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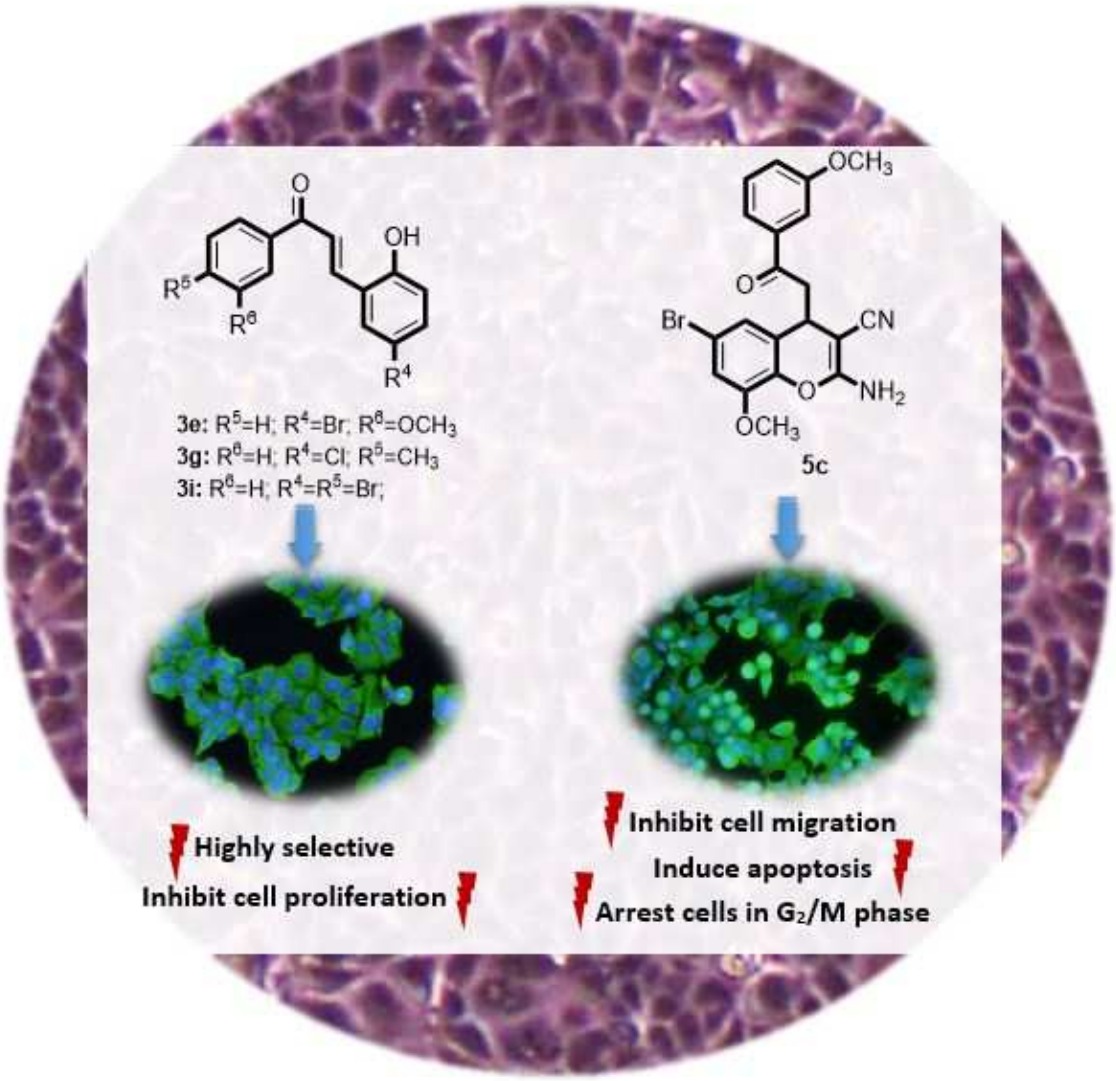
O
OH
R5
R6
R4
3e: R5=H; R4=Br; R6=OCH3
3g: R6=H; R4=Cl; R5=CH3
3i: R6=H; R4=R5=Br;
OCH3
O
Br
CN
O
NH2
OCH3
5c
Highly selective
Inhibit cell proliferation
Inhibit cell migration
Induce apoptosis
Arrest cells in G2/M phase

Exploitation of new chalcones and 4H-chromenes as agents for cancer treatment

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Anticancer activity
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Abstract

Chalcone and chromene derivatives were synthesized in good yield through simple and effective reactions using innocuous solvents such as water and ethanol and high yielding aldol condensations. Generally, the reactions were performed at room temperature, leading to the isolation of highly pure compounds. These compounds were tested on breast cancer cells (MCF-7 and Hs578T) and breast non-neoplastic cells (MCF-10A). After determination of IC_{50} value, specific assays were performed to analyze the potential of these compounds, and those bearing halogenated substituents presented enhanced activity comparing to methoxyl or methyl groups. More specifically, the bromine atom was often present in the bioactive molecules that proceeded to the final assays and showed to be promising candidates for further studies. The selected chromene acted as a cell migration inhibitory agent and triggered regulated cell death associated with G_2/M cell-arrest and microtubule destabilization. For chalcones, the results suggest an anti-proliferative activity. Also, results for combination-therapy potentiated the antitumor effect of doxorubicin and reduced cytotoxicity in MCF-10A cells.

1. Introduction

Cancer is a rather heterogeneous pathology related to defects in terms of the regulatory circuits that control cellular homeostasis, including proliferation, differentiation, cell migration and death and, due to this dynamic system, there is an explicit difficulty to reach an effective treatment [1, 2]. Breast cancer is the most frequent type of cancer in women worldwide and the fifth cause of death from cancer. Furthermore, with an estimated 1.67 million new cases, it is considered the second most common cancer in the world [3]. The treatment for breast cancer depends on the stage and grade of cancer and may include a combination of surgery and radiation, chemical, immunological and targeted therapies [4]. However, not all the cases of breast cancer have good prognosis leading to the importance of understanding the tumor heterogeneity and survival for the specific subtypes [5].

Chalcones and chromene derivatives have been studied for many years and several therapeutic applications were found including anti-HIV [6], antidiabetic [7], antibacterial [8], antifungal [9], anti-inflammatory [10, 11], anti-coagulant [12], antioxidant [10, 13], antidepressant [14, 15] and antileishmanial activity [16]. A general overview of recent works reveals an infinite search for naturally occurring compounds with anticancer activity [17, 18]. Additionally, newly synthetized molecules have proven their potential for cancer treatment. Crolibulin, a 4*H*-chromene, is currently in phase I/II of clinical trials on anaplastic thyroid cancer patients [19, 20]. Described as a tubulin inhibitor, its activity includes induction of apoptosis and disruption of neovascular endothelial cells with interruption of blood flow to the tumor.

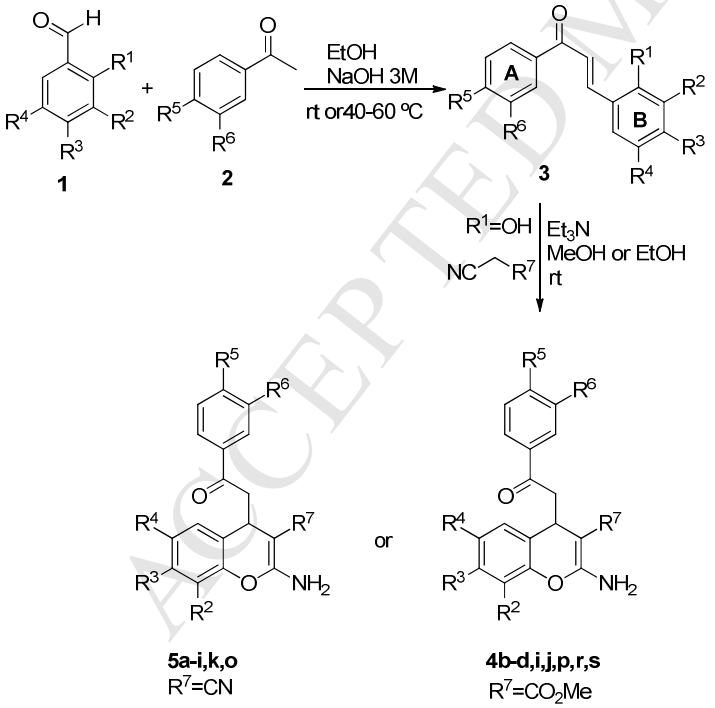
Further, many other chalcones and chromenes with promising anticancer effectiveness against a diversity of breast cancer cell lines have been identified and led to many synthetically inspired compounds with enhanced antitumor properties [21]. The mechanisms of action include triggering of apoptosis either via death receptor-mediated or mitochondrial-mediated pathways [22-24]. Other compounds are effective through cell-cycle arrest [25], estrogen receptor agonist and antagonist [26-28] degradation of Hsp90 client proteins [29], generation of reactive oxygen species (ROS) [30], inhibiting HIF-1 activation [31] or monoamine oxidase (MAO) A [32].

Since cancer is a disease that affects the population worldwide and chalcones and chromenes have already proven anticancer activity via a diversity of modes of action, this became an interesting research area [33]. Inspired by the important biological activity of these compounds, a series of chalcones and chromenes were synthesized using an eco-friendly methodology and a range of compounds were selected to assess their anticancer activity on breast cell lines.

2. Results and Discussion

2.1. Chemistry

Chalcone and chromene derivatives were synthesized as illustrated in Scheme 1. The synthesis of *α,β*-unsaturated carbonyl compounds **3** was performed under base-catalyzed aldol condensation conditions. The reaction of aromatic benzaldehydes **1** with acetophenones **2** occurred in EtOH and aqueous NaOH (3M), at room temperature or with gentle heating, leading to the isolation of the expected compounds **3** with yields between 46 and 100%.



Comp	R^1	R^2	R^3	R^4	R^5	R^6
3a	OH	OCH_3	H	Br	H	H
3b	OH	OCH_3	H	Br	Cl	H
3c	OH	OCH_3	H	Br	H	OCH_3
3d	OH	OCH_3	H	Br	Br	H
3e	OH	H	H	Br	H	OCH_3
3f	OH	F	H	F	H	H
3g	OH	H	H	Cl	CH_3	H
3h	OH	OCH_3	H	Br	CH_3	H
3i	OH	H	H	Br	Br	H
3j	OH	OCH_3	H	H	Cl	H
3k	OH	H	H	Br	CH_3	H
3l	OH	F	H	F	CH_3	H
3m	OH	H	H	Cl	H	OCH_3
3n	H	H	H	Br	CH_3	H
3o	OH	H	H	H	H	H
3p	OH	H	H	Br	H	H
3q	OH	H	H	Cl	Cl	H
3r	OH	H	H	Br	Cl	H
3s	OH	H	H	Cl	H	H
3t	OH	H	OCH_3	H	Cl	H

Scheme 1. Synthetic pathway for the synthesis of chalcones **3** and 4*H*-chromenes **4** and **5**.

In order to synthetize 4*H*-chromenes **4** and **5**, *α,β*-unsaturated carbonyl compounds **3** were combined with active methylene compounds (methyl cyanoacetate, R^7=CO_2Me or malononitrile, R^7=CN) (Scheme 1). Reactions were carried out in

methanol or ethanol and in the presence of triethylamine, leading to the expected products **4** and **5** by simple filtration in 12 to 94% yields.

2.2.Biological evaluation

2.2.1. *In vitro* cytotoxic activity

The 34 newly synthetized compounds **3**, **4** and **5** with good purity level were evaluated in an initial screening for their anticancer properties by the SRB assay, after 72 hours treatment. The first screening was performed on the Luminal-A subtype breast cancer cell line- MCF-7 - using two different concentrations (10 and 30 µM) for each compound (Tables 1 and 2 Supporting Information). By analysis of structure-activity, compounds with halogenated substituents presented better anticancer activity when compared to molecules substituted with methoxyl ($-OCH_3$) and methyl ($-CH_3$) groups. However, within the group of halogens, bromine (-Br) presented enhanced activity comparing with chlorine (-Cl) and, even more, for fluorine (-F) [34].

From this first screening a range of 20 compounds were selected due to the low cell survival rates induced in MCF-7 cells. Treatment of Hs578T cells (basal-like breast cancer subtype) was also performed to assess if the subtype of breast malignancy could lead to a different response to the treatment, given to the differential receptor expression [35, 36]. Cytotoxicity was also evaluated for the non-neoplastic mammary epithelial MCF-10A cell line aiming to determine if the promising molecules would be harmful for non-tumoral cells [37]. Since chemotherapy presents side effects, screening for cytotoxicity, could allow to manage the molecular scaffold attending to the reduction of these body-responses. In general, Hs578T cell's sensitivity to the compounds was inferior to MCF-7 cells, which could be explained by the different subtype of cancer, including differential hormone receptor expression. For MCF-10A cells, almost every compound assessed led to a 50% (or more) cell survival for the 30 µM concentration, which compared to the effect on breast cancer cells, corresponds to lower cytotoxicity.

After careful analysis of the results from this first screening, a range of chalcones **3** (**b**, **e**, **g** and **i**) and chromenes **5** (**c**, **e** and **i**) (Figure 1) were selected to further assess the survival curves and determine the respective IC_{50} values for viability (Table 1 and Figure 40 Supporting Information).

