Universidade do Minho
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New Chromene-Based Drug Candidates for Cancer Treatment

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Olivia Alexandra Elias Pontes
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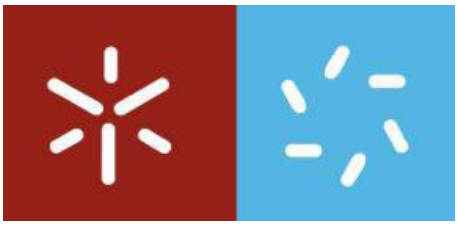

University of Minho

School of Sciences

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New chromene-based drug candidates for cancer treatment

Master Thesis

Applied Biochemistry

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Abstract

Cancer is a devastating disease worldwide, with millions of diagnoses per year and many people living with this pathology. Breast cancer is one of the major causes of death in women while male breast cancer represents less than 1% of the total male diagnosed population. Due to this increasing cancer incidence, research in this area has been growing at the same rate. Thus, there is an urgency in discovering new drugs for cancer treatment.

The chromene scaffold has been identified in several compounds with anticancer activity. The substitution pattern highly influences the activity and the mode of action and the synthesis of new derivatives is an important element in the search for improved drug candidates. The major aim of this Master thesis was the synthesis of new chromene derivatives with enhanced anticancer activity.

Chalcone and chromene derivatives were isolated in good yield through clean reactions using innocuous solvents such as water and ethanol and highly effective aldol condensations. Generally, the reactions were performed at room temperature, leading to the isolation of highly pure compounds.

Newly synthetized compounds were tested on cancer cells and a non-tumoral cell line. For the first screening, a range of 51 compounds was assessed for cell survival by SRB assay on MCF-7 cells. Then, only the 22 most active compounds were tested against another breast cancer cell line (HS578t) and cytotoxicity was evaluated for MCF-10 normal cells. Then, a selection of the eight most promising chalcones and chromenes had their IC_{50} determined for all three cell lines. Finally, some more specific assays were performed and it was found that a selected chromene acted as a cell migration inhibitory agent. Some preliminary results, with protein expression, also showed that this chromene might be causing its anticancer activity through induction of cell-apoptosis. For chalcones, the results suggest an anti-proliferative ability and reduction of membrane integrity.

Generally, compounds with halogenated substituents presented enhanced activity comparing to methoxy 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.

KEYWORDS anti-migratory | breast cancer | chalcones | 4*H*-chromenes | MCF-7 | Michael addition

Resumo

O cancro é uma doença devastadora que em todo o mundo apresenta milhões de diagnósticos por ano e muitas pessoas vivem com esta patologia. O cancro da mama é uma das maiores causas de morte entre a população feminina enquanto que, o cancro da mama masculino afeta menos de 1% de todos os diagnósticos oncológicos nos homens. Devido à crescente incidência de cancro, a investigação farmacológica nesta área tem vindo a crescer na mesma proporção. Assim, a descoberta de novas drogas para tratamento oncológico representa uma urgência.

O núcleo de cromeno foi identificado em diversos compostos com atividade anticancerígena. O padrão de substituição demonstra afetar a atividade e o modo de ação e a síntese de novos derivados é um elemento importante em investigação para melhorar a ação do candidato. O principal objetivo desta tese de Mestrado era sintetizar novos derivados de cromeno com atividade anticancerígena melhorada.

Vários derivados de calconas e cromenos foram isolados com bons rendimentos, através de reações limpas usando solventes inócuos tais como água e etanol, em condensações aldólicas altamente efetivas. Na generalidade, as reações foram realizadas à temperatura ambiente, levando ao isolamento de compostos altamente puros.

Os recém-sintetizados compostos foram testados em linhas celulares de cancro e numa linha celular não-tumoral. Num primeiro *screening*, um lote de 51 compostos foi testado para a viabilidade celular por ensaio com sulforodamina em células MCF-7. Então, apenas os 22 compostos mais ativos foram testados para outra linha celular (HS578t) e a citotoxicidade foi avaliada em células normais MCF-10. Uma série dos oito compostos mais promissores para as três linhas celulares, foi selecionada e teve o IC_{50} determinado. Finalmente, alguns ensaios mais específicos foram elaborados e registou-se que o único cromeno selecionado apresentou poder para inibição da migração celular. Alguns resultados preliminares, para expressão proteica, clarificaram que este cromeno poderá estar a agir como agente anticancerígeno através da indução da apoptose. Para as calconas testadas, os resultados sugerem uma capacidade antiproliferativa e a redução da integridade membranar.

Em geral, os compostos com substituintes halogenados exibiram melhor atividade em comparação com grupos metilo ou metoxilo. Mais especificamente, o átomo de bromo esteve por diversas vezes presente nas moléculas bioativas que procederam para os ensaios finais e demostraram ser candidatos promissores para estudos futuros.

PALAVRAS-CHAVE adição de Michael | anti-migratório | cancro da mama | calconas | 4*H*-cromenos | MCF-7

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Abbreviations

^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance spectroscopy
ºC	Celsius degree
Ar	Aromatic
comp	compound
CSC	Cancer stem cell
d	doublet (relative to NMR peaks)
dd	doublet of doublets (relative to NMR peaks)
dt	doublet of triplets (relative to NMR peaks)
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	half maximal effective concentration
ED_{50}	median effective dose
ER	Estrogen receptor
EWG	electron withdrawing group
GEP	gene expression profiling
GI_{50}	half maximal growth inhibition concentration
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
IC_{50}	half maximal inhibitory concentration
IHC	Immunohistochemistry
IR	Infrared Spectroscopy
LiHMDS	Lithium bis(trimethylsilyl)amide
m	Medium (relative to IR spectra absorption bands)
m	multiplet (relative to NMR peaks)
M	molar
MBC	Male breast cancer
MeOH	methanol
m.p.	melting point
ppm	parts per million
PR	Progesterone receptor
q	quartet (relative to NMR peaks
ROS	Reactive oxygen species
rt	room temperature
s	singlet (relative to NMR peaks)
s	Strong (relative to IR spectra absorption bands)
SRB	Sulforhodamine B

t	triplet (relative to NMR peaks)
td	triplet of doublets (relative to NMR peaks)
THF	tetrahydrofuran
TLC	Thin Layer Chromatograph
tt	triplet of triplets (relative to NMR peaks)
w	Weak (relative to IR spectra absorption bands)
η	yield
δ	chemical shift

CHAPTER I – INTRODUCTION

1. Cancer

Cancer is the name given to a group of related diseases where part of the body cells start to grow uncontrollably and spread into contiguous tissues [1,2]. Cancer is the second leading cause of death in contemporary society, preceded by cardiovascular diseases. In 2012, approximately 14 million people around the world were diagnosed with cancer, about 8.2 million deaths were registered due to this disease and 32.6 million people live with cancer (within 5 years of diagnosis) [1,3].

