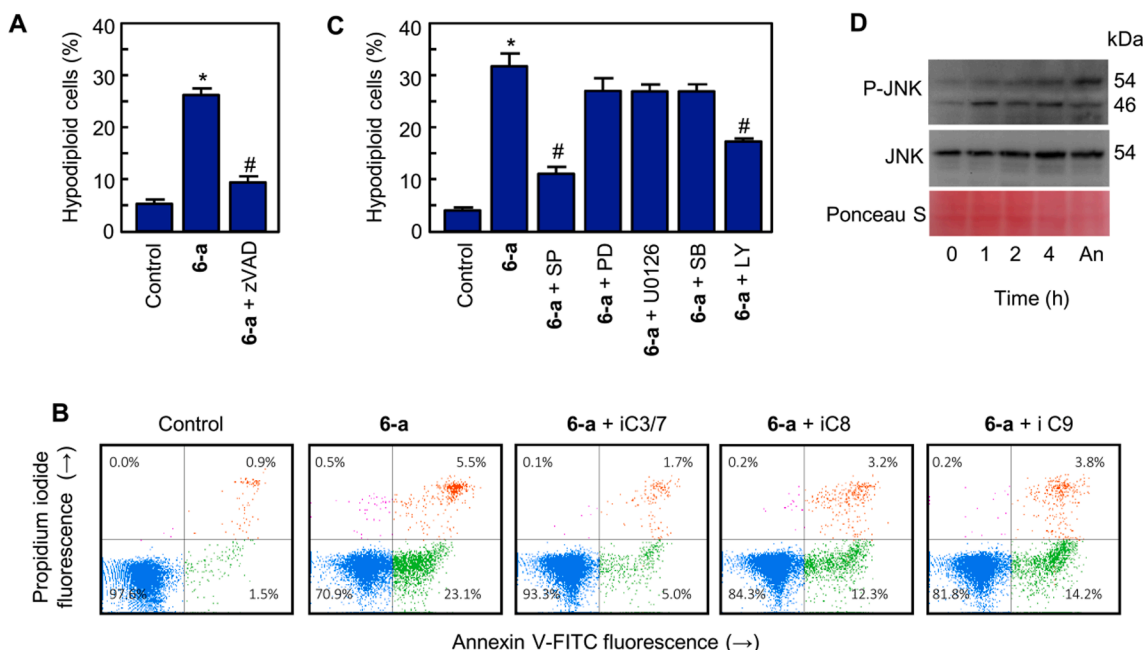


Fig. 6. Effect of the general caspase inhibitor and specific inhibitors on apoptosis induction triggered by compound **6-a** in U-937 cells. (A) Cells were treated with **6-a** or in combination with the pan-caspase inhibitor z-VAD-fmk (100 μ M) and the percentage of hypodiploid cells was determined by flow cytometry after propidium iodide staining. * $P < 0.05$, significantly different from control. # indicates $P < 0.05$ for comparison with compound **6-a** treatment alone. (B) Cells were preincubated in the absence or presence of the selective caspase inhibitors against caspase-3/7 (iC3/7, 50 μ M z-DEVD-fmk), caspase-8 (iC8, 50 μ M z-IETD-fmk) and caspase-9 (iC9, 50 μ M z-LEHD-fmk) for 1 h and then followed by treatment with 10 μ M **6-a** for 24 h and apoptosis was determined by flow cytometry analysis of annexin V-FITC and propidium iodide-stained U-937 cells. Representative data from two separate experiments are shown. (C) Cells were pretreated with the JNK inhibitor SP600125 (SP, 10 μ M), the MEK 1/2 inhibitors PD98059 (PD, 10 μ M), U0126 (10 μ M), the p38^{MAPK} inhibitor SB 203,580 (SB, 2 μ M) or the PI3K inhibitor LY294002 (LY, 20 μ M) and then with **6-a** for 24 h and the percentage of hypodiploid cells was determined by flow cytometry after propidium iodide staining. Bars represent means $\pm$ SE of two independent experiments performed in triplicate. *indicates $P < 0.05$ for comparison with untreated control. # indicates $P < 0.05$ for comparison with compound **6-a** treatment alone. (D) Representative Western blots show the time-dependent phosphorylation of JNK/SAPK by naphthylchalcone **6-a**. Cells were incubated with 30 μ M **6-a** for the time periods shown. Protein extracts were prepared and analysed on western blots probed with specific antibodies. As a positive control we used anisomycin (An, 25 ng/ml for 4 h). Equal protein loading was controlled by staining the membrane with Ponceau S (a representative section of the stained membrane is shown).