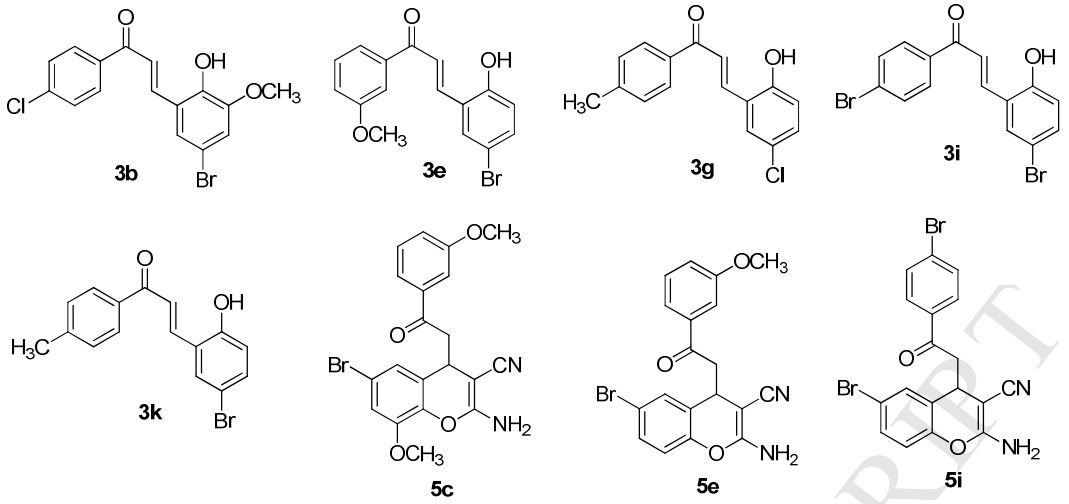


Figure 1. Selected compounds for IC_{50} determination in MCF-7, Hs578T and MCF-10A cell lines.

Table 1. Anticancer activity (IC_{50}, μM) of the selected compounds **3** and **5** against MCF-7, Hs578T and MCF-10A cell lines. Each compound was tested at least in triplicate and the data are presented as mean values±SD.

Compound	IC_{50} (μM)			Selectivity Index [38]	
	MCF-7	Hs578T	MCF-10A	(MCF-10A vs MCF-7)	(MCF-10A vs Hs578T)
Doxorubicin	1.32 ± 0.042	0.52 ± 0.006	1.54 ± 0.078	0.17	1.96
3b	5.15 ± 0.007	5.33 ± 0.010	12.11 ± 0.028	1.35	1.27
3e	**5.07** ± 0.009	**7.27** ± 0.016	**40.64** ± 0.319	**7.02**	**4.59**
3g	**3.98** ± 0.071	**4.80** ± 0.011	**24.25** ± 0.031	**5.09**	**4.05**
3i	**3.82** ± 0.022	**5.88** ± 0.012	**29.26** ± 0.026	**6.66**	**3.98**
3k	2.00 ± 0.033	1.60 ± 0.023	6.52 ± 0.017	2.26	3.08
5c	**3.65** ± 0.021	**4.52** ± 0.019	**18.87** ± 0.020	**4.17**	**3.08**
5e	5.94 ± 0.015	10.26 ± 0.017	18.93 ± 0.031	2.19	0.85
5i	7.24 ± 0.017	16.48 ± 0.027	14.66 ± 0.027	1.02	-0.11

Doxorubicin was used as reference compound helping to validate the *in vitro* results of IC_{50} determination. In general, the newly synthetized compounds presented lower cytotoxic effect than doxorubicin for malignant cells however with enhanced selectivity index. Chalcones **3** showed in general lower IC_{50} values than chromenes **5**, in both cancer cell lines. In 2013, a series of new chalcones and flavonol derivatives were synthetized and tested against HCT116 cells (colorectal carcinoma cell line) in our research group [34]. Comparing with compounds **3** and taking into account the different cell lines, these new molecules seem to present enhanced anticancer activity with lower IC_{50} values for malignant cells. This improvement in activity may be due to the 2-hydroxy group in ring B of the chalcone instead of, the previously described, ring A.

Compounds **3e**, **3g**, **3i** and **5c** were concluded to be the most promising due to their less aggressive effect on the non-malignant cell line MCF-10A and better selectivity index. These compounds proceeded to more specific tests such as cell migration capacity (wound-healing assay), proliferation (BrdU incorporation), protein expression using Western Blotting and Annexin/PI and cell cycle arresting assays by flow cytometry.

2.2.2. Effect of selected compounds on cell migration

Migration and invasion capacity of malignant cells are two of the main features involved in cancer progression and metastasis [39]. To assess if cell migration was being affected, compounds **3e**, **3g**, **3i** and **5c** were tested by treating MCF-7 cells with respective ½ IC_{50} and IC_{50} during 48 hours for each compound and performing the wound-healing assay (Figure 41 Supporting Information). At the beginning and regular timepoints (12, 24 and 48 hours), images were captured (Figure 41 Supporting Information) and the progression of the scratch closure for each compound was analyzed by be_Wound_1.7.1 and summarized in Figure 2.

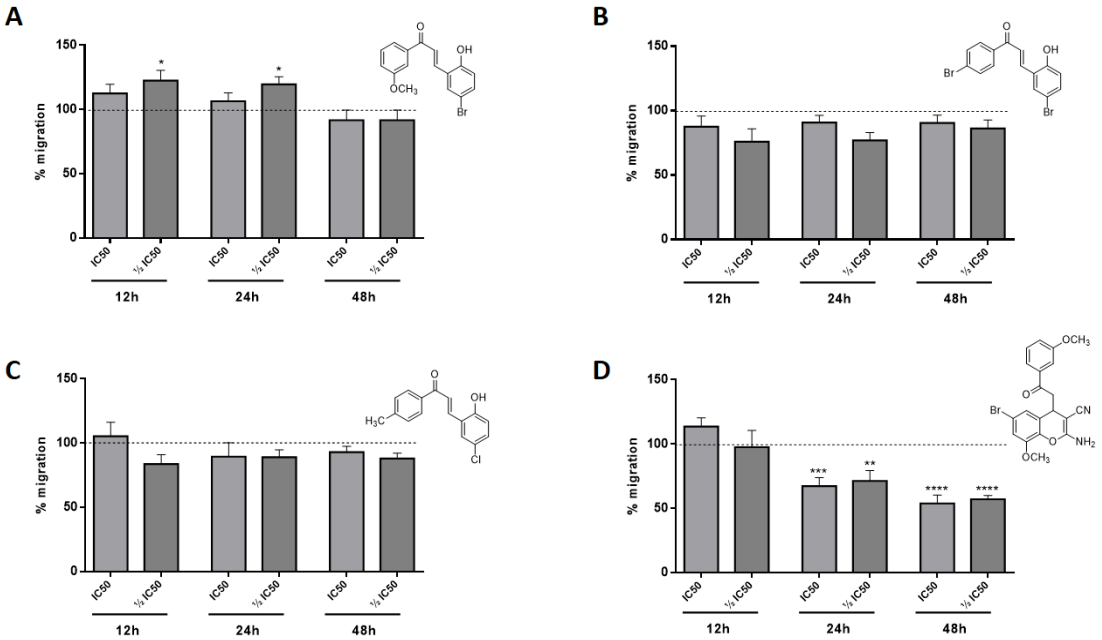


Figure 2. Effect of chalcones **3e** (A), **3g** (B) and **3i** (C) and chromene **5c** (D) on MCF-7 cell migration (12, 24 and 48 hours of treatment) by the wound-healing assay. Results are presented as mean ± SD of three independent experiments. *p=0.01, **p=0.0022, ***p=0.0001, ****p<0.0001 compared to control (DMSO).

As shown in Figure 41A of Supporting Information, MCF-7 cells presented a satisfactory migratory capacity since the scratch reduced significantly after 48 hours when cells were not treated. In general, chromene **5c** presented a much more promising inhibitory ability than chalcones (Figure 2 and Figure 41E Supporting Information). Treatment with compound **5c**, for 48 hours, was able to significantly decrease the percentage of cell migration relative to control (around 50%) as shown in Figure 2. Several chromenes were already reported as being good anti-migratory agents against breast cancer cell lines tested through wound-healing assay [40, 41]. Both apigenin and luteolin are naturally occurring chromenes with similar and simple structures as compound **5c**. In the case of apigenin, the inhibitory effect was reported as being caused by the downstream signaling blockage of PI3K/Akt pathway necessary for motility induced by HGF (Hepatocyte growth factor) as well as adherence junction disassembly [41]. However, as it can be seen in Figure 41E of Supporting Information, the majority of the cells were non-adherent indicating that they died by the treatment not allowing to determine if the inhibitory power was due to loss of migratory ability or by cell death induction.

Cell migration was not significantly affected by chalcones (Figure 2 and Figure 41B-D Supporting Information). In the literature, not many articles describe chalcones as inhibitors of breast cancer cell migration. In 2014, researchers found, through the wound-healing assay, a chalcone able to significantly inhibit cell migration of highly metastatic AGS cells (human gastric carcinoma) [42]. According to a quantitative assessment, treatment with 1 and 2 µg/ml of chalcone inhibited 30.1 and 50.6% of cell migration at 48 hours, respectively.

2.2.3. Effect of chalcones and chromenes on cell proliferation

The inhibitory influence on cell proliferation of the four best chalcones and chromenes was evaluated through the ability of BrdU incorporation during DNA synthesis. MCF-7 cells were treated with IC_{50} and ½ IC_{50} values, for 24 and 48 hours (Figure 3).

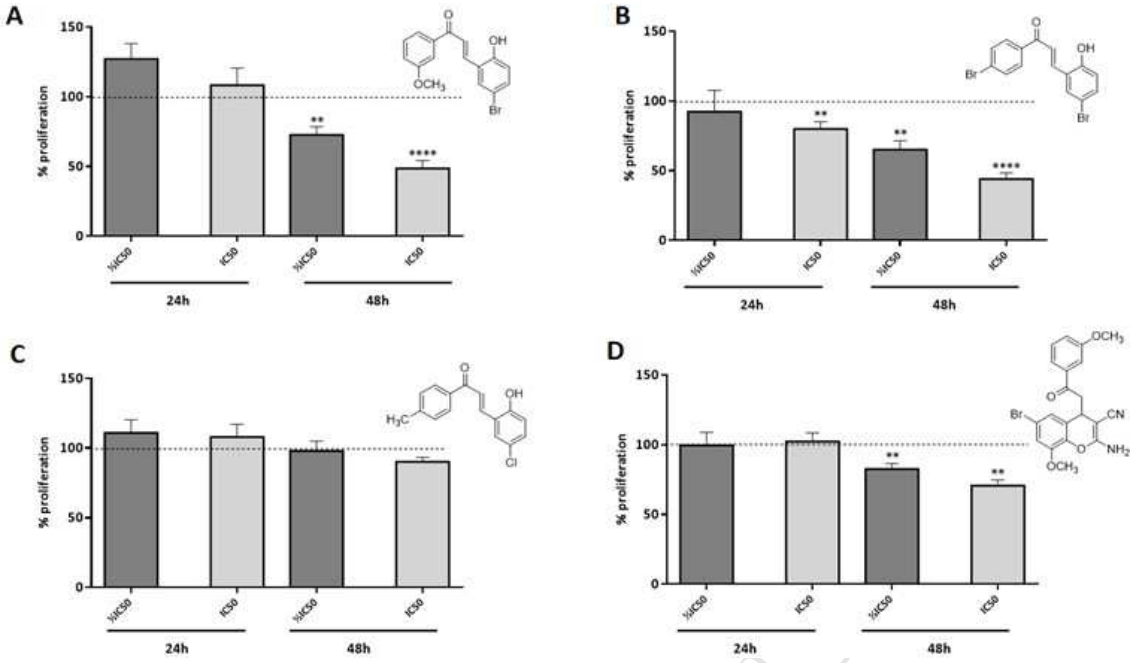


Figure 3. Effect of chalcones **3e** (A), **3g** (B) and **3i** (C) and chromene **5c** (D) on MCF-7 cell proliferation after 24 and 48 hours of treatment. Results are presented as mean ± SD of at least three independent experiments. **p<0.005, ****p<0.0001 compared to control (0.5% DMSO).

As shown by the analysis of Figure 3A and 3B, chalcones induced a significant decrease in cell proliferation, in a time- and concentration-dependent manner, for MCF-7 cells. The highest effect was observed for compounds **3e** and **3g** with more than 50% of reduction in cell proliferation at 48 hours, with the IC_{50} treatment. On the other hand, chalcone **3i** did not affect cell proliferation maintaining similar rates comparing to the control (0.5% DMSO) (Figure 3C) and evidencing the differences in activity of the two halogen atoms (-Br and -Cl) present in the aromatic moiety.

In 2015, a series of synthesized chalcones, very similar to those tested in this assay, were shown to present good anti-proliferative activity against several cell lines, including MCF-7 [43]. One chalcone of this range, substituted with a methoxy group in the aromatic moiety, was described to inhibit significantly BrdU incorporation against NCI-H460 cells (human lung cancer cell line) and, consequently, cell proliferation around 33% after 48 hours of treatment when compared to control.

A slightly weaker effect was observed for compound **5c** (Figure 3D) that reduced cell proliferation in about 20% with the IC_{50} treatment at 48 hours.

2.2.4. Effect of chalcones and chromenes on cell death

Annexin V is commonly combined with Propidium iodide (PI) to understand if cells are viable, apoptotic, or necrotic through differences in plasma membrane integrity and permeability [44]. To better understand the ability of compounds **3e**, **3g**, **3i** and **5c** to induce cell death, the AnnexinV/PI assay was performed. MCF-7 cells were treated with IC_{50} of each previously described compound for 48h and then harvested and stained with annexin V and PI. Analysis by flow cytometry showed that MCF-7 cells are affected by treatment with compounds **3e** and **5c** as revealed by the elevated percentage of early and late apoptotic cells when compared to untreated control cells (0.5% DMSO) (Figure 4). Treatment with the compounds **3g** and **3i** did not show major alterations on cell viability in comparison with control cells (Figure 4).