For the first time, in 2000, Hanahan and Weinberg defined the succession of acquired abilities which allow cancer cells to survive, proliferate and disseminate, as hallmarks of cancer [2,4]. Self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, unlimited replicative potential, sustained angiogenesis and tissue invasion and metastasis were the six hallmarks proposed [2].

Recently, these hallmarks were reviewed and modified by the same authors, including the ability to alter or reprogram cellular metabolism and the capability of evasion to immunological destruction [5]. Consequently, two more consequential features were defined: the inflammation by innate immune cells and the genomic instability and mutation. Additionally, in 2012, Floor and co-workers mentioned another essential characteristic – loss of differentiation [6].

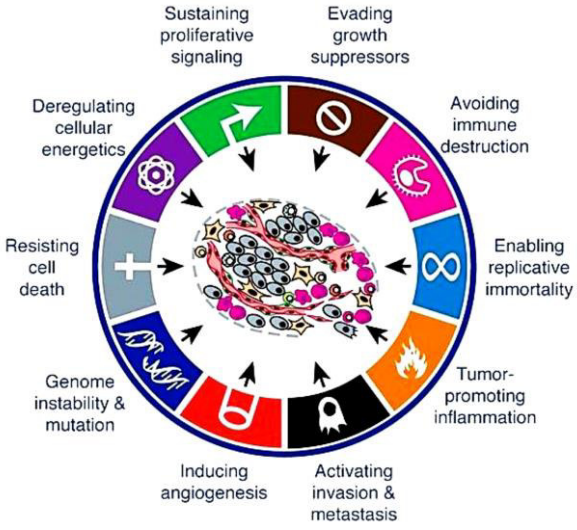


Figure 1. The Hallmarks of Cancer. Schematic illustration of the acquired capabilities of tumor cells essential for tumor growth and progression (adapted from [5]).

These hallmark abilities are acquired through deregulation of signaling pathways which, in combination with the interconnections and crosstalk between the individual sub-circuits, can lead to alteration of multiple capabilities through a certain oncogenic event [5].

Over the years, the knowledge about carcinogenesis has increased and two models were suggested to describe the development of cancer cells. The stochastic model defends that any type of cell is able of initiating and promoting tumor development and the cancer stem cell (CSC) model which suggests that tumors are hierarchically structured and cancer-promoting potential is only present in CSCs [7,8].

According to this information, cancer is a rather heterogeneous pathology related to defects in terms of the regulatory circuits that control cellular homeostasis, including death, proliferation, differentiation and cell migration and, due to this dynamic system, there is an explicit difficulty to reach an effective treatment to this disease [9,10].

1.1. Breast cancer

Breast cancer is the most frequent cancer in women worldwide and the fifth cause of death from cancer, with 522.000 deaths in 2012. Furthermore, with an estimated 1.67 million new cases, it is considered the second most common cancer in the world (Figure 2) [3]. Moreover, successive demographic and epidemiologic transitions, predominantly in less developed countries, have a huge contribution in the increased incidence of cancer and, until 2025, it is expected an occurrence of 20 million cancer cases every year [11]. On the other hand, male breast cancer (MBC), with less than 1% of the total cancer diagnoses in male population, is very uncommon [12,13]. Over the years, MBC mortality is increasing due to lack of male population concern about this pathology. Consequently, male patients are diagnosed in a more advanced stage of the disease [13,14].

In Portugal, for both sexes, breast cancer had an incidence and mortality of 12,4% and 6.5%, respectively, in 2012 [3]. Due to its high incidence, breast cancer is one of the diseases that shows more concern by the scientific community. This fact led to an increasing search for new anticancer drugs with increasing potential for prevention and treatment.

As prevention for breast cancer, a leading medical screening procedure was implemented: mammography. However, not all the cases of breast cancer have successful

endings so it is important to understand this cancer heterogeneity and improve prevention, diagnosis and survival for the specific subtypes [15–18].

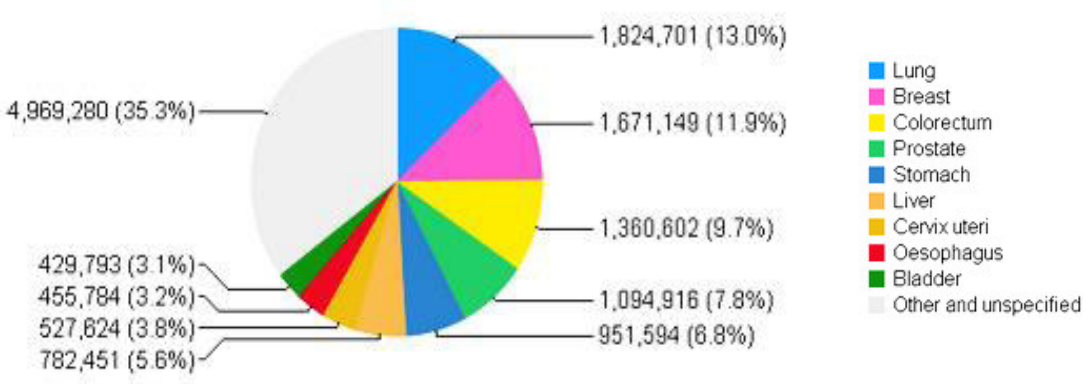


Figure 2. *Estimated incidence for all types of cancer worldwide (adapted from [3]).*

Over the last five decades, the inter and intravariability of breast cancer tumors have been studied by histopathology methods [19]. Recently, new techniques like microarrays and next-generation sequencing, by advances in genetic sequencing and molecular analysis, were combined and employed to describe the multiplicity of breast tumor subtypes [20,21]. Hence, luminal A, luminal B, HER2-overexpressing and triple negative were the breast cancer tumor subtypes defined through genetic and molecular methods (Table 1) [22–24]. This classification was possible according to the evaluation of progesterone (PR) or estrogen receptors (ER) by immunohistochemistry (IHC) and fluorescent *in situ* hybridization. In recent years, as a form to differentiate aggressiveness or tumor cell proliferation in the several types of breast cancer, new tumor procedures for classification, such as Ki-67 status or histologic grade, were labored [22]. This novel established approach aims at more effective treatments in terms of surgery, chemotherapy, radiotherapy or new targeted therapies [23,25,26].

According to the different subtypes of breast cancer, the treatment used will also be changed. From all the four subtypes of breast tumors, luminal A tends to have a best prognosis, with high rates of survival and equally low recurrence rates. Due to the presence of ER-positivity, treatment for these tumors generally includes a diversified hormone therapy as tamoxifen (Figure 3) and/or aromatase inhibitors [27,28].