IR spectra were recorded using a Thermo Nicolet 6700 FTIR spectrophotometer (ATR, cm⁻¹). Reagents and solvents were used without any additional purification.

4.1. Procedure for the preparation of compound **5-b**

A solution of 2'-hydroxy-6'-methoxyacetophenone (2.0 g; 12.04 mmol) and 2-naphthaldehyde (2.07 g; 1.1 eq) in degassed EtOH (100 mL) was cooled to a temperature between 0 °C and -5 °C and stirred for during 30 min at the same temperature. KOH (1.69 g; 2.5 eq/ H₂O 10 mL) solution, previously degassed, was added. The resulting mixture was then stirred for 1 h at a temperature between -5 °C and -10 °C and 16 h at room temperature. Crushed ice (100 g) was added and the mixture pH was adjusted to ~ 3 with HCl and then stirred for 1 h. The solid was isolated by filtration, washed with water, recrystallized from ethanol (50–60 °C) and dried under vacuum at a temperature below 40 °C. An off-white solid was obtained (1.76 g, 48% yield). m.p. 137.6 °C; ¹H NMR (400 MHz, CDCl₃) δ 12.99 (s, 1H, OH-C-2'), 7.90 (s, 1H, H-1), 7.88 – 7.82 (m, 3H, H-4, H-5, H-8), 7.53 (dd, J = 8.5, 1.6 Hz, 1H, H-3), 7.51 – 7.45 (m, 2H, H-6, H-7), 7.37 (t, J = 8.3 Hz, 1H, H-4'), 6.60 (dd, J = 8.4, 0.7 Hz, 1H, H-5'), 6.37 (dd, J = 8.4, 0.7 Hz, 1H, H-3'), 5.49 (dt, J = 8.7, 2.9 Hz, 1H, CH), 3.81 (s, 3H, OCH₃), 3.59 (dd, J = 18.5, 3.1 Hz, 1H, CH₂), 3.51 (dd, J = 18.5, 8.9 Hz, 1H, CH₂), 3.44 (d, J = 3.1 Hz, 1H, OH-CH). ¹³C NMR (101 MHz, CDCl₃) δ 206.34 (C=O), 164.85 (C-2'), 161.54 (C-6'), 140.68 (C_{quat}), 136.67 (C-4'), 133.39 (C_{quat}), 132.94 (C_{quat}), 128.29, 128.04, 127.69 (C-4, C-5, C-8), 126.17, 125.87 (C-6, C-7), 124.55 (C-1), 124.10 (C-3), 111.23 (C_{quat}), 111.03 (C-5'), 101.33 (C-3'), 70.20 (CH), 55.74 (OCH₃), 53.76 (CH₂). IR (ATR, cm⁻¹): 3058 (C–H, Ar), 1597 (C=O), 1505, 1450 (C=C, Ar), 1214 (C–O) 1180,

1119 (OCH₃).

4.2. General procedure for the preparation of naphthylchalcones

The chalcones were synthesized by mixing the aldehyde with the acetophenone in adequate solvent (ethanol or methanol or PEG-200) with an alkaline base [NaOH or KOH or Ca(OH)₂]. The mixture was vigorously stirred at the temperature and time reported in Table 1. The progress of the reaction was monitored by TLC or by HPLC. After completion of the reaction, crushed ice (50 w/w) was added and the mixture pH was adjusted with concentrated HCl and then stirred for 1 h. The solid precipitated was filtered and washed with water. Recrystallization from ethanol afforded pure chalcone.

Compounds **6-c** and **6-e** did not precipitate upon pH adjustment, instead, the mixture was extracted with dichloromethane (3x 12.5 v/v). The combined organic phase was washed with brine (12.5 v/v), dried over anhydrous MgSO₄ and concentrated to dryness. The residue obtained was recrystallized from ethanol affording a pure chalcone.