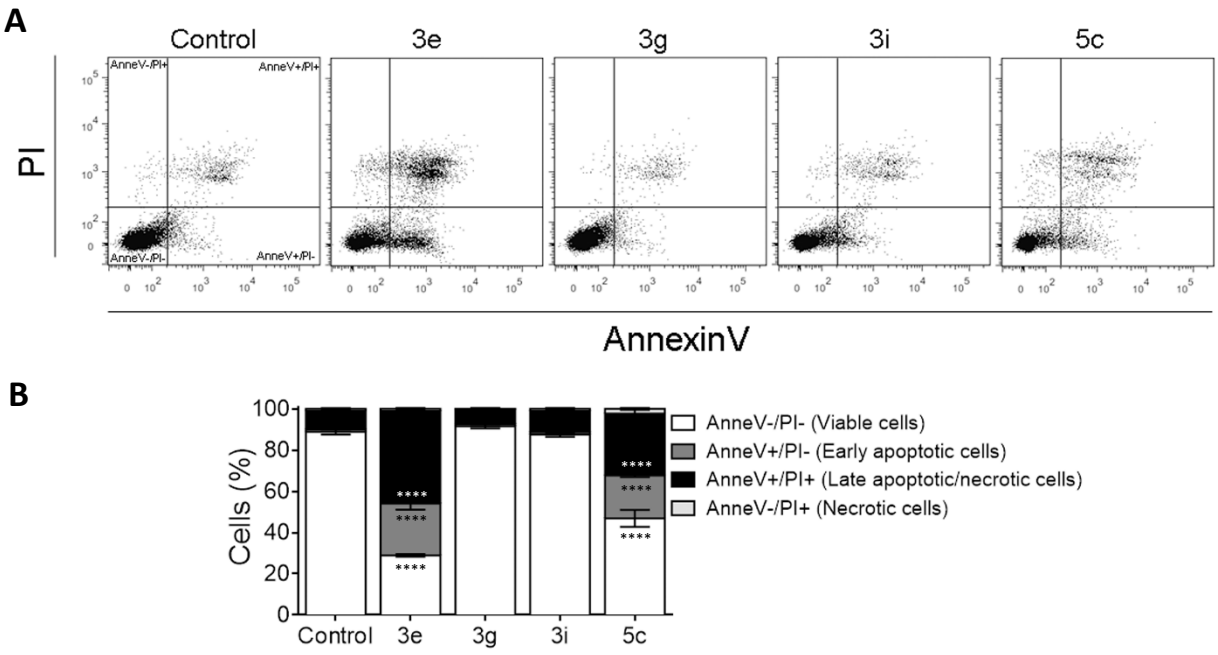


Figure 4. Flow cytometry analysis of MCF-7 cell's viability assessed by annexin V/PI assay. **(A)** Representative dot plots of MCF-7 cells untreated (control) or treated for 48 h with IC_{50} concentrations for chalcones (**3e**, **3g** or **3i)** or the chromene **5c**. **(B)** Graphical representation after quantification of the percentage of cells in each quadrant of the dot plot. The results were obtained using the non-treated cells as control (100 %) and mean±SEM of two independent experiments. Annexin V/PI data was analyzed by two-way ANOVA and Bonferroni post hoc test. ****p<0.0001.

Many other chromenes were previously described as apoptosis-inducers both by receptor-mediated extrinsic or mitochondrial-mediated intrinsic pathway. For example, the newly synthetized Gen-27 was demonstrated to be a bioactive molecule increasing apoptotic cell rates in human breast cancer cells, including MCF-7 cells [22].

Researchers found that the release of cytochrome c from mitochondria to cytosol activates caspases and induces PARP cleavage. Another study showed that the new thiadiazole hybrid (IPTBC) inhibits cell growth of human breast adenocarcinoma cells *in vitro* mainly due to apoptosis rather than necrosis, as showed by multiple apoptosis assays [23]. Therefore, chalcones and chromenes are recognized as anticancer agents through pro-apoptotic mechanisms. Since tumor cells are highly adaptable to different conditions due to their ability of proliferate and escape apoptosis, further studies to better understand the nature of the apoptotic behavior would be important. Thus, the levels of several proteins involved in cell death pathways were determined by Western-blot.

As represented in Figure 5, regulated cell death seems to be mainly induced by compound **5c**. The level of cleaved PARP (c-PARP) after 24 hours of treatment is considerably enhanced with this chromene comparing with compounds **3**. Nevertheless, molecule **3e** seems also to induce a discreet increase in the levels of c-PARP. This data is in agreement with the data above described related to the evaluation of cell survival by AnnexinV/PI.

Regarding Bim, a BH-3 only protein from Bcl-2 family, an evident increase of its levels is noted (Figure 5). Moreover, biophysical studies have indicated that BH3-domain proteins, of which Bim is a member, were described to interact with tubulin that is also overexpressed upon treatment with chalcones and chromene [45]. In an initial phase, microtubules are described to sequester Bim by binding to the dynein light chain, thereby preventing initiation of apoptotic signaling. When released from the microtubules, Bim migrates to the mitochondria, interacting with several proteins (e.g. Bax, Bcl-2 and Bcl-xL) and, finally, promoting apoptosis [45]. Finally, levels of Bcl-xL (Figure 5), an anti-apoptotic protein from the Bcl-2 family, seem to considerably decrease upon treatment at 48 hours thereby promoting apoptosis [46]. Together, this information suggests that an early apoptosis signal is released from cells affecting then the levels of pro- and anti-apoptotic proteins involved in mitochondrial-induced apoptosis.

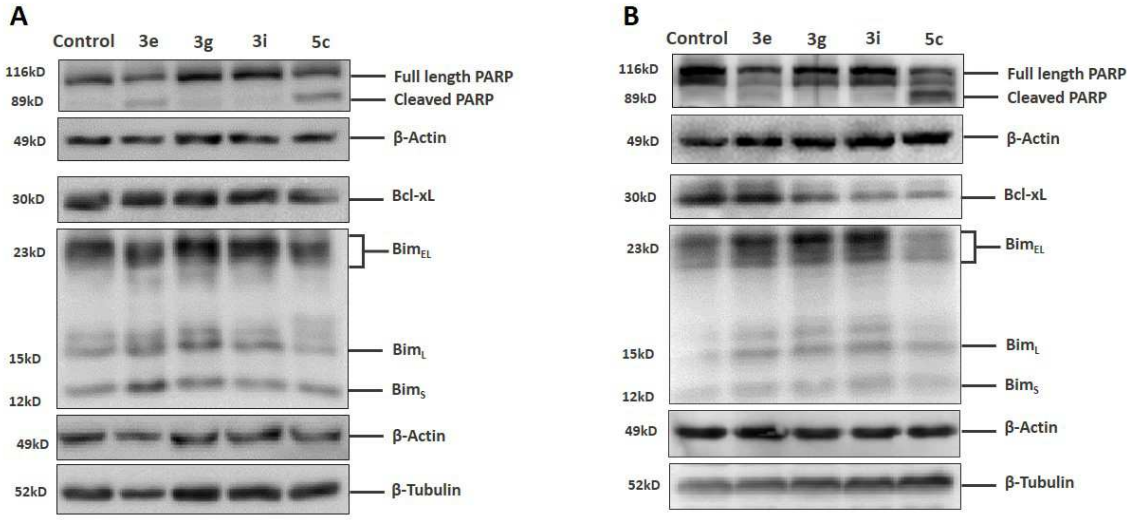


Figure 5. Representative immunoblots MCF-7 cells treated for 24h (**A**) and 48h (**B**) with compounds **3e**, **3g**, **3i** and **5c**. Similar blots were obtained from at least two independent experiments.

2.2.5. Effect on cell cycle arrest

Inhibition of cell cycle progression, allied to apoptosis, is an important factor to control cancer cell growth. To investigate whether the four selected compounds affect the cell cycle of MCF-7 cells, the cell cycle distribution of the cells stained with PI were analyzed using flow cytometry for determination of the DNA content. As shown in Figure 6, compared to the control group, cells treated with compound **5c** showed a statistically significant accumulation of cells in G_2/M cell cycle phase accompanied by a notorious decrease of cells in G_0/G_1 phase of the cell cycle. Furthermore, treatment of MCF-7 cells with the compound **3e** resulted in an extensive fragmentation of DNA and accumulation of cells with a sub-G_0/G_1 content of DNA (Figure 6A). On the contrary, the other chalcones did not presented such marked effect revealing a very similar pattern to control cells.

In 2014, a newly synthetized flavonoid (LL-202) revealed anticancer effects against human breast cells *in vitro* inhibiting growth and proliferation as well as an apoptosis-inducing behavior both by mitochondrial- and death-receptor-mediated pathways [24]. By flow cytometry, it was reported that LL-202 induced cell cycle arrest at G_2/M phase in MCF-7 cells. Finally, the newly synthetized flavonoid significantly inhibited the growth of MCF-7 breast cancer xenograft tumors in a dose-dependent manner, with low systemic toxicity.

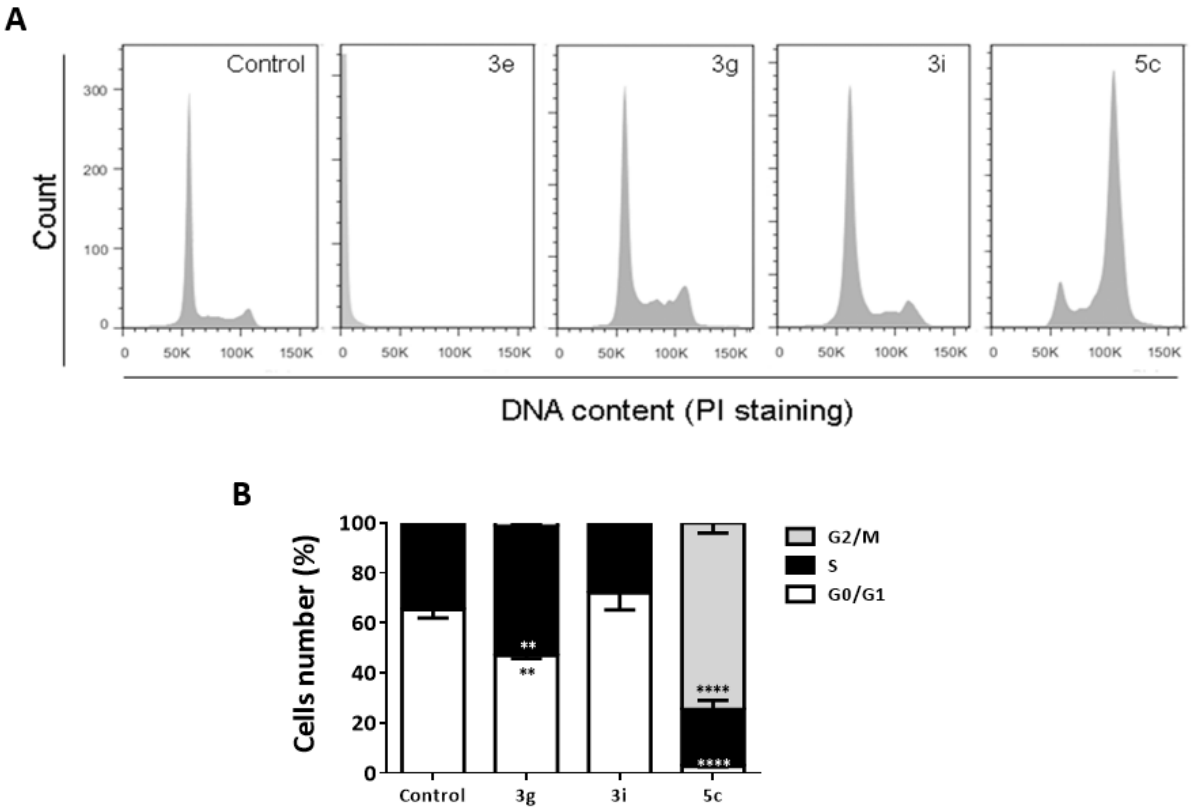


Figure 6. Flow cytometry analysis of the DNA content of MCF-7 cells. **(A)** Cell cycle profile of MCF-7 cells untreated (control) or treated for 48 hours with the IC_{50} concentrations for chalcones (**3e**, **3g** or **3i)** or the chromene **5c**. **(B)** Quantification of the cells in different phases of cell cycle. Results are presented as mean ± SD of 2 independent experiments. Data was analyzed by two-way ANOVA and Bonferroni post hoc test. **p<0.005, ****p<0.0001 compared to control.

2.2.6. Effects of chalcones and chromene on microtubule dynamics

It is well-known that the tubulin binding agents are divided into two types: microtubule stabilizer agents (MSAs) as paclitaxel and microtubule destabilizer agents (MDAs) as colchicine [47]. To evaluate to which type compounds **3e**, **3g**, **3i** and **5c** are related to, newly synthetized compounds were employed at their IC_{50} value. Also, paclitaxel (at 2 µM) was administrated to malignant cells to assess its effect and visualize the main differences in comparison with our compounds.