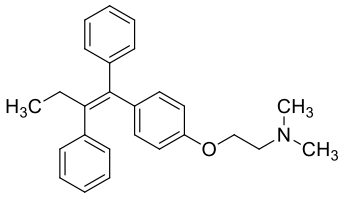


Figure 3. *Hormone therapy agent: tamoxifen.*

Before discovering that HER2 subtype tumors could have a target for treatment, women with these tumors had a very poor prognosis. However, nowadays, for tumors HER2-positive, it is common to treat this cancer subtype with anti-HER2 drugs such as trastuzumab [29].

Table 1. *Molecular subtypes of breast cancer (adapted from* [30]*).*

Intrinsic subtypes (GEP)	***IHC classification (St Gallen)***	***Agreement IHC/GEP***
Luminal A	"Luminal A" ER and/or PR positive HER2 negative Ki-67<14%	73%-100%
Luminal B	"Luminal B (HER2 negative)" ER and/or PR positive HER2 negative Ki-67≥14% "Luminal B (HER2 positive)" ER and/or PR positive Any Ki-67 HER2 over-expressed or amplified	73%-100%
HER2-enriched	"HER2 positive (non-luminal)" HER2 over-expressed or amplified ER and PR absent	41%-69%
Basal-like	"Triple negative" ER and PR absent HER2 negative	80%

(ER, estrogen receptor; PR, progesterone receptor; GEP, gene expression profiling; HER2, human epidermal growth factor receptor 2)

Basal-like tumors are the most aggressive subtype of breast cancer. Because of their negativity for ER and HER2, hormone therapy and trastuzumab cannot be used as treatments. So, generally, a combination of surgery, radiotherapy and chemotherapy is used, making the treatment with larger scope but also more aggressive to the patients [31]. Targeted therapies for basal-like tumors do not exist however treatment options are currently being studied. As

possible targets for coming therapies, epidermal growth factor receptor and androgen receptor were mentioned [32].

1.1.1. Breast cancer treatments

Treatment for breast cancer depends on the general health of the patient as well as the stage and grade of cancer and may include a combination of surgery and radiation, chemical, immunological and targeted therapies. The typical treatment for the patients with stage I tumors involves surgery to remove the cancer and a small amount of tissue around the tumor (lumpectomy) [33]. Stage II patients are considered for adjuvant chemotherapy as a preventive strategy against cancer recurrence [33]. Patients with stage III disease, having a high rate of recurrence and cancer related death, are usually considered for adjuvant chemotherapy [28,29]. However, as referred previously, breast cancer is divided into four different subgroups according to the expression of PR, ER or both. This differential expression on the surface of cancer cells will condition adjuvant chemotherapy. Regarding targeted therapies, tumors with over-expression of HER2 are treated with an anti-HER2 antibody, such as trastuzumab, or a tyrosine kinase inhibitor, such as lapatinib (Figure 4) [30,31]. Triple negative tumors, with absent expression of PR, ER and HER2 and increased rates of metastasis, relapse and death, compared to other breast cancer types, are treated with cytotoxic chemotherapy due to the lack of targeted therapies [31].

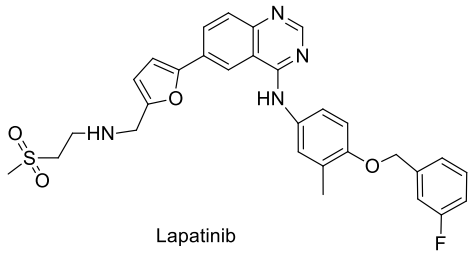


Figure 4. *Chemical structure of drugs used in targeted therapy: lapatinib.*

Radiotherapy treatment uses X-ray or other types of radiation to kill and inhibit the continuous proliferation of cancer cells [37]. Two types of radiation therapy may be performed: external radiation which sends radiation to the cancer from a machine outside the body and internal radiation, that injects radioactive substances into or near the tumor [38]. This procedure will vary depending on the type and stage of treating cancer [35–37].

The cancer treatment that uses drugs to stop tumor cellular growth is defined as chemotherapy. This procedure acts essentially in the hallmark of cancer characterized by the evasion from growth suppressors and its action leads to cellular division arrest (mitosis) or cell death (apoptosis) [42]. This type of therapy has two different ways of administration: systemic chemotherapy, in which the drug enters into the bloodstream and regional chemotherapy, that places the drug directly in the local of action [38,39]. However, as tumor cells share mechanisms of action with normal cells, there are many side effects that limit the patient life during the treatment [44]. Chemotherapy, because of its cytotoxic and non-selective properties, has limited clinical applications [45]. So, hematological malignancies, metastatic solid cancers and primary cancers as an aide to surgery, by their high proliferative index and as an attempt of localized treatment, are the main applications of this type of treatment [42].

The drugs administrated through chemotherapy are divided into classes according to the drug target and its mechanism of action [43]. Hence, some of the highlighted classes with current and common use are:

i. Alkylating agents which damage the DNA by formation of covalent bonds, inducing single-strand or double-strand breaks that lead to DNA cross-linking, preventing cellular proliferation. Chlorambucil [46] and busulfan [47] are two examples of the many alkylating agents currently available (Figure 5).

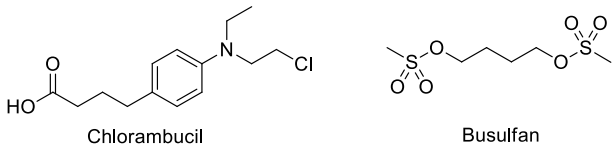


Figure 5. *Chemical structures of chlorambucil and busulfan, two examples of alkylating agents.*

ii. Antimetabolites affect the RNA and DNA growth by the introduction of similar pyrimidine and purine analogues into the strand. S phase is generally affected by these drugs. As examples, methotrexate [48], pemetrexed [49] and 5-fluorouracil (5-FU) are represented in Figure 6 [48].