4.2.1. 1-(2-hydroxy-6-methoxyphenyl)-3-(naphthalen-2-yl)prop-2-en-1-one (**6-a**)

Yield: 66%; yellowish powder; m.p. 121 °C. ¹H NMR (400 MHz, DMSO) δ 10.37 (s, 1H, OH-C-2'), 8.19 (s, 1H, H-1), 8.00 – 7.87 (m, 4H, H-3, H-4, H-5, H-8), 7.62 – 7.54 (m, 2H, H-6, H-7), 7.49 (d, J = 16.1 Hz, 1H, H- β), 7.29 (m, 2H, H- α , H-4'), 6.61 (d, J = 8.3 Hz, 1H, H-5'), 6.58 (d, J = 8.2 Hz, 1H, H-3'), 3.77 (s, 3H, OCH₃). ¹³C NMR (101 MHz, DMSO) δ 194.41 (C=O), 158.03 (C_{quat}), 156.81 (C_{quat}), 143.86 (C- β), 133.81 (C_{quat}), 132.92 (C_{quat}), 131.98 (C_{quat}), 131.67, 128.73 (C- α , C-4'), 130.43 (C-1), 128.59, 128.52, 127.66, 127.43, 126.76 (C-6, C-7),

123.88, 115.80 (C_{quat}), 108.96 (C-3'), 102.29 (C-5'), 55.76 (OCH₃). IR (ATR, cm⁻¹): 3058 (C—H, Ar), 1597 (C=O), 1505 (C=C, Ar), 1450 (C=C, Ar), 1214 (C—O), 1180, 1119 (OCH₃). HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd for C₂₀H₁₆O₃ [M]⁺: 304.1099; found for 334.1173.

4.2.2. 1-(2-hydroxy-6-methoxyphenyl)-3-(6-methoxynaphthalen-2-yl)prop-2-en-1-one (6-b)

Yield: 65%; dark yellow powder; m.p. 130.5 °C. ¹H RMN (400 MHz, CDCl₃) δ 13.22 (s, 1H, OH-C-2'), 7.89 (m, 6H, H-α, H-β, H-1, H-4, H-7, H-8), 7.37 (t, *J* = 8.3 Hz, 1H, H-4'), 7.18 (dd, *J* = 8.86, 3.2 Hz, 1H, H-3), 7.15 (s, 1H, H-5), 6.63 (d, *J* = 8.3 Hz, 1H, H-5'), 6.45 (d, *J* = 8.3 Hz, 1H, H-3'), 3.98 (s, 1H, OCH₃), 3.95 (s, 1H, OCH₃). ¹³C RMN (101 MHz, CDCl₃) δ 194.38 (C=O), 164.88 (C-2'), 160.99 (C-6'), 158.93 (C-6), 143.56 (C-β), 135.81 (C_{quat}), 135.78 (C-4'), 130.74 (C_{quat}), 130.61, 130.26, 127.49, 126.64, 124.46 (H-α, H-1, H-4, H-7, H-8) 128.80 (C_{quat}), 119.45 (C-3), 106.06 (C-5), 112.06 (C_{quat}), 111.0, 101.58 (C-3', C-5'), 56.02 (OCH₃), 55.42 (OCH₃). IR (ATR, cm⁻¹): 3059 (C—H, Ar), 1626 (C=O), 1505, 1451 (C=C, Ar), 1172 (OCH₃). HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd for C₂₁H₁₈O₄ 334.1205; found for 334.1282.

4.2.3. 1-(2-hydroxy-4-methoxyphenyl)-3-(naphthalen-2-yl)prop-2-en-1-one (6-c)