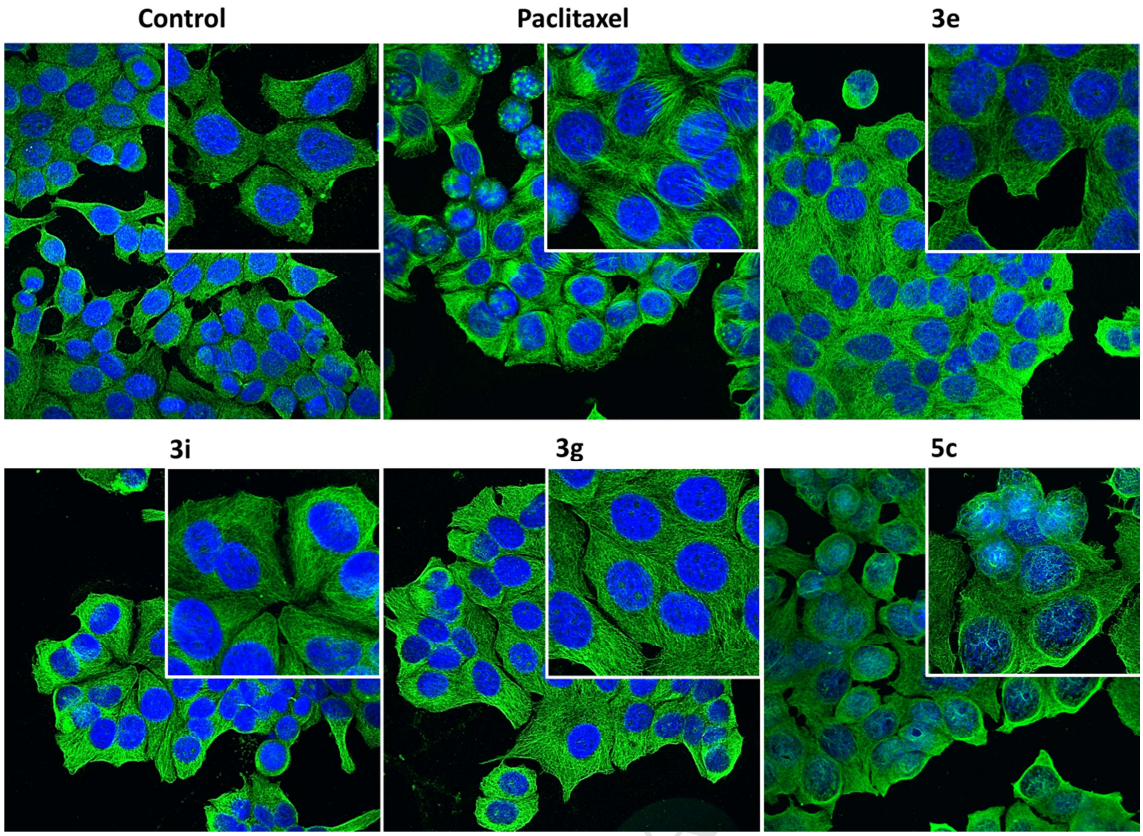


Figure 7. Effect of **3e, 3g, 3i and 5c** on the microtubule network of MCF-7 cells. Untreated (control), paclitaxel (2 µM) and cells treated with compounds **3e, 3g, 3i and 5c** at the IC_{50} value for 12h were stained with β-tubulin and counterstained with 4,6-diamidino-2-phenylindole (DAPI). Microtubules and unassembled tubulin are shown in green. DNA, stained with DAPI, is shown in blue.

As shown in Figure 7, chalcones treatment did not significantly affect cells maintaining morphological similarities to the control. Also, no tubulin network disturbance seems to happen when MCF-7 cells are treated with compounds **3e**, **3g** and **3i**.

On the other hand, compound **5c** induced significant morphological alterations compatible with a disruption of the microtubules dynamics after 12 hours treatment. Images suggest that compound **5c** promotes cell detachment, shrinkage and round up in contrast with the elongated shape of the chalcone treated cells that are similar to the control. Also, nuclei seem to be contracted and present condensed chromatin (Figure 7). These cell characteristics point to a regulated cell death phenotype in accordance with previous results.

In fact, crolibulin, a chromene with similar scaffold to compound **5c**, has proved its activity as a MDA binding to the colchicine-binding site on β-tubulin and being on clinical trials for anaplastic thyroid cancer treatment [48]. Moreover, this small molecule activity leads to the cell cycle arrest, induction of apoptosis, and inhibition of tumor cell proliferation, being found a comparable mode of action for this compound [20]. Our results demonstrate that compound **5c**

seems to inhibit tubulin dynamics and might be a promising novel tubulin polymerization inhibitor agent. The increased levels of β-tubulin assessed by immunoblot (Figure 5) together with the analysis of Bim levels, further support the activation of the intrinsic apoptosis signal pathway.

2.2.7. Effect of the compounds in combination with doxorubicin

Anthracyclines are antitumor antibiotics that alter the cancer cell DNA, preventing uncontrolled cell proliferation and replication, by DNA intercalation or formation of free radicals. Doxorubicin (Figure 8) has demonstrated a great treatment potency, being considered one of the most potent chemotherapeutic drugs approved by FDA [49]. Slow disease progression by interfering with dividing cells has been broadly acknowledged for several decades. Doxorubicin is used as therapy for hematological malignancies and solid tumors, including breast cancer. On the other hand, its high cytotoxicity on noncancerous cells in the human body complicates cancer treatment [50]. The literature reports that doxorubicin toxicity affects several organs including the heart, brain, kidney and liver.

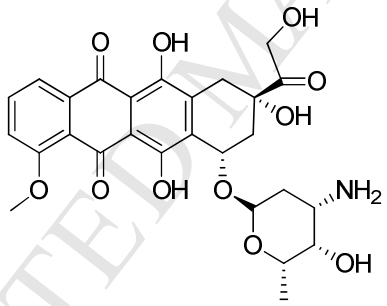


Figure 8. Doxorubicin.

Aiming to enhance doxorubicin anticancer potential and, possibly, diminish its side effects, a combinatory study with compounds **3e**, **3g**, **3i** and **5c** and doxorubicin was performed on MCF-7 cancer cells and MCF-10A non-malignant cells (Table 2 and Figure 42 Supporting Information). The combined treatment was performed using a combination of half the IC_{50} of doxorubicin (0.66 and 0.77 µM for MCF-7 and MCF-10A cell lines, respectively) and varying the concentration (from 30 to 0.005 µM) of each selected compound (**3e**, **3i**, **3g** and **5c**) (Figure 43 Supporting Information). Comparing to the IC_{50} of doxorubicin (Table 1), the combination with compounds **3e**, **3g**, **3i** and **5c** improves the anticancer effect by decreasing the minimal effective concentration need to reduce cell viability in 50% of the malignant cells. Furthermore, IC_{50} for MCF-10A cells is greatly improved by the combined treatment, increasing the selectivity index of

the newly synthetized compounds. These results suggest that this combined-therapy may decrease the toxicity of non-neoplastic cells, anticipating a potential reduction of side effects.

Table 2. Antiproliferative activity (IC_{50}, µM) of the selected compounds **3** and **5** against MCF-7 and MCF-10A cell lines in combination with doxorubicin after 72h treatment.

Compound	IC_{50} (µM)		Selectivity index
	MCF-7	MCF-10A	
Doxo + 3e	0.42 ± 0.050	>60	>141.9
Doxo + 3g	0.27 ± 0.025	1.10 ± 0.063	3.07
Doxo + 3i	0.28 ± 0.034	15.11 ± 0.872	52.96
Doxo + 5c	0.11 ± 0.009	1.58 ± 0.091	13.36

3. Conclusions

The search for novel and effective breast cancer treatments is increasing with the particular concern to reduce side effects. These advances improve the patient's quality of life and lead to higher survival rates.

Good yields through clean reactions were obtained for chalcone and chromene derivative synthesis. Innocuous solvents such as water and ethanol were used and aldol condensations were highly effective. Generally, the reactions were performed at room temperature, leading to the isolation of many pure compounds.

Synthetic chalcones and chromenes demonstrated to be powerful anticancer agents through all the assays performed. Halogenated substituents presented more interesting capacities than methoxyl or methyl groups when they were the only substituents in the molecular scaffold. The bromine atom was the most present halogen in the final molecules that proceeded to the final assays and showed to be more promising candidates for further studies.

Specifically, for chalcones, the results suggest an anti-proliferative activity. On the other hand, chromene **5c** demonstrated to be involved in the inhibition of cell migration and induction of apoptosis, apparently triggering cell death associated with a G_2/M phase cell cycle arrest. The increased levels of β-tubulin together with the analysis of PARP and Bim levels, support the activation of the intrinsic apoptosis signal pathway. Also, our results demonstrate that compound **5c** seems to alter tubulin polymerization and might be a promising novel MDA.

Finally, when combining these novel compounds with the commercially available drug doxorubicin, results lead to a decrease in the IC_{50} value of MCF-7 malignant cells and, more importantly, increase in the selectivity index.

4. Experimental

4.1. Chemistry

All chemicals, reagents and solvents for compounds synthesis were analytical grade, purchased from commercial sources and used without further purification, unless otherwise specified. Reactions were monitored by thin layer chromatography (TLC) using silica gel 60 plates purchased from Macherey-Nagel with a 0.2 mm and a fluorescence indicator. An UV chamber (CN-6 Vilber Lourmat) with a 254 nm lamp was used for revelation. Dry flash chromatography was performed with silica gel (particle size <0.063 mm) from MN Kieselgel 60 (230 ASTM). For reactions at high temperatures, a hot plate stirrer IKAMAG RCT was used, with magnetic stirring at 40 rpm and at different temperatures according to the specific procedure. For solvent evaporation, a Buchi RE 11 rotary evaporator was used with vacuum and variable bath temperature. NMR spectra were obtained in Bruker Avance III (at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR), at 25ºC, with deuterated dimethylsulfoxide (DMSO-d_6) as solvent. Chemical shifts were recorded in parts per million (ppm) using the residual solvent peak as an internal standard. IR spectra were recorded in FT-IR Bomem MB 104 using nujol mulls and NaCl cells. Melting points were determined in a Stuart SMP3 melting point apparatus and are uncorrected. Elemental analysis was performed on a LECO CHNS-932 instrument. 3,5-Difluoro-2-hydroxybenzaldehyde was synthesized as described before [51].

*4.1.1. General procedure for the synthesis of chalcone derivatives **3***

Aqueous 3M NaOH was added to a solution of substituted salicylaldehyde **1** (1.1 equiv.) and acetophenone **2** in ethanol. The solution was stirred at room temperature (4-31 hours) or heated at 40-60 ºC (for **3a**, **b, q** and **u**; 18-27 hours). The solution was neutralized with concentrated HCl (37%) and a suspension was obtained, cooled at 4 ºC for 1-16 hours. The solid was filtered and washed with ethanol and water leading to the respective *α,β*-unsaturated carbonyl compound **3**. This procedure was adapted from reference [34].

*4.1.1.1. 3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-phenylprop-2-en-1-one (**3a**).* Yellow solid, yield 100%, mp 175-177 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.85 (s, 3H, -OCH_3), 7.17 (d, J = 2.0 Hz, 1H, H4), 7.65 (tt, J = 1.3 Hz, 1H, H4'), 7.77 (d, J = 2.4 Hz, 1H, H6), 7.88 (d, J = 15.6 Hz), 8.03 (d, J = 15.6 Hz), 8.13 (d, J = 6.8Hz, 1H, H6'), 9.74 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 56.36 (-OCH3), 110.50 (C5), 116.14 (C4), 121.49 (C6), 122.09, 123.20

(C1), 128.51 (C2'+C6'), 128.72 (C3'+C5'), 137.34, 133.08, (C4'), 137.60 (C1'), 145.93 (C3), 149.04 (C2), 189.17. Anal. Calcd for $C_{16}H_{15}O_3Br$: C, 57.33; H, 4.52. Found: C, 57.02; H, 4.77.

*4.1.1.2. 3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one (**3b**).* Light brown solid, yield 66%, mp 163-165 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.85 (s, 3H, -OCH_3), 7.17 (d, *J* = 2.4 Hz, 1H, H4), 7.63 (dt, *J* = 8.4Hz, 1H, H5'), 7.77 (d, *J* = 2.0 Hz, 1H, H6), 7.91 (d, *J* = 16.0 Hz), 8.00 (d, *J* = 16.0 Hz), 8.18 (d, *J* = 6.0Hz, 1H, H6'), 9.77 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 56.37 (-OCH_3), 110.59 (C5), 116.26 (C4), 121.47 (C6), 121.67, 123.09 (C1), 128.84 (C2'+C6'), 130.45 (C3'+C5'), 136.24 (C4'), 137.75, 138.06 (C1'), 146.03 (C2), 149.05 (C3), 188.01. Anal. Calcd for $C_{16}H_{15}O_3BrCl$: C, 51.99; H, 4.10. Found: C, 52.19; H, 4.04.

*4.1.1.3. 3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (**3c**).* Yellow solid, yield 100%, mp 148-150 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.84 (s, 3H, -OCH3), 7.17 (d, *J* = 2.4Hz, 1H, H4), 7.23 (dd, *J* = 8.4, 2.8 Hz, 1H, H4'), 7.47 (t, *J* = 8.0 Hz, 1H, H5'), 7.59 (dd, *J* = 2.8, 1.6 Hz, 1H, H2'), 7.75 (d, *J* = 1.2Hz, 1H, H6'), 7.77 (d, *J* = 2.4 Hz, 1H, H5), 7.88 (d, *J* = 15.6Hz), 7.98 (d, *J* = 15.6Hz), 9.75 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.36 (-OCH3), 56.36 (-OCH3), 110.57 (C5), 113.06 (C2'), 116.16 (C4), 119.06 (C6'), 121.07 (C4'), 121.51 (C6), 122.12, 123.19 (C1), 129.87 (C5'), 137.43, 139.05 (C1'), 145.94 (C2), 149.03 (C3), 159.52 (C3'), 188.92. Anal. Calcd for $C_{17}H_{17}O_4Br$: C, 55.91; H, 4.70. Found: C, 56.06; H, 4.69.