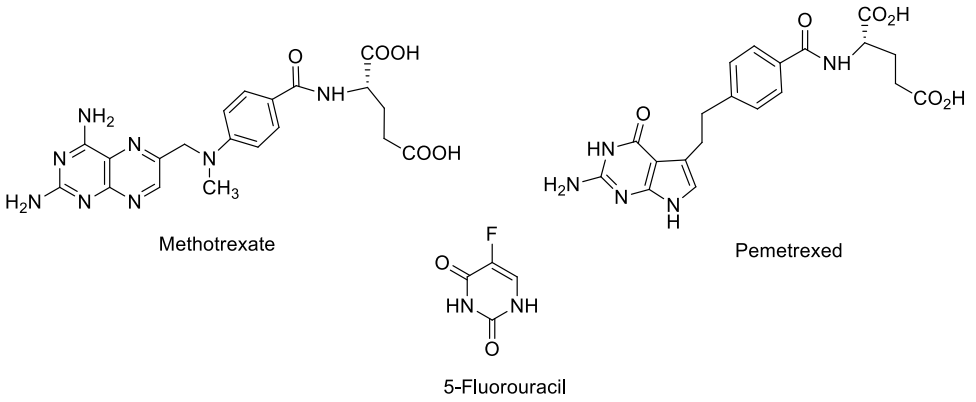


Figure 6. *Antimetabolites chemical structures: the examples of methotrexate, pemetrexed and 5-fluorouracil.*

iii. Anthracyclines are anti-tumor antibiotics that alter the cancer cells DNA, keeping them from proliferate and replicate uncontrollably by DNA intercalation or formation of free radicals. Two of these antibiotics are doxorubicin [50] and daunorubicin [51] (Figure 7).

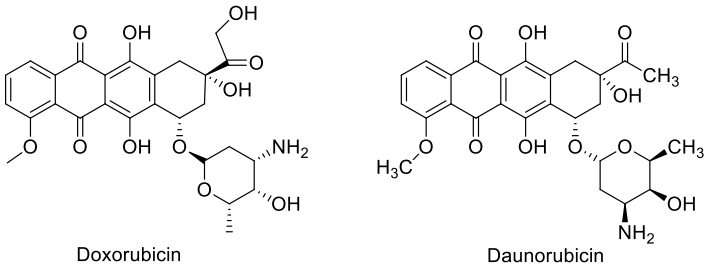


Figure 7. *Chemical structures of doxorubicin and daunorubicin. Two anti-tumor antibiotics.*

iv. Topoisomerase inhibitors influence the DNA strand separation, done by topoisomerase enzymes, that arises during S phase and would result on DNA copies at the end of cell cycle. Etoposide [52] and teniposide [53] are both inhibitors of this enzyme (Figure 8).

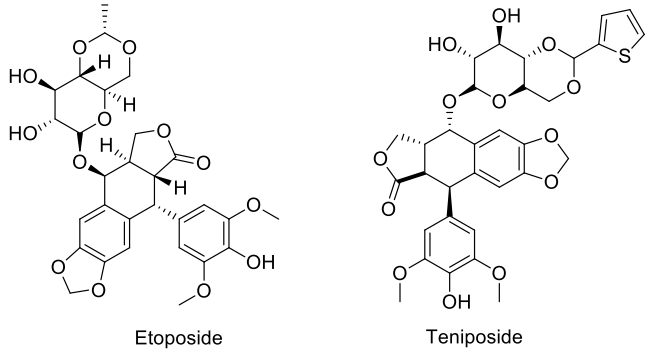


Figure 8. *Two examples of topoisomerase inhibitors: chemical structures of etoposide and teniposide.*

v. Mitotic inhibitors, also known as tubulin-binding drugs, interfere with numerous functions of the cell such as mitosis, meiosis, intracellular transport and others. Paclitaxel [54], docetaxel [55] and eriblin [56] illustrate this class of drugs (Figure 9).

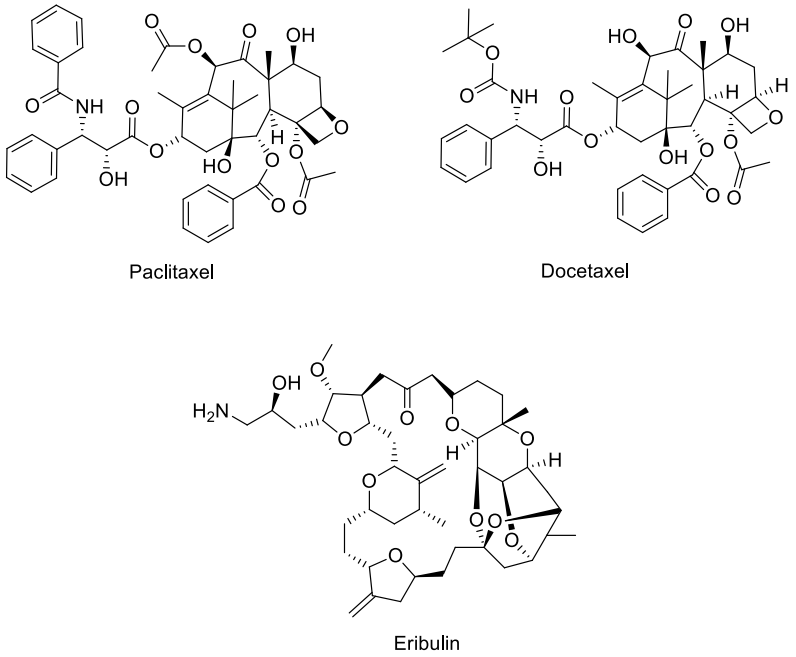


Figure 9. *Chemical structures of paclitaxel, docetaxel and eribulin, some mitotic inhibitors.*

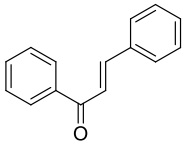
Due to the heterogeneity of most cancers, chemotherapy is usually administrated as a combination of the above referred classes of drugs. This procedure intends to overcome cell resistance and prevents the generation of new resistant subclones [57]. As a benefit to patients, it reduces the side effects of single agents that would have to be used in higher doses in monotherapy [38,53].

In the last few years, research for cancer cures and treatments have made progresses. Targeted agents were recently discovered as potential drug candidates [54,55]. These agents widen the therapeutic window, reducing many side effects of traditional procedures as chemotherapy and radiotherapy [58]. In 2013, der Hollander and research group described that targeted drugs have potential uses as preventive agents [59]. It was revealed that ER-targeting drugs, such as tamoxifen, have shown preventive efficacy for ER-positive breast cancer leading to and effective reduction in incidence. However, the major problem still remains for triple negative breast cancer in which it is necessary to identify possible novel targets, develop non-

toxic drugs that successfully interrupt the activity of these targets and find possible delivery systems for these novel preventive therapies [59].

2. Chalcones

Chemically described as 1,3-diphenyl-2-propen-1-ones, chalcones **1.1** result from the connection of two aromatic rings by a three-carbon α,β-unsaturated carbonyl bond [60] (Figure 10). An aldol condensation between benzaldehyde and acetophenone in the presence of sodium hydroxide as a catalyst is the most efficient method that leads to the preparation of chalcones.



1.1: (*E*)-chalcone

Figure 10. *Chalcone.*

Chalcones are naturally occurring compounds in edible plants that exhibit a wide-range spectrum of biological activities as anti-inflammatory [57,58], antioxidant [63], anti-tubercular [64], anti-HIV [65], antimalarial [66], anti-allergic [67], anti-diabetic [68], cardioprotective [69] and also anticancer agents through multiple mechanisms [70].