Yield: 41%; crystalline dark yellow powder; m.p. 146.8 °C; ¹H NMR (400 MHz, CDCl₃) δ 13.49 (s, 1H, OH-C-2'), 8.09–8.00 (m, 2H, H-1, H-β), 7.93–7.82 (m, 4H, H-4, H-5, H-6', H-8), 7.80 (d, *J* = 8.6 Hz, 1H, H-3), 7.69 (d, *J* = 15.5 Hz, 1H, H-α), 7.57–7.50 (m, 2H, H-6, H-7), 6.54–6.47 (m, 2H, H-5', H-3'), 3.87 (s, 3H, OCH₃). ¹³C NMR (101 MHz, CDCl₃) δ 191.80 (C=O), 166.77 (C_{quat}), 166.26 (C_{quat}), 144.51 (C-β), 134.44 (C_{quat}), 133.38 (C_{quat}), 132.30 (C_{quat}), 130.81 (C-1), 128.79, 131.28, 128.71, 127.83 (C-4, C-5, C-6', C-8), 127.49, 126.84 (C-6, C-7), 123.71 (C-3), 120.44 (C-α), 114.18 (C_{quat}), 107.82, 101.10 (C-3', C-5'), 55.63 (OCH₃). IR (ATR, cm⁻¹): 3062 (C—H, Ar), 1632 (C=O), 1560, 1464 (CH = CH, Ar), 1215 (C—O), 1178 (OCH₃). HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd para C₂₀H₁₆O₃ 304.1099; found for 304.1174.

4.2.4. 1-(2-hydroxy-5-methoxyphenyl)-3-(naphthalen-2-yl)prop-2-en-1-one (6-d)

Yield: 34%; Orange powder. m.p. 118.2 °C; ¹H NMR (400 MHz, CDCl₃) δ 12.42 (s, 1H, OH-C-2'), 8.08 (d, *J* = 15.5 Hz, 1H, H-β), 8.06 (s, 1H, H-1) 7.92–7.84 (m, 3H, H-4, H-5, H-8), 7.80 (d, *J* = 8.6 Hz, 1H, H-3), 7.70 (d, *J* = 15.4 Hz, 1H, H-α), 7.60–7.50 (m, 2H, H-6, H-7), 7.42 (d, *J* = 3.0 Hz, 1H, H-6'), 7.16 (dd, *J* = 9.0, 3.0 Hz, 1H, H-4'), 6.99 (d, *J* = 9.0 Hz, 1H, H-3'), 3.86 (s, 3H, OCH₃). ¹³C NMR (101 MHz, CDCl₃) δ 193.32 (C=O), 157.99 (C_{quat}), 151.75 (C_{quat}), 145.71 (C-β), 134.58 (C_{quat}), 133.35 (C_{quat}), 132.08 (C_{quat}), 131.16 (C-1), 128.87, 128.77, 127.67 (C-4, C-5, C-8), 127.87, 126.92 (C-6, C-7), 123.84 (C-4'), 123.67 (C-3), 120.21 (C-α), 119.74 (C_{quat}), 119.39 (C-3'), 113.07 (C-6'), 56.21 (OCH₃). IR (ATR, cm⁻¹): 3057 (C—H, Ar), 1641 (C=O), 1565, 1493 (C=C, Ar), 1223 (C—O), 1171 (—OCH₃). HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd C₂₀H₁₆O₃ 304.1099; found for 304.1171.

4.2.5. 1-(2-hydroxy-5-methoxyphenyl)-3-(6-methoxynaphthalen-2-yl)prop-2-en-1-one (6-e)

Yield: 64%; orange powder; m.p. 141.5 °C; ¹H NMR (400 MHz, CDCl₃) δ 12.47 (s, 1H, OH-C-2'), 8.07 (d, *J* = 15.4 Hz, 1H, H-β), 8.00 (s, 1H, H-1), 7.79 (m, 4H, H-4, H-5, H-7, H-8), 7.67 (d, *J* = 15.4 Hz, 1H, H-α), 7.42 (d, *J* = 3.0 Hz, 1H, H-6'), 7.16 (dd, *J* = 9.0, 3.0 Hz, 1H, H-4'), 6.99 (d, *J* = 9.0 Hz, 1H, H-3'), 3.95 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃). ¹³C NMR (101 MHz, CDCl₃) δ 193.32 (C=O), 159.21 (C_{quat}), 157.96 (C_{quat}), 151.72 (C_{quat}), 146.04 (C-β), 136.15 (C_{quat}), 131.05 (C-1), 130.37, 127.65, 124.43 (H-4, H-5, H-7, H-8), 129.97 (C_{quat}), 128.74 (C_{quat}), 123.68 (C-4'), 119.81 (C_{quat}), 119.68, 119.34 (C-3'), 119.04 (C-α), 113.06 (C-6'), 56.21 (OCH₃), 55.44 (OCH₃). IR (ATR, cm⁻¹): 3059 (C—H, Ar), 1637 (C=O), 1560, 1481 (C=C, Ar), 1257 (C—O), 1171 (OCH₃). HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd para C₂₁H₁₈O₄ 334.1205; found for 334.1280.