*4.1.1.4. 3-(5-chloro-2-hydroxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (**3d**).* Yellow solid, yield 86%, mp 158-160 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.84 (s, 3H, -OCH3), 6.95 (d, *J* = 8.8 Hz, 1H, H3), 7.24 (dd, *J* = 5.6, 2.8 Hz, 1H, H6'), 7.30 (dd, *J* = 8.8, 2.4 Hz, 1H, H4), 7.37 (d, *J* = 7.6Hz, 1H, H4'), 7.48 (t, *J* = 8.0Hz, 1H, H5'), 7.60 (dd, *J* = 1.6, 1.2Hz, 1H, H2'), 7.92 (d, *J* = 16.0 Hz), 7.96 (d, *J* = 16.0 Hz), 8.03 (d, *J* = 2.8Hz, 1H, H5), 10.55 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.28 (-OCH_3), 113.05 (C2'), 117.85 (C3), 119.05 (C6'), 121.04 (C4'), 122.05, 123.05 (C1), 123.23 (C5), 127.47 (C6), 129.88 (C5'), 131.40 (C4), 137.67, 139.08 (C1'), 155.95 (C2), 159.53 (C3'), 188.95. Anal. Calcd for $C_{16}H_{15}O_3Cl$: C, 66.24; H, 5.22. Found: C, 65.98; H, 4.95. The ^{1}H- and ^{13}C-NMR spectra were identical to those reported for the compound synthesized through a previously published procedure [52].

*4.1.1.5. 3-(5-bromo-2-hydroxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (**3e**).* Yellow solid, yield 82%, mp 162-164 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.84 (s, 3H, -OCH3), 6.90 (d, *J* = 8.8Hz, 1H, H3), 7.23 (dd, *J* = 5.6, 2.8Hz, 1H, H6'), 7.37 (dt, *J* = 7.6, 1.6Hz, 1H, H4'), 7.42 (dd, *J* = 8.8, 2.4Hz, 1H, H4), 7.48 (t, *J* = 8.0 Hz, 1H, H5'), 7.60 (q, *J* = 1.6 Hz, 1H, H2'), 7.92 (d, *J* = 15.6 Hz), 7.96 (d, *J* = 15.6Hz), 8.13 (d, *J* = 2.4Hz, 1H, H5), 10.60 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.37 (-OCH3), 113.06 (C2'), 117.86 (C3), 119.05 (C6'), 121.07 (C4'), 122.03, 123.13 (C1), 123.19 (C5), 127.40 (C6), 129.89 (C5'), 131.24 (C4), 137.60, 139.09

(C1'), 155.92 (C2), 159.54 (C3'), 188.95. Anal. Calcd for $C_{16}H_{15}O_3Br$: C, 57.33; H, 4.52. Found: C, 57.44; H, 4.75.

*4.1.1.6. 3-(3,5-difluoro-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (**3f**).* Yellow solid, yield 75%, mp 180-182 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.33 (m, 1H, H4), 7.57 (t, *J* = 7.2 Hz, 2H, H3'+H5'), 7.65 (m, 1H, H4'), 7.97 (d, *J* = 15.6 Hz), 7.77 (m, 1H, H6), 8.03 (dd, *J* = 15.6, 1.2 Hz), 8.14 (d, *J* = 7.2 Hz, 2H, H2'+H6'), 10.33 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 109.08 (dd, J=23.0, 4.0Hz, C6), 106.28 (dd, J=23.0, 28.0Hz, C4), 123.29, 136.67 (t, *J* = 3.0 Hz), 124.94 (dd, *J* = 9.0, 4.0 Hz, C1), 128.54 (C2'+C6'), 128.80 (C3'+C5'), 133.34 (C4'), 137.44 (C1'), 141.19 (dd, *J* = 11.0, 3.0 Hz, C2), 151.59 (dd, *J* = 241.0, 13.0 Hz, C3), 154.33 (dd, *J* = 235.7, 11.6 Hz, C5), 188.17. Anal. Calcd for $C_{15}H_{14}O_2F_2$: C, 68.73; H, 5.39. Found: C, 68.58; H, 5.18.

*4.1.1.7. 3-(5-chloro-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one (**3g**).* Yellow solid, yield 87%, mp 179-181 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, -CH3), 6.97 (d, *J* = 8.8 Hz, 1H, H3), 7.28 (dd, *J* = 8.8, 2.8 Hz, 1H, H4), 7.37 (d, *J* = 8.0 Hz, 2H, H3'+H5'), 7.93 (d, *J* = 16.0 Hz), 7.94 (d, *J* = 16.0 Hz), 8.06 (q, *J* = 6.4, 1.6 Hz, 2H, H2'+H6'), 10.58 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.17 (-CH3), 117.86 (C3), 122.04, 123.13 (C1), 123.19 (C5), 127.40 (C6), 128.65 (C2'+C6'), 129.31 (C3'+C5'), 131.24 (C4), 135.12 (C1'), 137.24, 143.50 (C4'), 155.92 (C2), 188.63. Anal. Calcd for $C_{16}H_{15}O_2Cl$: C, 69.94; H, 5.51. Found: C, 69.88; H, 5.36. The ^{1}H- and ^{13}C-NMR spectra were identical to those reported for the compound synthesized through a previously published procedure [52].

*4.1.1.8. 3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(p-tolyl)prop-2-en-1-one (**3h**).* Yellow solid, yield 77%, mp 193-195 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.39 (s, 3H, -CH3), 3.85 (s, 3H, -OCH3), 7.17 (d, *J* = 2.4Hz, 1H, H4), 7.37 (d, *J* = 8.0Hz, 2H, H3'+H5'), 7.76 (d, *J* = 2.0 Hz, 1H, H6), 7.90 (d, 16.0 Hz), 7.97 (d, *J* = 16.0 Hz), 8.07 (d, *J* = 8.0 Hz, 2H, H2'+H6'), 9.71 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.18 (-CH3), 53.36 (-OCH3), 110.57 (C5), 116.06 (C4), 121.45 (C6), 122.11, 123.28 (C1), 128.67 (C2'+C6'), 129.30 (C3'+C5'), 135.08 (C1'), 136.96, 143.52 (C4'), 145.85 (C2), 149.03 (C3), 188.58. Anal. Calcd for $C_{17}H_{17}O_3Br$: C, 58.47; H, 4.92. Found: C, 58.27; H, 4.99.

*4.1.1.9. 3-(5-bromo-2-hydroxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one (**3i**).* Yellow solid, yield 70%, mp 171-173 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.79 (d, *J* = 8.8 Hz, 1H, H3), 7.31 (dd, *J* = 8.8, 2.4 Hz, 1H, H4), 7.67 (d, *J* = 6.8 Hz, 2H, H3'+H5'), 7.83 (d, *J* = 15.6 Hz), 7.86 (d, *J* = 15.6 Hz), 7.98 (d, *J* = 6.4 Hz, 2H, H2'+H6'), 8.04 (d, *J* = 2.4 Hz, 1H, H6), 10.49 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 110.85 (C5), 118.33 (C3), 121.56, 123.56 (C1), 127.25 (C4'), 130.34 (C6), 130.55 (C2'+C6'), 131.80 (C3'+C5'), 134.38 (C4), 136.59 (C1'), 137.94, 156.43 (C2), 188.24. Anal. Calcd for $C_{15}H_{12}O_2Br_2$: C, 46.90; H, 3.16. Found: C, 47.12; H, 3.10.

*4.1.1.10. 1-(4-chlorophenyl)-3-(2-hydroxy-5-methoxyphenyl)prop-2-en-1-one (**3j**).* Orange solid, yield 84%, mp 142-134 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.83 (s, 3H, -OCH3), 6.83 (t, *J* = 8.0 Hz, 1H, H6), 6.89 (d, *J* = 8.8 Hz, 1H, H4), 7.05 (dd, *J* = 8.0, 0.8 Hz, 1H, H5), 7.63 (d, *J* = 8.8 Hz, 2H, H2'+H6'), 7.81 (d, *J* = 16.0 Hz), 8.09 (d, *J* = 16.0 Hz), 8.12 (d, *J* = 6.4 Hz, 2H, H3'+H5'), 9.47 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 55.95 (-OCH3), 113.76 (C4), 119.05 (C5), 119.82 (C6), 120.73, 121.51 (C1), 128.85 (C2'+C6'), 130.25 (C3'+C5'), 136.49 (C4'), 137.82 (C1'), 139.70, 146.72 (C2), 148.01 (C3), 188.28. Anal. Calcd for $C_{16}H_{15}O_3Cl$: C, 66.10; H, 5.21. Found: C, 66.00; H, 5.27.

*4.1.1.11. 3-(5-bromo-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one (**3k**).* Yellow solid, yield 87%, mp 191-193 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.40 (s, 3H, -CH3), 6.89 (d, *J* = 8.8 Hz, 1H, H3), 7.37 (d, *J* = 8.0 Hz, 2H, H3'+H5'), 7.40 (dd, *J* =8.8, 2.4 Hz, 1H, H4), 7.93 (d, *J* = 16.0Hz), 7.94 (d, *J* = 16.0 Hz), 8.06 (d, *J* = 8.0 Hz, 2H, H2'+H6'), 8.13 (d, *J* = 2.4Hz, 1H, H6), 10.55 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.17 (-CH3), 110.83 (C5), 118.28 (C3), 122.01, 123.75 (C1), 128.66 (C2'+C6'), 129.30 (C3'+C5'), 130.26 (C6), 134.09 (C1'), 134.09 (C4), 137.11, 143.51 (C4'), 156.28 (C2), 188.59. Anal. Calcd for $C_{16}H_{15}O_2Br$: C, 60.20; H, 4.75. Found: C, 59.93; H, 4.77.

*4.1.1.12. 3-(3,5-difluoro-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one (**3l**).* Yellow solid, yield 79%, mp 202-204 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.40 (s, 3H, -CH3), 7.32 (m, 1H, H4), 7.38 (d, *J* = 8.0 Hz, 2H, H3'+H5'), 7.75 (m, 1H, H6), 7.95 (d, *J* = 15.6 Hz), 8.01 (dd, *J* = 15.6, 1.6 Hz), 8.07 (d, *J* = 8.4 Hz, 2H, H2'+H6'), 10.30 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.17 (-CH_3), 106.16 (dd, *J* = 23.0, 28.0 Hz, C4), 109.02 (dd, *J* = 23.0, 4.0 Hz, C6), 123.30, 125.02 (dd, *J* = 10.0, 4.0 Hz, C1), 128.69 (C2'+C6'), 129.36 (C3'+C5'), 134.93 (C1'), 136.27 (t, *J* = 4.0 Hz), 141.10 (dd, *J* = 15.0, 3.0 Hz, C 2), 143.72 (C4'), 151.53 (dd, *J* = 240.0, 13.0 Hz, C3), 154.34 (dd, *J* = 236.0, 12.0 Hz, C5), 188.55. Anal. Calcd for $C_{16}H_{14}O_2F_2$: C, 69.55; H, 5.12. Found: C, 69.80; H, 4.93.

*4.1.1.13. 3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one (**3m**).* Yellow solid, yield 80%, mp 189-191 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.86 (s, 3H, -OCH3), 7.19 (d, *J* = 2.4 Hz, 1H, H4), 7.78 (d, *J* = 3.2 Hz, 1H, H6), 7.91 (d, J = 16.0 Hz), 7.79 (d, *J* = 8.8 Hz, 2H, H3'+H5'), 8.05 (d, *J* = 16.0 Hz), 8.11 (d, *J* = 8.4 Hz, 2H, H2'+H6'), 9.79 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 56.37 (-OCH_3), 110.59 (C5), 116.27 (C4), 121.48 (C6), 121.65, 123.09 (C1), 127.27 (C4'), 130.56 (C2'+C6'), 131.80 (C3'+C5'), 136.57 (C1'), 137.74149.06 (C2), 146.04 (C3), 188.23. Anal. Calcd for $C_{16}H_{14}O_3Br_2$: C, 46.40; H, 3.41. Found: C, 46.55; H, 3.47.

*4.1.1.14. 3-(3-bromophenyl)-1-(p-tolyl)prop-2-en-1-one (**3n**).* Yellow solid, yield 80%, mp 127-129 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.40 (s, 3H, -CH3), 7.38 (d, *J* = 7.2 Hz, 2H, H3'+H5'), 7.42 (d, *J* = 8.0 Hz, 1H, H3), 7.63 (m, 1H, H4), 7.69 (d, *J* = 15.6 Hz), 7.85 (d, *J* = 7.6

Hz, 1H, H2), 8.00 (d, *J* = 15.6 Hz), 8.04 (t, *J* = 1.6Hz, 1H, H6), 8.10 (d, *J* = 8.4 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.18 (-CH_3), 122.36 (C5), 123.51, 128.78 (C2'+C6'), 128.22 (C2), 129.34 (C3'+C5'), 130.76 (C6), 130.89 (C3), 132.93 (C4), 134.82 (C1'), 137.23 (C1), 141.83, 143.79 (C4'), 188.40. Anal. Calcd for $C_{16}H_{15}OBr$: C, 54.71; H, 4.31. Found: C, 54.59; H, 4.33.

*4.1.1.15. 3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one (**3o**).* Yellow solid, yield 48%, mp 169-171 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.87 (t, *J* = 7.6 Hz, 1H, H5), 6.95 (dd, *J* = 8.0, 0.8 Hz, 1H, H3), 7.27 (td, *J* = 7.2, 1.6, 1H, H4), 7.56 (t, *J* = 7.2 Hz, 2H, H2'+H6'), 7.79 (d, *J* = 8.8 Hz, 2H, H3'+H5'), 7.84 (dd, *J* = 8.0, 1.6 Hz, 1H, H6), 7.86 (d, *J* = 16.0 Hz), 8.02 (d, *J* = 16.0 Hz), 10.29 (s, 1H, -OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 116.22 (C3), 119.38 (C5), 120.92, 121.33 (C1), 128.31 (C2'+C6'), 128.70 (C6), 128.75 (C3'+C5'), 132.03 (C4), 132.86 (C4'), 137.90 (C1'), 139.54, 157.25 (C2), 189.50. Anal. Calcd for $C_{15}H_{14}O_2$: C, 79.61; H, 6.25. Found: C, 79.64; H, 6.22. The ^{1}H- and ^{13}C-NMR spectra were identical to those reported for the compound synthesized through a previously published procedure [53].