2.1. Anticancer properties of chalcones

A general overview of recent works reveals a constant search for naturally occurring chalcones with anticancer activity and novel mechanisms of action. Thus, many natural chalcones with promising anticancer effectiveness against a diversity of cancer cell lines have been identified.

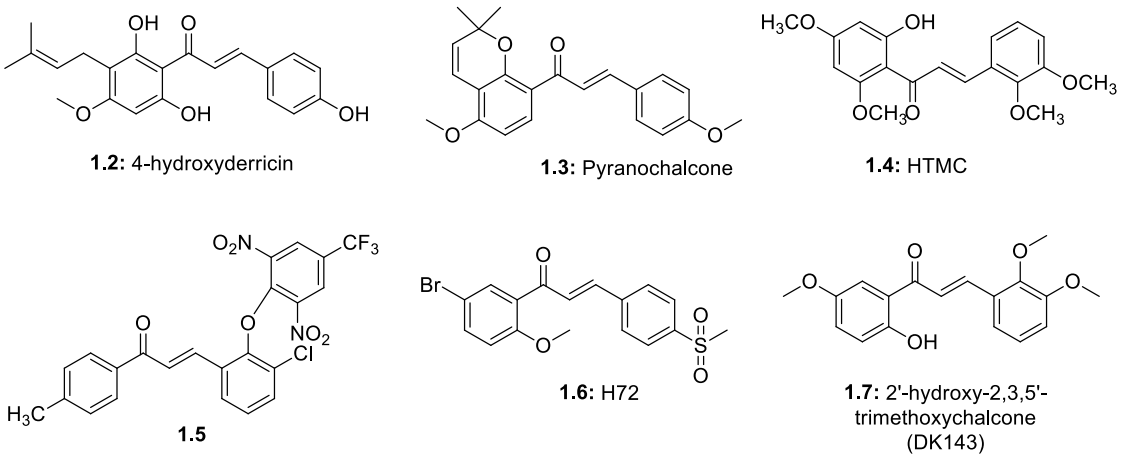


Figure 11. *Natural and synthetic chalcones with anticancer properties.*

Found in *Angelica keiskei* roots, 4-hydroxyderricin **1.2** (Figure 11) was described as an inducer of apoptotic cell death via death receptor-mediated and mitochondrial pathways by topoisomerase II inhibition in leukemia cells (HL60) [71]. Furthermore, this chalcone also exhibited great cytotoxic properties against another three cancer cell lines: A459 (lung), AZ521 (stomach) and CRL1579 (melanoma).

Ye *et al.* found a novel pyranochalcone **1.3** (Figure 11) with strong cytotoxic effect isolated from the seeds of a Chinese medicinal plant, *Milletia pachycarpa*, showing great minimal effective concentration in several cancer cell lines [72]. Moreover, this pyranochalcone showed strong apoptosis inducing effects at a concentration of 2 μM within 36h.

HTMC **1.4** (2'-hydroxy-2,3,4',6'-tetramethoxychalcone) (Figure 11) was extracted from a medicinal plant *Caesalpinia pulcherrima* and demonstrated great *in vitro* selectivity for many human lung cancer cell lines [73]. In A549 (lung adenocarcinoma cells), HTMC presented a cytotoxic activity by causing G_1 phase cell-cycle arrest.

Naturally occurring chalcones revealed promising anticancer properties that led to many synthetically inspired chalcones with enhanced anticancer activity. The manipulation of the aryl rings or replacement by scaffolds with heteroaryl/steroidal/alicyclic and conjugation of pharmacologically interesting scaffolds through molecular hybridization are the three main used strategies to manipulate and enhance the characteristics of chalcones as anticancer agents [74].

Zhang *et al.* described that chalcone **1.5** (Figure 11) presented a remarkable antiproliferative potential with very low IC_{50} values of 0.03 and 0.95 μg/mL for MCF-7 (breast) and A549 (lung) cell lines, respectively [75]. At 1.42 μg/mL, this compound also demonstrated to be a promising antitubulin polymerization inhibitor.

A series of 20 brominated chalcones were designed, prepared and tested in four different gastric cancer cell lines [76]. From all the compounds, H72 **1.6** (Figure 11) was the one that presented more potential with lower IC_{50} and less cytotoxicity to the normal gastric cell line GES-1. The treatment with H72 in MGC803 and HGC27 seems to induce the generation of ROS which causes mitochondria mediated apoptosis and activates the caspase 9/3 cascade.

A synthetic flavonoid derivative, 2'-hydroxy-2,3,5'-trimethoxychalcone **1.7** (DK143) (Figure 11) showed evidences to be involved in apoptosis induction by unfolded protein response in the breast cancer cell line MDA-MB-231 [77]. The production of ROS (Reactive Oxygen Species) and upregulation of ER stress sensors expression are the mechanisms involved in the anticancer activity of this synthetic chalcone. DK143 does not affect viability for the non-transformed cell line MCF10A. *In vivo*, DK143 suppressed mouse tumor growth.

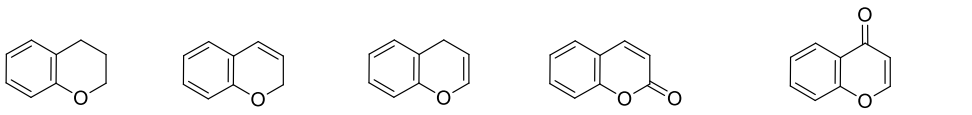
3. Chromenes and coumarines

The fusion of a benzene ring with a heterocyclic pyran ring results in a polycyclic compound named benzopyran. Chromene was the nomenclature adopted by IUPAC to define these compounds. 1-benzopyran (**1.8** and **1.9** in Figure 12) and 2-benzopyran (**1.10** and **1.11** in Figure 11) were the two classes of benzopyrans named by the position of the oxygen atom on the pyran ring [78]. Depending on the level of oxidation and saturation, 1-benzopyran may be named chroman **1.12**, 2*H*-chromene (2*H*-1-benzopyran) **1.13**, 4*H*-chromen (4*H*-1-benzopyran) **1.14**, 2*H*-chromen-2-one **1.15** and 4*H*-chromen-4-one **1.16** (Figure 12) [79].

1.8: 2*H*-1-benzopyran **1.9:** 4*H*-1-benzopyran **1.10:** 1*H*-2-benzopyran **1.11:** 3*H*-2-benzopyran

Figure 12. *1-benzopyrans and 2-benzopyrans.*

Coumarin, also known as 2*H*-1-benzopyran-2-one or 1,2-benzopyrone (Figure 13), and its derivatives are largely disseminated in nature [80]. Present as secondary metabolites in the roots, leaves and seeds of several plant species, coumarines have various and advantageous biological activities [81]. Regulation of plant growth, bacteriostasis, fungistasis and waste products exemplify the not yet completely clear functions of coumarines [81].