4.2.6. 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-(6-methoxynaphthalen-2-yl)prop-2-en-1-one (6-f)

Yield: 30%; yellow powder; m.p. 153.8 °C; ¹H NMR (400 MHz, CDCl₃) δ 14.41 (s, 1H, OH-C-2'), 7.99–7.69 (m, 6H, H-α, H-β, H-1, H-4, H-7, H-8), 7.17 (dd, *J* = 8.9, 1.3 Hz, 1H, H-3), 7.14 (s, 1H, H-5), 6.12 (s, 1H, H-5'), 5.98 (s, 1H, H-3'), 3.94 (s, 6H, OCH₃), 3.84 (s, 3H, OCH₃). ¹³C NMR (101 MHz, CDCl₃) δ 192.58 (C=O), 168.44 (C_{quat}), 166.14 (C_{quat}), 162.51 (C_{quat}), 158.82 (C_{quat}), 142.92, 130.32, 130.20, 127.43, 126.57, 124.48, (C-α, C-β, H-1, C-4, H-7, H-8), 135.67 (C_{quat}), 130.97 (C_{quat}), 128.82 (C_{quat}), 119.38 (C-3), 106.43 (C_{quat}), 106.05 (C-5), 93.83 (C-3'), 91.29 (C-5'), 55.91 (OCH₃), 55.60 (OCH₃), 55.41 (OCH₃). IR (ATR, cm⁻¹): 3055 (C—H, Ar), 1622 (C=O), 1556, 1480 (C=C, Ar), 1216 (C—O), 1177 (—O-CH₃). HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd para C₂₂H₂₀O₅ 364.1311; found for 364.1388.

4.2.7. 1-(2-hydroxy-3,5-dimethylphenyl)-3-(6-methoxynaphthalen-2-yl)prop-2-en-1-one (6-g)

Yield: 30%; yellowish powder: m.p. 144.1–145.6 °C; ¹H NMR (400 MHz, CDCl₃) δ 13.07 (s, 1H, OH em C-2'), 8.04 (d, *J* = 15.4 Hz, 1H, H-β), 7.99 (s, 1H, H-1), 7.79 (m, 3H, H-3, H-4, H-8), 7.73 (d, *J* = 15.4 Hz, 1H, H-α), 7.59 (s, 1H, H-4' ou H-6'), 7.21 (s, 1H, H-4' ou H-6'), 7.19 (dd, *J* = 9.0, 2.6 Hz, 1H, H-7), 7.15 (d, *J* = 2.6 Hz, 1H, H-5), 3.95 (s, 3H, OCH₃), 2.34 (s, 3H, CH₃), 2.27 (s, 3H, CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 193.79 (C=O), 160.09 (C_{quat}), 159.10 (C_{quat}), 145.36 (C-β), 138.35 (C-4' ou C-6'), 130.84 (C-1), 130.33, 127.58, 124.49 (C-3, C-4, C-8), 130.15 (C_{quat}), 128.76 (C_{quat}), 127.30 (C_{quat}), 127.05 (C_{quat}), 126.84 (C-4' ou C-6'), 119.59 (C-α ou C-7), 119.49 (C-α ou C-7), 119.09 (C_{quat}), 106.07 (C-5), 55.42 (OCH₃), 20.66 (CH₃), 15.58 (CH₃). IR (ATR, cm⁻¹): 3059 (C—H, Ar), 1626 (C=O), 1560, 1479 (C=C, Ar), 1172 (OCH₃). HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd para C₂₂H₂₀O₃ 332.1412; found for 332.1484.