*4.1.1.16. 3-(5-bromo-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (**3p**).* Yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.90 (d, *J* = 8.8 Hz, 1H, H3), 6.40 (dd, *J* = 8.8, 2.4 Hz, 1H, H4'), 7.42 (dd, *J* = 8.8, 2.4 Hz, 1H, H4), 7.56 (t, *J* = 6.4Hz, 1H, H5), 7.92 (d, *J* = 8.0 Hz, 2H, H3'+H5'), 7.95 (d, *J* = 16.0 Hz), 7.97 (d, *J* = 16.0 Hz), 8.13 (d, *J* = 4.8 Hz, 2H, H2'+H6'), 8.15 (d, *J* = 4.8Hz, 1H, H6), 10.56 (s, 1H, -OH). The product was contaminated with aldehyde in a 1:0.13 molar ratio according to ^{1}H NMR data and was used as starting material in the next step, without further purification. The ^{1}H- and ^{13}C-NMR spectra were identical to those reported for the compound synthesized through a previously published procedure [53].

*4.1.1.17. 3-(5-chloro-2-hydroxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one (**3q**).* Yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.98 (d, *J* = 8.8Hz, 1H, H_3), 7.31 (dd, *J* = 8.8, 2.4, 1H, H_4), 7.63 (d, *J* = 8.4Hz, 1H, H_6), 7.57 (d, *J* = 8.4Hz, 2H, $H_{3'}$+$H_{5'}$), 8.17 (d, *J* = 8.8Hz, 2H, $H_{2'}$+$H_{6'}$), 10.64 (s, 1H, -OH). The product was contaminated with aldehyde in a 1:0.25 molar ratio according to ^{1}H NMR data and was used as starting material in the next step, without further purification.

*4.1.1.18. 3-(5-bromo-2-hydroxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one (**3r**).* Yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.84 (d, *J* = 8.4 Hz, 1H, H3), 7.27 (dd, *J* = 8.4, 2.0, 1H, H4), 7.63 (dd, *J* = 8.4, 2.0Hz, 1H, H6), 7.93 (d, *J* = 15.6 Hz), 7.95 (d, *J* = 7.6 Hz, 2H, H2'+H6'), 7.97 (d, *J* = 15.6 Hz), 8.18 (d, *J* = 8.8 Hz, 2H, H3'+H5'), 10.05 (s, 1H, -OH). The product was contaminated with aldehyde in a 1:0.6 molar ratio according to ^{1}H NMR data and was used as starting material in the next step, without further purification.

*4.1.1.19. 3-(5-chloro-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (**3s**).* Yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.96 (d, *J* = 8.0 Hz, 1H, H3), 7.30 (dd, *J* = 8.8, 2.8 Hz, 1H, H6), 7.56

(t, *J* = 7.6 Hz, 2H, H2'+H6'), 7.65 (t, *J* = 7.2 Hz 1H, H4), 7.94 (d, *J* = 16.0 Hz), 7.97 (d, *J* = 16.0 Hz), 8.14 (d, *J* = 8.8 Hz, 2H, H3'+H5'), 10.57 (s, 1H, -OH). The product was contaminated with traces of aldehyde according to ^{1}H NMR data and was used as starting material in the next step, without further purification.

*4.1.1.20. 1-(4-chlorophenyl)-3-(2-hydroxy-4-methoxyphenyl)prop-2-en-1-one (**3t**)*. Isolates as sodium salt, neutralization with HCl was not performed. Red solid, mp 156-159 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.75 (s, 3H, -OCH3), 6.48 (d, *J* = 2.4 Hz, 1H, H5), 6.50 (d, *J* = 2.4 Hz, 1H, H4), 7.62 (dd, *J* = 8.8, 2.0 Hz, 2H, H3'+H5'), 7.72 (d, *J* = 15.6 Hz), 7.82 (d, *J* = 9.6 Hz, 1H, H6), 8.02 (d, *J* = 15.6 Hz), 8.09 (dd, *J* = 8.8, 2.0 Hz, 2H, H2'+H6'), 10.43 (s, 1H, -OH). The product was contaminated with traces of aldehyde according to ^{1}H NMR data and was used as starting material in the next step, without further purification.

*4.1.2. General procedure for the synthesis of 4H-chromene-3-carboxylate derivatives **4***

Triethylamine (2-6 equiv.) was added to a solution of chalcone **3** and methyl 2-cyanoacetate (1.1-2 equiv.) in methanol. The mixture was stirred for 4-24 hours at room temperature, cooled and then filtered, leading to the expected chromene derivative **4**.

*4.1.2.1. Methyl 2-amino-6-bromo-4-(2-(4-chlorophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carboxylate (**4b**)*. Pink solid, yield 69%, mp 183-186 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.13 (m, 2H), 3.49 (s, 3H, -OCH_3), 3.81 (s, 3H, -OCH_3), 4.24 (dd, *J* = 4.8, 2.0 Hz, 1H), 7.03 (d, *J* = 2.0, 1H, H5), 7.08 (d, *J* = 2.0 Hz, 1H, H7), 7.53 (dd, *J* = 8.4, 1.6 Hz, 2H, H3'+ H5'), 7.62 (s, 2H), 7.86 (dd, *J* = 6.8, 2.0 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.74, 47.45, 50.38, 56.19 (-OCH_3), 73.84 (C3), 113.72 (C7), 115.44 (C6), 122.08 (C5), 128.67 (C3'+C5'), 128.67 (C4a), 129.87 (C2'+C6'), 135.38 (C4'), 137.98 (C1'), 138.28 (C8a), 147.77 (C8), 161.56 (C2), 168.07, 197.46. Anal. Calcd for $C_{20}H_{18}NO_5Br$: C, 55.66; H, 4.21; N, 3.24. Found: C, 55.39; H, 4.37; N, 3.40.

*4.1.2.2. Methyl 2-amino-6-bromo-8-methoxy-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4H-chromene-3-carboxylate (**4c**)*. White solid, yield 62%, mp 159-161 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.18 (m, 2H), 3.52 (s, 3H, -OCH3), 3.79 (s, 3H, -OCH_3), 3.81 (s, 3H, -OCH_3), 4.25 (dd, *J* = 6.8, 4.8 Hz, 1H), 7.00 (d, *J* = 2.0, 1H, H5), 7.08 (d, *J* = 2.0 Hz, 1H, H7), 7.16 (dd, *J* = 8.0, 2.0 Hz, 1H, H4'), 7.37 (t, *J* = 1.6 Hz, 1H, H2'), 7.38 (t, *J* = 8.0Hz, 1H, H5'), 7.44 (d, *J* = 7.6 Hz, 2H, H6'), 7.63 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.60, 47.94, 50.41, 55.25 (-OCH3), 56.10 (-OCH_3), 73.98 (C3), 112.25 (C2'), 113.67 (C7), 115.36 (C6), 119.27 (C4'), 120.46 (C6'), 122.06 (C5), 128.71 (C4a), 129.73 (C5'), 138.14 (C1'), 138.29 (C8a), 147.76 (C8), 161.55 (C2), 168.12, 198.21. Anal. Calcd for $C_{21}H_{20}NO_6Br$: C, 54.56; H, 4.37; N, 3.03. Found: C, 54.46; H, 4.33; N, 3.28.

*4.1.2.3. Methyl 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carboxylate (**4d**).* White solid, yield 57%, mp 164-166 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.15 (m, 2H), 3.49 (s, 3H, -OCH_3), 3.81 (s, 3H, -OCH_3), 4.24 (dd, *J* = 6.4, 4.8 Hz, 1H), 7.03 (d, *J* = 2.0 Hz, 1H, H5), 7.08 (d, *J* = 2.0 Hz, 1H, H7), 7.63 (s, 2H) 7.67 (dd, *J* = 8.4, 1.6 Hz, 2H, H3'+ H5'), 7.77 (dd, *J* = 5.2, 1.6 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.71, 47.43, 50.36, 56.11 (-OCH_3), 73.82 (C3), 113.71 (C7), 115.43 (C6), 122.06 (C5), 127.16 (C4'), 128.62 (C4a), 129.97 (C2'+C6'), 131.61 (C3'+C5'), 135.69 (C1'), 138.27 (C8a), 147.76 (C8), 161.54 (C2), 168.05, 197.65. Anal. Calcd for $C_{20}H_{17}NO_5Br_2$: C, 46.99; H, 3.36; N, 2.74. Found: C, 46.72; H, 3.28; N, 2.69.

*4.1.2.4. Methyl 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-4H-chromene-3-carboxylate (**4i**).* White solid, yield 94%, mp 140-141 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.16 (m, 2H), 3.50 (s, 3H, -OCH_3), 4.27 (dd, *J* = 6.8, 5.2 Hz, 1H), 6.96 (d, *J* = 8.4 Hz, 1H, H8), 7.36 (dd, *J* = 8.8 2.4 Hz, 1H, H7), 7.48 (d, *J* = 2.4, 1H, H5), 7.56 (s, 2H), 7.68 (dd, *J* = 6.8, 2.0 Hz, 2H, H3'+ H5'), 7.79 (dd, *J* = 6.4, 2.0 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.62, 50.37, 47.33, 73.80 (C3), 115.63 (C6), 117.79 (C8), 127.22 (C4'), 128.52 (C4a), 130.45 (C7), 130.97 (C5), 130.00 (C2'+C6'), 131.65 (C3'+C5'), 135.66 (C1'), 148.92 (C8a)161.61 (C2), 168.03, 197.68. Anal. Calcd for $C_{19}H_{15}NO_4Br_2$: C, 47.43; H, 3.15; N, 2.91. Found: C, 47.52; H, 3.26; N, 2.86.

*4.1.2.5. Methyl 2-amino-4-(2-(4-chlorophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carboxylate (**4j**).* Beige solid, yield 72%, mp 141-143 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.13 (m, 2H), 3.50 (s, 3H, -OCH_3), 3.78 (s, 3H, -OCH_3), 4.27 (dd, *J* = 4.8, 2.4 Hz, 1H), 6.80 (dd, *J* = 7.6, 1.2 Hz, 1H, H5), 6.90 (dd, *J* = 8.0, 1.6 Hz, 1H, H7), 6.97 (t, *J* = 8.0 Hz, 1H, H6), 7.53 (dd, *J* = 6.4, 2.0 Hz, 2H, H2'+H6'), 7.59 (m, 1H, H4'), 7.62 (s, 2H), 7.86 (dd, *J* = 6.8, 2.0Hz, 2H, H3'+ H5'). ^{13}C NMR (100 MHz, DMSO-d_6): δ38.18, 48.09, 50.31, 55.59 (-OCH3), 74.30 (C3), 110.61 (C7), 119.57 (C5), 124.04 (C6), 126.88 (C4a), 128.88 (C3'+C5'), 129.88 (C2'+C6'), 135.34 (C4'), 137.89 (C1'), 138.78 (C8a), 146.88 (C8), 161.94 (C2), 168.23, 197.45. Anal. Calcd for $C_{20}H_{18}NO_5Cl$: C, 61.94; H, 4.69; N, 3.61. Found: C, 61.78; H, 4.68; N, 3.57.

*4.1.2.6. Methyl 2-amino-6-bromo-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carboxylate (**4p**).* Yellow solid, yield 72%, mp 145-147 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ3.17 (m, 2H), 3.51 (s, 3H, -OCH_3), 4.29 (dd, *J* = 4.8, 2.4 Hz, 1H), 6.95 (d, *J* = 8.8 Hz, 1H, H8), 7.35 (dd, *J* = 8.8, 2.4 Hz, 1H, H7), 7.45-7.49 (m, 3H), 7.48 (dd, *J* = 6.4, 2.0 Hz, 2H, H3'+ H5'), 7.88 (dd, *J* = 8.0, 0.8Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.51, 47.34, 50.37, 73.94 (C3), 115.58 (C6), 117.77 (C8), 127.94 (C2'+C6'), 128.60 (C3'+C5'), 128.67 (C4a), 130.39 (C7), 130.95 (C5), 133.10 (C4'), 136.67 (C1'), 148.93 (C8a), 161.62 (C2), 168.08, 198.40. Anal.

Calcd for $C_{19}H_{16}NO_4Br$: C, 56.74; H, 4.02; N, 3.48 Found: C, 56.48; H, 4.04; N, 3.50.

*4.1.2.7. Methyl 2-amino-6-bromo-4-(2-(4-chlorophenyl)-2-oxoethyl)-4H-chromene-3-carboxylate (**4r**).* Yellow solid, yield 57%, mp 115-117 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.16 (m, 2H), 3.50 (s, 3H, -OCH_3), 4.27 (dd, *J* = 4.8, 2.4 Hz, 1H), 7.36 (dd, *J* = 8.8, 2.4 Hz, 1H, H7), 7.35 (d, *J* = 8.4Hz, 1H, H8), 7.49 (d, *J* = 2.4 Hz, 1H, H5), 7.54 (dd, *J* = 6.4, 1.6 Hz, 2H, H3'+ H5'), 7.59 (s, 2H), 7.88 (dd, *J* = 11.2, 2.4 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.65, 47.36, 50.38, 73.82 (C3), 117.80 (C8), 128.70 (C3'+C5'), 128.75 (C4a), 128.75 (C6), 129.90 (C2'+C6'), 130.46 (C7), 130.98 (C5), 135.36 (C1'), 138.03 (C4'), 148.93 (C8a), 161.63 (C2), 168.05, 197.48. Anal. Calcd for $C_{19}H_{15}NO_4ClBr$: C, 52.25; H, 3.47; N, 3.21. Found: C, 52.34; H, 3.47; N, 2.93.