1.12: Chroman **1.13:** 2*H*-chromen **1.14:** 4*H*-chromen **1.15:** 2*H*-chromen-2-one **1.16:** 4*H*-chromen-4-one

Figure 13. *Chroman* ***1.12****, 2H-chromene (2H-1-benzopyran)* ***1.13****, 4H-chromen (4H-1-benzopyran)* ***1.14****, 2H-chromen-2-one* ***1.15*** *and 4H-chromen-4-one* ***1.16****.*

Owing to their natural occurrence, chromenes and coumarin derivatives were studied and many therapeutic applications were found including antitumor [60,61], anti-HIV therapy [84], antibacterial [85], antifungal [86], anti-inflammatory [87], anti-coagulant [88], antioxidant [65,67] and antidepressant [68,69]. It is also reported that coumarines present lipid-lowering properties with moderate triglyceride-lowering potential [70,71]. Previously used as fixative and flavoring agents, Food and Drug Administration regulated coumarines as food adulterants because of their side effects including hepatotoxicity. Several European countries use coumarin type drugs for therapeutic purposes and the compounds are administrated to patients with lymphedema [72–74]. Additionally, perfumes, cosmetics, dyes and herbicides were also some applications of coumarine derivatives in industry [97].

3.1. Anticancer activity of chromenes

As described above, several studies were conducted to determine the potential therapeutic applications of chromenes. Since cancer is a disease that affects the population worldwide and chromenes have already proved some anticancer activity, this became an interesting research area. Several natural chromenes and synthetic derivatives were tested for their anticancer properties both *in vivo* and *in vitro*. Their cytotoxic effects and mechanism of action were also studied.

3.1.1. Natural chromenes as anticancer agents

Isolated from *Calophyllum dispar* fruits and stem barks, coumarins **1.17** and **1.18** (Figure 14) inhibited 50% of cellular growth in nasopharyngeal carcinoma KB cell lines. The cytotoxic effect was found to be more effective in a 4 to 8 μg/mL concentration range [98].

Scio *et al.* extracted coumarins **1.19a** and **1.19b** with dichloromethane from the stem bark of a Brazilian plant, *Kielmeyera albopunctata* (Figure 14). These compounds showed active anticancer activity against numerous and varied human cancer cell lines such as Lu1 (lung), Col2 (chondrosarcoma), KB (nasopharyngeal) and LNCaP (prostate), with ED_{50} from 11.1 to 19.8 μg/mL [99].

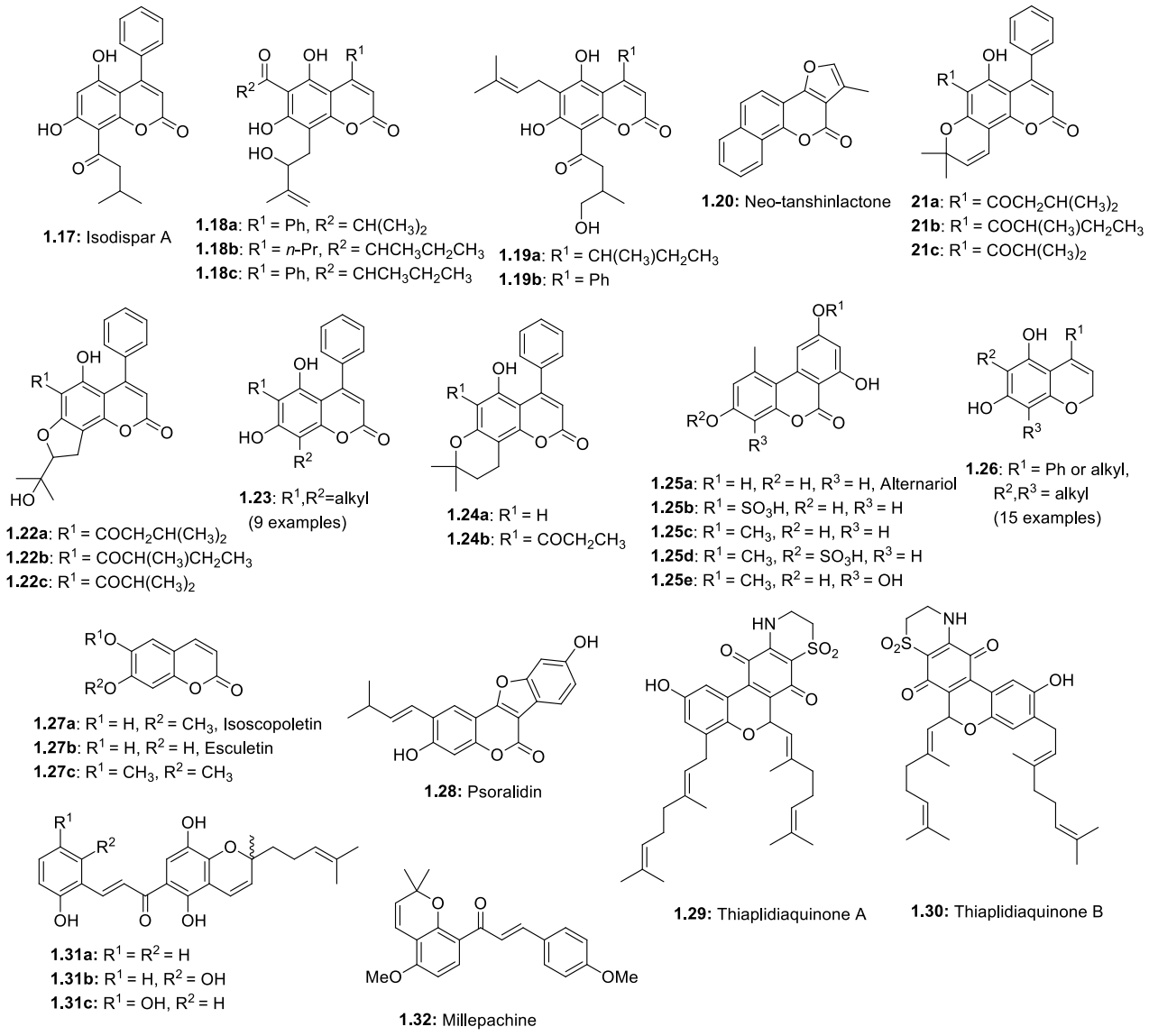


Figure 14. *Series of natural products with observed anticancer activity.*

The ethanol extract from the root of *Salvia miltiorrhiza* led to the isolation of a minor compound, neo-tanshinlactone **1.20** (Figure 14). The anticancer properties of this chromene were assessed for many breast cancer cell lines and a molecular route was proposed for this chromene. In terms of ED_{50}, the molecule showed low values with

concentrations ranging from 0.2 to 0.6 μg/mL for MCF-7 and ZR-75-1, both ER+ overexpressing cell lines, and for one ER-/HER2 (SK-BR-3) [78,79].