4.3. Preparation of naphthylflavanone

4.3.1. 6-Hydroxy-2-(6-methoxynaphthalen-2-yl)chroman-4-one (6-h → 7-a)

To a solution of 2', 5'-dihydroxyacetophenone (2.0 g; 13.15 mmol) and 6-methoxynaphthaldehyde (4.04 g; 1.65 eq) in EtOH (100 mL) was added NaOH (1.9 g; 3.60 eq) and the mixture was stirred for 16 h at room temperature. The resulting mixture was added to ice (150 g), acidified to pH ~ 2 and extracted with DCM (3x 25 mL). The combined organic phase was dried with anhydrous MgSO₄ and concentrated to dryness. The residue obtained was purified by column chromatography using as eluent EtOAc/heptane 8:2. 1-(2,5-Dihydroxyphenyl) –3- (6-methoxynaphthalene-2-yl) prop-2-en-1-one was obtained as a light brown solid. Part of this solid (0.2 g; 1.31 mmol) was added to glacial acetic acid (15 mL). The mixture was refluxed for 72 h. The resulting mixture was added to water (15 mL) and extracted with ethyl ether (3x 15 mL). The combined organic phase was washed with brine, dried with MgSO₄ anhydrous and concentrated to dryness. The residue was purified by column chromatography using as eluent a mixture of EtOAc/heptane in a proportion of 8:2. Yield: 34%; orange powder; m.p. 211 °C; ¹H NMR (400 MHz, DMSO) δ 9.46 (s, 1H, C=O), 7.97 (s, 1H, H-1'), 7.87 (dd, *J* = 8.6, 6.1 Hz, 2H, H-4', H-8'), 7.65 (dd, *J* = 8.6, 1.3 Hz, 1H, H-3'), 7.36 (d, *J* = 2.3 Hz, 1H, H-5'), 7.21 (dd, *J* = 9.0, 2.5 Hz, 1H, H-7'), 7.16 (d, *J* = 3.0 Hz, 1H, H-5), 7.07 (dd, *J* = 8.9, 3.0 Hz, 1H, H-7), 7.00 (d, *J* = 8.8 Hz, 1H, H-8), 5.68 (dd, *J* = 13.0, 2.6 Hz, 1H, CH), 3.90 (s, 3H, OCH₃), 3.29 (dd, *J* = 16.9, 13.1 Hz, 1H, CH₂), 2.86 (dd, *J* = 16.9, 2.8 Hz, 1H, CH₂). ¹³C NMR (101 MHz, DMSO) δ 191.78 (C=O), 157.59 (C_{quat}), 154.43 (C_{quat}), 151.58 (C_{quat}), 134.22 (C_{quat}), 134.15 (C_{quat}), 129.51, 127.01 (C-4', C-8'), 127.99 (C_{quat}), 125.39 (C-1', C-3'), 124.96 (C-7), 120.86 (C_{quat}), 119.01, 118.95 (C-7', C-8), 109.92 (C-5), 105.83 (C-5'), 78.88 (CH), 55.18 (OCH₃), 43.62 (CH₂). IR (ATR, cm⁻¹): 2948 (C—H, Ar), 1663 (C=O), 1472 (CH = CH, Ar), 1224 (C—O), 1173 (—OCH₃). HRMS (ESI-TOF) *m/z*: [M + H]⁺ Calcd C₂₀H₁₆O₄ 320.1049; found for 320.1115.

4.4. Biological activity

4.4.1. Cell culture and cytotoxicity assays

U-937 cells were from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin, incubated at 37 °C in a humidified atmosphere containing 5% CO₂ as described.[24] The doubling time of these cells were 30 h and the viability was always greater than 95% in all experiments as determined by the trypan blue exclusion method.

Naphthylchalcones and the flavanone **7a** were dissolved in DMSO and kept under dark conditions at 25 °C. The effects on cell viability was evaluated by colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described.[25] Before each experiment naphthylchalcones were dissolved in culture media at 37 °C and the final concentration of DMSO did not exceed 0.3% (v/v).