*4.1.2.8. Methyl 2-amino-6-chloro-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carboxylate (**4s**).* White solid, yield 56%, mp 116-118 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ3.20 (m, 2H), 3.51 (s, 3H, -OCH_3), 4.29 (dd, *J* = 6.8, 4.4 Hz, 1H), 7.00 (d, *J* = 8.8 Hz, 1H, H8), 7.23 (dd, *J* = 8.8, 2.4 Hz, 1H, H7), 7.35 (d, *J* = 2.8 Hz, 1H, H5), 7.57 (s, 2H), 7.59 (m, 1H, H4'), 7.48 (t, *J* = 7.2 Hz, 2H, H3'+ H5'), 7.88 (dd, *J* = 8.0, 0.8 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.62, 47.33, 50.39, 73.92 (C3), 117.40 (C8), 127.53 (C6), 127.65 (C7), 127.96 (C2'+C6'), 128.07 (C5), 128.24 (C4a), 128.63 (C3'+C5'), 133.13 (C4'), 136.68 (C1'), 148.49 (C8a), 161.69 (C2), 168.12, 198.43. Anal. Calcd for $C_{19}H_{16}NO_4Cl$: C, 63.78; H, 4.52; N, 3.92. Found: C, 63.79; H, 4.61; N, 3.82.

*4.1.3. General procedure for the synthesis of 4H-chromene-3-carbonitrile **5***

Triethylamine (3-5 equiv) was added to a solution of chalcone **3** and malononitrile (2 equiv.) in ethanol. The solution was stirred for 2-23 hours, at room temperature and filtered or first cooled to -20 ºC for 1 hour to 7 days and then filtered and identified as the respective product **5**.

*4.1.3.1. 2-Amino-6-bromo-8-methoxy-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carbonitrile (**5a**).* White solid, yield 42%, mp 177-179 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ3.39 (m, 2H), 3.81 (s, 3H, -OCH_3), 4.06 (t, *J* = 5.2 Hz, 1H), 6.90 (s, 2H), 7.05 (d, *J* = 1.6 Hz, 1H, H7), 7.10 (d, *J* = 2.0 Hz, 1H, H5), 7.61 (tt, *J* = 7.6, 1.2 Hz, 1H, H4'), 7.48 (t, *J* =8.0 Hz, 2H, H3'+H5'), 7.91 (d, *J* = 4.8 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.88, 46.27, 53.82 (C3), 56.15 (-OCH_3), 113.89 (C5), 115.62 (C6), 120.26, 121.62 (C7), 126.57 (C4a), 127.99 (C2'+C6'), 128.65 (C3'+C5'), 133.26 (C4'), 136.61 (C1'), 138.18 (C8a), 147.88 (C8), 161.19 (C2), 197.65. Anal. Calcd for $C_{19}H_{15}N_2O_3Br$: C, 57.16; H, 3.80; N, 7.02. Found: C, 57.35; H, 3.86; N, 6.92.

*4.1.3.2. 2-Amino-6-bromo-4-(2-(4-chlorophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carbonitrile (**5b**).* White solid, yield 68%, mp 169-171 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ

3.38 (m, 2H), 3.81 (s, 3H, -OCH_3), 4.05 (t, *J* = 5.6 Hz, 1H), 6.91 (s, 2H), 7.06 (d, *J* = 2.4 Hz, 1H, H5), 7.10 (d, *J* = 2.4 Hz, 1H, H7), 7.54 (d, *J* = 8.4 Hz, 2H, H3'+H5'), 7.91 (d, *J* = 8.4 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.95, 46.31, 53.65 (C3), 56.13 (-OCH3), 113.89 (C7), 115.63 (C6), 120.20, 121.61 (C5), 126.40 (C4a), 128.70 (C3'+C5'), 129.92 (C2'+C6'), 135.26 (C1'), 138.15 (2C, C8a and C4'), 147.85 (C8), 161.19 (C2), 196.78. Anal. Calcd for $C_{19}H_{14}N_2O_3ClBr$: C, 52.61; H, 3.26; N, 6.46. Found: C, 52.69; H, 3.15; N, 6.34.

*4.1.3.3. 2-Amino-6-bromo-8-methoxy-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4H-chromene -3-carbonitrile (**5c**).* White solid, yield 64%, mp 157-160 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.41 (m, 2H), 3.79 (s, 3H, -OCH_3), 3.81 (s, 3H, -OCH_3), 4.04 (t, *J* = 5.2 Hz, 1H), 6.91 (s, 2H), 7.05 (d, *J* = 2.0 Hz, 1H, H5), 7.10 (d, *J* = 2.0 Hz, 1H, H7), 7.18 (dd, *J* = 8.4, 1.2 Hz, 1H, H6'), 7.37 (t, *J* = 2.4 Hz, 1H, H2'), 7.39 (d, *J* = 8.0 Hz, 1H, H5'), 7.49 (dt, *J* = 8.0, 1.2Hz, 1H, H4'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.92, 46.30, 53.77 (C3), 55.28 (-OCH3), 56.14 (-OCH3), 112.23 (C2'), 113.87 (C7), 115.59 (C6), 119.53 (C6'), 120.27, 120.48 (C4'), 121.61 (C5), 126.52 (C4a), 129.78 (C5'), 138.04 (C1'), 138.18 (C8a), 147.86 (C8), 159.34 (C3'), 161.18 (C2), 197.48. Anal. Calcd for $C_{20}H_{17}N_2O_4Br$: C, 55.95; H, 4.00; N, 6.52. Found: C, 56.09; H, 4.25; N, 6.39.

*4.1.3.4. 2-Amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carbonitrile (**5d**).* White solid, yield 57%, mp 161-163 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.37 (m, 2H), 3.81 (s, 3H, -OCH_3), 4.05 (t, *J* = 5.2 Hz, 1H), 6.91 (s, 2H), 7.06 (d, *J* = 2.0 Hz, 1H, H5), 7.10 (d, *J* = 2.4 Hz, 1H, H7), 7.70 (dd, *J* = 6.8, 2.0 Hz, 2H, H3'+H5'), 7.82 (dd, *J* = 6.8, 2.0 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.97, 46.33, 56.16 (C3), 56.16 (-OCH_3), 113.91 (C7), 115.65 (C6), 120.21, 121.63 (C5), 126.43 (C4a), 127.28 (C4'), 130.04 (C2'+C6'), 131.67 (C3'+C5'), 135.60 (C1'), 138.17 (C8a), 147.87 (C8), 161.21 (C2), 197.02. Anal. Calcd for $C_{19}H_{14}N_2O_3Br_2$: C, 47.73; H, 2.96; N, 5.86. Found: C, 47.84; H, 2.85; N, 5.60.

*4.1.3.5. 2-Amino-6-bromo-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4H-chromene-3-carbonitrile (**5e**).* White solid, yield 25%, mp 134-135 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ3.44 (m, 2H), 3.79 (s, 3H, -OCH_3), 4.08 (t, *J* = 5.2 Hz, 1H), 6.89 (s, 2H), 6.94 (d, *J* = 8.8 Hz, 1H, H8), 7.18 (ddd, *J* = 8.0, 2.4, 0.8 Hz, 1H, H4'), 7.36 (dd, *J* = 7.2, 2.8 Hz, 1H, H7), 7.38 (t, *J* = 2.4Hz, 1H, H2'), 7.42 (d, *J* = 8.0 Hz, 1H, H5'), 7.49 (m, 1H, H5), 7.50 (m, 1H, H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.75, 46.18, 53.73 (C3), 55.31(-OCH3), 112.27 (C2'), 115.83 (C6), 118.03 (C8), 119.55 (C4'), 120.30, 120.51 (C6'), 126.41 (C4a), 129.81 (C5'), 130.58 (C5), 130.68 (C7), 138.04 (C1'), 148.71 (C8a), 159.36 (C3'), 161.25 (C2), 197.52. Anal. Calcd for $C_{19}H_{15}N_2O_3Br$: C, 57.16; H, 3.80; N, 7.02. Found: C, 57.06; H, 3.68; N, 6.92.

*4.1.3.6. 2-Amino-6,8-difluoro-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carbonitrile (**5f**).* Yellow solid, yield 21%, mp 149-151 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.49 (m, 2H), 4.11 (t, *J* = 5.2 Hz, 1H), 7.03 (s, 2H), 7.07 (dt, *J* = 9.2, 4.4, 2.0 Hz, 1H, H5), 7.25 (m, 1H, H7), 7.48

(t, *J* = 8.0 Hz, 2H, H3'+H5'), 7.60 (tt, *J* = 8.8, 6.8, 1.6 Hz, 1H, H4'), 7.92 (dd, *J* = 8.4, 1.2 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.96, 45.61, 53.69 (C3), 103.04 (dd, *J* = 27.0, 21.0 Hz, C7), 109.70 (dd, *J* = 24.0, 3.0 Hz, C5), 120.02, 127.63 (d, *J* = 9.0 Hz, C4a), 127.98 (C2'+C6'), 128.68 (C3'+C5'), 133.35 (C4'), 134.44 (dd, *J* = 11.0, 4.0Hz, C8a), 136.53 (C1'), 149.25 (dd, *J* = 248.0, 14.0 Hz, C6), 157.13 (dd, *J* = 240.0, 11.0 Hz, C8), 160.84 (C2), 197.58. Anal. Calcd for $C_{18}H_{12}N_2O_2F_2$: C, 66.25; H, 3.71; N, 8.59. Found: C, 65.99; H, 3.77; N, 8.40.

*4.1.3.7. 2-Amino-6-chloro-4-(2-oxo-2-(p-tolyl)ethyl)-4H-chromene-3-carbonitrile (**5g**).* White solid, yield 40%, mp 175-177 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ2.35 (s, 3H, -CH_3), 3.39 (m, 2H), 4.08 (t, *J* = 5.6 Hz, 1H), 6.89 (s, 2H), 7.00 (d, *J* = 8.8 Hz, 1H, H8), 7.24 (dd, *J* = 8.4, 2.4 Hz, 1H, H7), 7.30 (d, *J* = 8.0 Hz, 2H, H3'+H5'), 7.37 (d, *J* = 2.4 Hz, 1H, H5), 7.82 (d, *J* = 8.0 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ21.11 (-CH_3), 30.81, 46.03, 53.74 (C3), 117.64 (C8), 120.25, 126.08 (C4a), 127.68 (C5), 127.87 (C6), 127.76 (C7), 128.11 (C2'+C6'), 129.18 (C3'+C5'), 134.64 (C1'), 148.20 (C8a), 143.64 (C4'), 161.26 (C2), 197.13. Anal. Calcd for $C_{19}H_{15}N_2O_2Cl$: C, 67.37; H, 4.47; N, 8.27. Found: C, 67.51; H, 4.23; N, 8.08. The ^{1}H- and ^{13}C-NMR spectra were identical to those reported for the compound synthesized through a previously published procedure [52].

*4.1.3.8. 2-Amino-6-bromo-8-methoxy-4-(2-oxo-2-(p-tolyl)ethyl)-4H-chromene-3-carbonitrile (**5h**).* Yellow solid, yield 12%, mp 129-131 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ2.35 (s, 3H, -CH_3), 3.35 (m, 2H), 3.81 (s, 3H, -OCH_3), 4.04 (t, *J* = 5.2 Hz, 1H), 6.89 (s, 2H), 7.04 (d, *J* = 2.0Hz, 1H, H5), 7.09 (d, *J* = 2.4 Hz, 1H, H7), 7.28 (d, *J* = 8.0 Hz, 2H, H3'+H5'), 7.81 (d, *J* = 8.0 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ21.12 (-CH_3), 30.91, 46.19, 53.85 (C3)56.15 (-OCH_3), 113.87 (C7), 115.60 (C6), 120.25, 121.60 (C5), 126.65 (C4a), 128.11 (C2'+C6'), 129.18 (C3'+C5'), 134.19 (C1'), 138.15 (C8a), 143.62 (C4'), 147.88 (C8), 161.17 (C2), 197.13. Anal. Calcd for $C_{20}H_{17}N_2O_3Br$: C, 58.12; H, 4.15; N, 6.78. Found: C, 57.90; H, 4.29; N, 6.48.

*4.1.3.9. 2-Amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-4H-chromene-3-carbonitrile (**5i**).* White solid, yield 50%, mp 186-188 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ3.45 (m, 2H), 4.08 (t, *J* = 5.6 Hz, 1H), 6.90 (s, 2H), 7.38 (dd, *J* = 8.8, 2.4 Hz, 1H, H7), 6.92 (d, *J* = 8.4Hz, 1H, H8), 7.52 (d, *J* = 2.4 Hz, 1H, H5), 7.70 (d, *J* = 8.8 Hz, 2H, H3'+H5'), 7.84 (d, *J* = 8.8 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ30.76, 46.20, 53.62 (C3), 115.86 (C6), 118.04 (C8), 120.22, 126.30 (C4a), 127.41 (C4'), 130.61 (C5), 130.05 (C2'+C6'), 130.72 (C7), 131.69 (C3'+C5'), 135.58 (C1'), 148.67 (C8a), 161.26 (C2), 197.01. Anal. Calcd for $C_{18}H_{12}N_2O_2Br_2$: C, 52.31; H, 2.93; N, 6.78. Found: C, 52.35; H, 2.69; N, 6.53.