17 Chromene-based compounds were extracted from the leaves of a tree native from Panama, Costa Rica and Colombia, *Marila pluricostata*. From this set of compounds, some mammeisin derivatives were recognized (chromenes **1.21**, **1.22** and 9 examples of chromenes **1.23**) (Figure 14) and some molecules were newly discovered (compounds **1.21, 24a** and **1.24b** with R^1 = H and R^2 = $CH_2CHC(CH_3)_2$). This range of compounds exhibited great cytotoxic effect (0.1 μg/mL GI_{50} value) in human cancer cell lines MCF-7, H-480 and SF-268 leading to a promising anticancer activity [102].

Naturally occurring in the Egyptian plant *Polygonum senegalense*, 5 chromenes **1.25** (Figure 14) were isolated from the fungal endophyte *Alternaria sp*. The cytotoxic activity of these molecules was studied against lymphoma cells L5178Y and relevant results were found for compounds **1.25a-c**, with EC_{50} values of 1.7 to 7.8 μg/mL. The inhibitory potential of these chromenes was also assessed in 24 different protein kinases leading to an effective inhibitory power for several enzymes. An interesting inhibition of cellular growth was also found for concentrations lower than 10 μg/mL [81,82].

Du *et al.* isolated 15 coumarins **1.26** (Figure 14) from *Mammea Americana*, a tropical/subtropical plant. These compounds showed anticancer activity in human breast and prostate cancer cell lines through the inhibition of the activation of HIF-1 (hypoxia-inducible factor-1). These molecules demonstrated to have EC_{50} values lower than 10 µM for human breast tumor (T47D) and prostate tumor (PC-3) cell lines. The inhibition of HIF-1 seemed to be enhanced by coumarins bearing a 6-prenyl-8-(3-methyloxobutyl) substituent [105].

Extracted from the roots of *Mallotus resinosus*, coumarins **1.27a-c** (Figure 14) demonstrated a potent antitumor effect by their activity in cleaving DNA. The effectiveness of these molecules for DNA cleavage was not extraordinary, however Ma *et al.* considered that these compounds were valuable leads for future synthetic approaches, because of the simplicity of their structure and also due to their importance as DNA strand scission agents [106].

In 1996, a potent anticancer chromene was isolated from *Psoralea corylifolia*, psoralidin **1.28** (Figure 14). This molecule exhibited cytotoxic effects on colon and breast cancer cell lines however its activity was more effective against gastric tumor cell lines (SNU-1 and SNU-16) [107]. This chromene was also found to have antioxidant [108], antibacterial [109] and antidepressant activity [90].

Aiello *et al.* isolated two new prenylated benzoquinones, thiaplidiaquinones A (**1.29**) and B (**1.30**) (Figure 14), from *Aplidium conicum* an ascidian from the Sardinia coast (Italy) [110]. These compounds were studied by propidium iodide (PI) staining and flow cytometry assessing their cytotoxicity against a human T lymphoma cell line. Thiaplidiaquinones **1.29** and **1.30** were demonstrated to have a pro-apoptotic character. The treated cells showed that both compounds induced a strong production of intracellular ROS that led to the appearance of apoptotic cells.

Recently, flemingins were isolated from leaves of a tropical native plant of Africa, *Flemingia grahamiana* [111]. Flemingins **1.31a** and **1.31c** (Figure 14) seemed to present cytotoxic activity when tested against MCF-7 breast human cancer cell line with IC_{50} of 8.9 and 7.6 µg/mL, respectively. For this reason, authors describe that these compounds could be interesting for the development of novel anticancer agents.

Millepachine **1.32** (Figure 14) is a compound isolated from a leguminosae, *Millettia pachycarpa*, for the first time in 2013 [112]. This compound showed a great anti-proliferative activity in HepG2 (human hepatocellular carcinoma) cell line and flow cytometry showed that millepachine presents the ability of arresting cells in G_2/M phase. When this arrest was investigated, the authors found a relation with the inhibition of cyclin-dependent kinase 1 activity and suggested millepachine induced apoptosis through a mitochondrial apoptotic pathway. Consequently, this compound also induced the formation of ROS. Hence, compound **1.32** may be a potential lead compound for antitumor drugs.

3.1.2. Synthetic chromenes as anticancer agents

A general overview of some recent articles shows that a large number of chromene derivatives have been prepared with enhanced anticancer activity comparing to the natural analogues. The main advantage of synthetic chromenes is the reduction of concentrations needed to induce an improved cytotoxic effect.

4-Arylcoumarins **1.33** (Figure 15) were synthetized and tested for their anticancer potential [113]. The best results were found for coumarins bearing the substituents R^2 = H, R^3 = OMe, R^4 = OH and R^1 = H or R^1 = OMe that inhibited cellular growth of human T cell leukemia cell line (CEM) at a reduced concentration (0.083 and 0.52 µM, respectively). These two compounds showed inhibition of microtubule formation through a pro-apoptotic mechanism, however a nonapoptotic pathway also demonstrated to be involved in the induction of cell death.