4.4.2. Quantification of hypodiploid cells and flow cytometry analysis of annexin V-FITC and propidium iodide-stained cells

Flow cytometric analysis of propidium iodide-stained cells was performed as previously described.[26] After treatments, cells were centrifuged at 500x g for 10 min, washed with cold PBS, fixed with ice-cold 75% ethanol and stored at -20 °C overnight. Samples were then centrifuged (500x g for 10 min at 4 °C), washed with PBS, resuspended in 200 µL of PBS containing 100 µg/mL RNase A and 50 µg/mL propidium iodide and incubated for 1 h in the dark. The DNA content was analyzed by flow cytometry with a BD FACSVerser™ cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometric analysis of annexin V-FITC and propidium iodide-stained cells was performed as described.[27]

4.4.3. In vitro tubulin polymerization assay

In vitro tubulin polymerization assays were performed with reagents as described by the manufacturer (Cytoskeleton Inc., Denver, CO, USA). Briefly, naphthylchalcone **6-a** was incubated with purified bovine tubulin in 80 mM PIPES buffer (pH 7.0) containing 10% glycerol, 1 mM GTP, 1 mM MgCl₂ and 1 mM EGTA at 37 °C, and the increase in absorbance was measured at 340 nm in a Beckman Coulter DTX880 microplate reader at 37 °C and recorded every 60 s for 60 min. Colchicine (5 µM) was used as positive control of inhibition of tubulin polymerization.

4.4.4. Assay of caspase activity

Caspase activity was determined by measuring proteolytic cleavage of the chromogenic substrates Ac-DEVD-pNA (for caspase-3 like protease activity), Ac-IETD-pNA (for caspase-8 activity) and Ac-LEHD-pNA (for caspase-9 activity) as previously described.[25]

4.4.5. Western blot analysis

Cells were harvested by centrifugation (500x g, 10 min, 4 °C) and pellets were resuspended in lysis buffer [20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 1% Triton X-100, 10 mM sodium fluoride, 2 mM EDTA, 2 mM tetrasodium pyrophosphate, 10% glycerol, 20 mM sodium β-glycerophosphate], with the protease inhibitors phenylmethylsulfonyl fluoride (PMSF, 1 mM), aprotinin, leupeptin, and pepstatin A (1 µg/mL each) and kept on ice during 15 min. Cells were sonicated on ice five times (5 s each, with intervals between each sonication of 5 s) with a Braun Labsonic 2000 microtip sonifier and centrifuged (11,000x g, 10 min, 4 °C). Bradford's method was used to determine protein concentration.

Subcellular fractionation was performed for the cytochrome c release. Briefly, cells were harvested as above and the pellets were resuspended in buffer [20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 0.1 mM

phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 µg/mL leupeptin, aprotinin, and pepstatin A] with 250 mM sucrose. The samples were incubated for 15 min on ice and lysed 10 times with a 22-gauge needle. The lysates were centrifuged (1,000x g, 5 min, 4 °C). The supernatants were centrifuged at 105,000x g for 45 min at 4 °C, and the resulting supernatant was used as the soluble cytosolic fraction.

The samples that were loaded in sodium dodecyl sulphate-polyacrylamide gel (from 7.5 to 15% depending on the molecular weight of interest) were prepared with the same amount of protein and boiled for 5 min. The proteins were transferred to a poly(vinylidene difluoride) membrane for 20 h at 20 V. The membrane was blocked with 10% nonfat milk in Tris-buffered saline [50 mM Tris-HCl (pH 7.4), 150 mM NaCl] containing 0.1% Tween-20 (TBST) for 1 h, followed by incubation with specific antibodies against caspase-3, caspase-8, caspase-9 and poly(ADP-ribose)polymerase overnight at 4 °C. The cytosolic fractions were analysed by immunoblotting with an specific antibody against cytochrome c. Membranes were washed three times with TBST and incubated for 1 h with the specific secondary antibody and the antigen-antibodies complexes were visualized by enhanced chemiluminescence using the manufacturer's protocol.

4.4.6. Statistical methods

Statistical differences between means were tested using (i) Student's *t*-test (two samples) or (ii) one-way analysis of variance (ANOVA) (3 or more samples) with Tukey's test used for *posteriori* pairwise comparisons of means. A significance level of *P* < 0.05 was used.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Professor Christopher David Maycock for its support and Hovione for funding this research. The NMR spectrometers used are part of the National NMR Facility, supported by Fundação para a Ciência e Tecnologia (RECI/BBB-BQB/0230/2012). This work was also partially supported by Fundação para a Ciência e a Tecnologia (FCT, Portugal), through funding UIDB/04567/2020 and UIDP/04567/2020.

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