*4.1.3.10. 2-Amino-6-bromo-4-(2-oxo-2-(p-tolyl)ethyl)-4H-chromene-3-carbonitrile (**5k**).* White solid, yield 62%, mp 183-185 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ2.35 (s, 3H, -CH_3), 3.43

(m, 2H), 4.08 (t, *J* = 5.6 Hz, 1H), 6.89 (s, 2H), 6.94 (dd, *J* = 8.8 Hz, 1H, H8), 7.23 (dd, *J* = 2.4, 0.4 Hz, 1H, H5), 7.28 (d, *J* = 8.0 Hz, 2H, H3'+H5'), 7.38 (dd, *J* = 8.8, 2.4 Hz, 1H, H7), 7.81 (dd, *J* = 6.4, 1.6 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ21.12 (-CH_3), 30.74, 46.06, 53.80 (C3), 115.82 (C6), 118.03 (C8), 120.25, 126.52 (C4a), 128.11 (C2'+C6'), 129.18 (C3'+C5'), 130.56 (C5), 130.65 (C7), 134.18 (C1'), 143.64 (C4'), 148.67 (C8a), 161.23 (C2), 197.14. Anal. Calcd for $C_{19}H_{15}N_2O_2Br$: C, 59.55; H, 3.95; N, 7.31. Found: C, 59.39; H, 3.96; N, 7.38.

*4.1.3.11. 2-Amino-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carbonitrile (**5o**).* White solid, yield 27%, mp 136-138 ºC. ^{1}H NMR (400 MHz, DMSO-d_6): δ3.36 (m, 2H), 4.10 (t, *J* = 5.6 Hz, 1H), 6.82 (s, 2H), 6.98 (dd, *J* = 8.0, 0.8 Hz, 1H, H8), 7.05 (td, *J* = 7.2, 1.2 Hz, 1H, H7), 7.23 (m, 2H, H5+H6), 7.47 (t, *J* = 8.0 Hz, 2H, H3'+H5'), 7.60 (tt, *J* = 14.8, 7.6, 1.2 Hz, 1H, H4'), 7.90 (dd, *J* = 8.4, 1.2 Hz, 2H, H2'+H6'). ^{13}C NMR (100 MHz, DMSO-d_6): δ31.09, 46.90, 54.18 (C3), 115.94 (C8), 120.64, 123.92 (C4a), 124.54 (C7), 128.08 (C2'+C6'), 128.10 (C6), 128.11 (C5), 128.76 (C3'+C5'), 133.34 (C4'), 136.74 (C1'), 149.35 (C8a), 161.64 (C2), 197.94. Anal. Calcd for $C_{18}H_{14}N_2O_2$: C, 74.47; H, 4.87; N, 9.65. Found: C, 74.18; H, 4.77; N, 9.91. The ^{1}H- and ^{13}C-NMR spectra were identical to those reported for the compound synthesized through a previously published procedure [52].

4.2. Biological evaluation

4.2.1 Cell lines and culture conditions

Two human breast cancer cell lines Hs578T (basal subtype) and MCF-7 (luminal-A subtype), and a normal breast cell line MCF-10A, were obtained from ATCC (American Type Culture Collection). The two cancer cell lines were cultured in Dulbecco's modified Eagle medium, 4.5g/l glucose (DMEM, Gibco) supplemented with 10% heating activated Foetal Bovine Serum (FBS, Gibco) and 1% antibiotic solution (Penicillin-Streptomycin, Gibco). The normal cell line was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco) supplemented with 5% heating activated Foetal Bovine Serum (FBS, Gibco), 1% antibiotic solution (Penicillin-Streptomycin, Gibco), 1% steroid hormone (Hydrocortisone, Sigma-Aldrich), 0.1% peptide hormone (Insulin, Sigma-Aldrich) and 0.01% protein complex (Cholera Toxin, Gibco). Cells were grown in a humidified incubator at 37 ºC and 5% CO_2. For all assays, DMSO (Dimethyl Sulfoxide, Sigma-Aldrich) controls were used.

To cultivate cells for any assay, sub-confluent cells were reaped by gently washing flasks with phosphate-buffer saline (PBS 1x) and then detaching with trypsin (TrypLE™ Express, Gibco) at 37ºC. To inactivate trypsin DMEM 10% FBS or DMEM/F12 5% FBS were added and cells were collected and centrifuged 5 minutes at 900 rpm. Cells were suspended in new medium and 10µl of cell suspension were collected, in which 20 µl of trypan blue (Trypan Blue Solution,

0.4%, Gibco) was added. Cells were counted in a Neubauer chamber for posterior density calculation.

4.2.2. Cell viability by the Sulforhodamine B assay

For the first screen and to determine the compounds IC_{50} value for the different breast cancer cell lines, cells were plated in 96-well plates, at a 3000 cells/100 µL density per well for all the cell lines (Hs578T, MCF-7 and MCF-10A). Cells were allowed to adhere for 18 to 20 hours (overnight) and then were exposed to various compound concentrations used for a total of 72 hours treatment. Controls were performed with 0.3% or 0.5% of DMSO (compound vehicle) for the first screening and IC_{50} determination, respectively. The compound cytotoxic effect was evaluated by the Sulforhodamine B assay (SRB, TOX-6, Sigma-Aldrich). Spectrophotometric measurement of absorbance was read at 490 nm, using 690 nm as background absorbance (Tecan Infinite M200). GraphPad Prism 5 software was used for the analysis and IC_{50} values from three independent experiments (at least), each one in triplicate, applying a sigmoidal dose-response (variable slope) non-linear regression, after logarithmic transformation.

4.2.3.Proliferation assay

MCF-10A and MCF-7 cells were plated in 96-well plates at a density of 8000cells/100µl, and grown overnight at 37°C in a 5% CO_2 humidified atmosphere. Then, adherent cells were treated with compounds **3e**, **3g**, **3i** and **5c** at IC_{50} and ½ IC_{50} concentrations or 0.5% DMSO (controls) for 24 and 48 hours. After incubation, cells were labelled by addition of 5µl/well BrdU labelling solution (final concentration: 20 µM BrdU) reincubating for 6 hours to allow BrdU to incorporate into the proliferating cell DNA, replacing thymidine. After labelling, culture medium was removed, cells were fixed and DNA was denatured through incubation with 100 µl of FixDenat solution at room temperature. Denaturation of DNA is essential for antibody conjugate binding to the incorporated BrdU. After 30 minutes, FixDenat solution was removed, and 50 µl of Anti-BrdU-POD antibody was incubated for 90 minutes at room temperature. The Anti-BrdU-POD antibody binds to the incorporated BrdU in the newly synthesized cellular DNA. The antibody conjugate was removed and wells were washed three times with PBS 1x. The immune complexes were detected by the addition of Substrate solution (100 µl/well) and the plate was incubated at room temperature until colour development was satisfactory for photometric detection (5-10 minutes). Substrate reaction was stopped by adding 25 µl of 1M H_2SO_4 to each well and softly mixed. The reaction product was quantified by measuring absorbance at 450 nm (reference wavelength: 690 nm) in a microplate reader (Tecan Infinite M200). A blank test was used in each experimental time point, without cells, performing all steps described above. The results of at least three independent experiments (in triplicate) were evaluated with GraphPad Prism 5 software.

4.2.4. Migration assay

MCF-7 were plated in 6-well plates at a density of $9.0x10^5$/2 ml and grown overnight at 37ºC in a 5% CO_2 humidified atmosphere. Two scratches were made with a 200 µl pipette tip in confluent cells. Cells were gently washed with 500 µL PBS 1x and were treated with compounds **3e**, **3g**, **3i** and **5c** at the respective IC_{50}, ½ IC_{50} or 0.5% DMSO (control), for 72 hours. At 0, 12, 24 and 48 hours specific wound sites (four positions for each wound) were photographed at 100x magnification using an Olympus IX51 inverted microscope equipped with an Olympus DP20 Digital Camera System. Assessment of five migration distances were performed with the MeVisLab platform and the percentage of cell migration normalized to the control was evaluated with the GraphPad Prism 5 software. Three independent experiments were performed for each compound.

4.2.5. Cell viability assessed by Annexin/PI assay

MCF-7 cells were seeded in 6-well culture plates and allowed to adhere overnight in DMEM. Cells were treated with IC_{50} concentration of the tested compounds or 0.5% DMSO (controls) for 48h. Triplicates were performed in each experiment. Floating and adherent cells were collected and combined by centrifugation. Elimination of the supernatant was followed by addition of 1 mL of Binding Buffer. To 300 mL of this solution, 8 µL FITC annexin V (BD Pharmingen) and 30 µL of PI (50µg/mL, P1304MP, Invitrogen), were added. Samples were incubated for 15 min at room temperature, in the dark. A further 200 mL of Binding Buffer was added to each sample. PI signal was measured using a FACS LSRII flow cytometer (BD Biosciences®) with a 488 nm excitation laser. The annexin V signal was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic with a 525 nm band pass. Signals were captured and FACS Diva was used as the acquisition software. The percentage of cells in each phase was analysed using the FlowJo 7.6 (Tree Star®) software. Two independent biological replicates were performed.

4.2.6. Protein extraction and Western blot

Cells (MCF-7) were grown in T25 flasks and, at 70-80% confluence, cells were treated with compounds **3e**, **3g**, **3i** and **5c** at the respective IC_{50} or 0.5% DMSO (controls) for 24 hours. After treatment, cells were collected by scraping and centrifuged at 2000 rpm for 5 minutes at room temperature. The supernatant was discarded and the pellet was suspended in lysis buffer (50mM Tris pH 7.6-8, 150mM NaCl, 5mM EDTA, 1mM Na_3VO_4, 10mM NaF, 1% NP-40, 1 % Triton-X100 and 1/7 protease inhibitor cocktail (Roche Applied Sciences)) and incubated for 15 minutes on ice. Lysates were centrifuged at 13000 rpm for 15 minutes at 4 ºC and the

supernatants collected for protein concentration determination using the DC Protein Assay Kit (BioRad).

Twenty µg of total protein of each sample were separated on 12% polyacrylamide gel (100V for 90 minutes) and transferred to a nitrocellulose membrane (100V for 30 minutes). Membranes were blocked with 5% milk in 1x TBS for 60 minutes before overnight incubation with the specific primary antibodies at 4 ºC (rabbit anti-PARP antibody (#9542, Cell Signalling), 1:500 5% milk; mouse anti-caspase-9 antibody, 1:500 5% milk (#9508, Cell Signalling); rabbit anti-BIM antibody, 1:1000 5% BSA (Cell Signalling, #2933), rabbit anti-β-Tubulin antibody, 1:200000 5% BSA (Abcam, ab6046), mouse anti-β-Actin antibody (Santa Cruz Biotechnology, #E1314), 1:500 5% milk). After washing 5 minutes (twice) and more 15 minutes (once) with 0.1% Tween 20, blots were incubated for 1 hour with the respective secondary antibodies at room temperature (anti-rabbit IgG-HRP (#7074) and anti-goat IgG-HRP (#7076) secondary antibodies, 1:2000 5% milk, Cell Signaling). After washing 5 minutes (twice) and further 15 minutes (once) with TBS/0.1% Tween 20, immunoreactive bands were detected with chemiluminescent SuperSignal West Femto Kit (Pierce, Thermo Scientific) on ChemiDoc XRS+ system (BioRad).

4.2.7. Cell Cycle Analysis

MCF-7 cells were plated in 6-well plates at a density of 2.5×10^5cells/2mL/well, and grown overnight at 37°C in a 5% CO_2 humidified atmosphere. Cells were treated with compounds **3e**, **3g**, **3i** and **5c** at the respective IC_{50} or 0.5% DMSO (controls) for 48 hours. Aliquots of cells were collected, pelleted, washed, and fixed with ethanol (70% v/v) for at least 30 min at 4 °C. Cells were twice resuspended in PBS and after centrifugation and elimination of the supernatant, resuspended in a solution containing PBS, PI (50 µg/mL, Invitrogen), RNase A (20 mg/mL, Invitrogen) and Triton X 100. After a final incubation for 1 h in the dark at 50 °C, PI signal was measured using a FACS LSRII flow cytometer (BD Biosciences®) with a 488nm excitation laser, captured and FACS Diva was used as the acquisition software. The percentage of cells in each phase was analysed using the FlowJo 7.6 (Tree Star®) software. Two independent biological replicates were performed.

4.2.8. Immunofluorescence Assay

MCF-7 cells (2.0×10^5cells/mL/well) were seeded in 6-well culture plate and incubated overnight. Cells were treated with 0.5% DMSO (drug vehicle), paclitaxel or compounds **3e**, **3g**, **3i** and **5c** for 12 and 24 hours. Then, cells were fixed with methanol for 20 min at -20ºC and blocked for 30 min in 5% BSA/PBS. Cells were incubated 2 hours with β-tubulin primary antibody (Abcam, ab6046), 1:200 at room temperature. After washing three times with PBS-Tween 0.05%, cells were incubated with the corresponding fluorescence-conjugated secondary

antibody (1:500) for 1 h. The nuclei of cells were labeled with DAPI. All images were captured on an Olympus FV1000 (Japan) confocal microscope, under a 60x oil objective (NA = 1.35). Z-series imaging was acquired using a 405nm laser excitation line for DAPI staining and a 488 nm laser excitation line laser for the AF488 microtubule antibody. The pinhole was adjusted to 1.0 Airy unit of optical slice, and a scan was acquired every 0.5 um along the z-axis. All the images were analyzed using the Olympus software FLUOVIEW Ver.4.2b Viewer (Olympus Corporation).

4.2.9. Statistical analysis

All graphs and statistical analysis were performed with the GraphPad Prism 6 software. Statistical significance was assessed by the t-test and results are presented as normalized means ± SEM

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Highlights

- A series of new chalcone and chromene derivatives were synthesized using efficient procedures;
- Compounds were tested for their anticancer properties in the breast cancer cell model;
- Multiple biological effects were associated with new chalcone- and chromene-based compounds;
- Chalcones showed to be bioactive molecules as cell proliferation inhibitors;
- Chromene **5c** inhibited cell migration, induced apoptosis and triggered cell death by G_2/M cell-arrest.