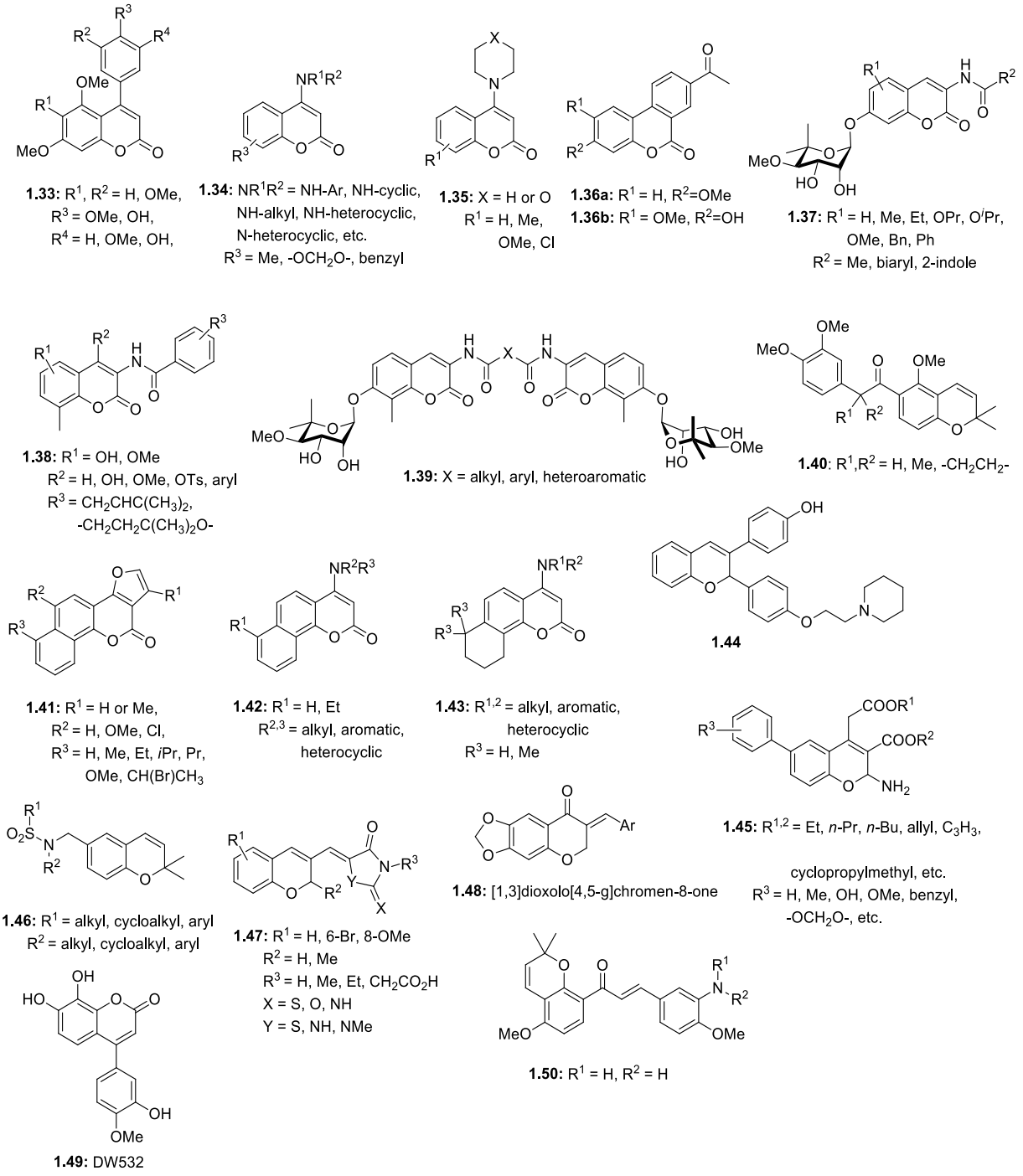


Figure 15. *Numerous synthetic chromene derivatives with reported anticancer properties.*

Di Braccio *et al.* prepared chromene derivatives **1.34** (Figure 15) and assessed their ability to inhibit proliferation and cellular growth in two cell lines (Ehrlich and HeLa cells) [114]. In general, the compounds were only active in one of these tests. However, several molecules were recognized for their antiproliferative and cytotoxic activities, with low IC_{50} values in the 1.74 to 12.9 µM concentration range. The amine moiety and the substituent in the aromatic ring affected the activity of the chromenes. The phenylamino and cycloalkylamino groups proved to

be the most active amino substituents and the tricyclic nucleus, formed by an extra aromatic ring in the chromene moiety, also favored the results.

A series of DNA-dependent protein kinase (DNA-PK) inhibitors was prepared as an attempt to enhance cytotoxicity of DNA-damaging therapies used in cancer therapy [115]. Chromenes **1.35** (Figure 15) carrying a heterocyclic amine in position 4 were studied and the IC_{50} varied between 1.75 and 11.31 μM. The lower IC_{50} value (1.75 μM) was found for the chromene bearing a morpholine group in C_4 and a methoxyl group in position 6 of the aromatic ring.

Schmidt and co-workers prepared nonesteroidal chromene derivatives **1.36a** and **1.36b** (Figure 15) and described, after assessment against primary human vein endothelial cells, that these compounds presented specific inhibitory activity for endothelial cell proliferation [116]. These molecules have such relevance because inhibitors of endothelial cell proliferation are used as treatment of angiogenic pathologies, including solid tumors.

Blagg *et al.* [95–97] and Bras and co-workers [120] prepared and evaluated novobiocin derivatives **1.37** and **1.38** (Figure 15) for their antiproliferative ability and potential heat shock protein 90 (Hsp90) inhibitory properties, both as potential targets for new anticancer agents. Blagg *et al.* proved that derivative **1.37**, bearing a methyl group in R^2 and in C_8 of R^1, were potent inhibitors of the protein-folding mechanism for Hsp90 [117]. These authors studied the antiproliferative action of chromenes **1.37** carrying a substituted biaryl or 2-indolyl group using numerous cell lines of breast (SkBr3 and MCF-7), prostate (LNCaP and PC-3), pancreatic (PL45) and colon (HCT116) cancer and several promising compounds with low IC_{50} values were identified [96,97]. On the other hand, Bras and co-workers tested derivatives **1.38** without the noviose moiety. Varying the substitution pattern in positions 4 and 7 was critical and a tosyl moiety in both positions led to the best results. These compounds not only led to degradation of Hsp90 client proteins, but also to cell death in MCF-7 cell line [120]. Coumermycin A1 derivatives **1.39** (Figure 15) were also tested and showed inhibitory power against Hsp90 protein folding process [121].

Compounds **1.40** (Figure 15) showed antiproliferative effect and inhibitory activity for Hsp90, by suppressing HIF-1α (hypoxia inducible factor alpha) client protein. These chromenes exhibited excellent antiproliferative activity in the nano-molar concentration range for H1299 cell line (lung carcinoma). The antiangiogenic properties were also studied using zebrafish embryos and a dose dependent manner was observed (0.25-1.25 μM) [122].

A variety of neo-tanshinlactone analogues **1.41** (Figure 15) were synthetized and tested for their anticancer activity in several breast cancer cell lines (MCF-7, ZR-75-1, MDA-MB-231,

HS587T and SK-BR-3), non-small cell lung cancer (A549), skin cancer (A431), prostate cancer (LN-CaP and PC-3), colon cancer (SW620), nasopharyngeal carcinoma (KB) and vincristine-resistant KB subline (KB-VIN) [123]. Chromene **1.41** bearing a methyl group in the R^1 position and a methyl or ethyl group in the R^3 position led to the best inhibition of cell growth and also to the best selectivity values, showing that the substitution pattern in the furan and the neighboring aromatic ring strongly influenced the activity. This compound showed ED_{50} of 0.45 μg/mL for MCF-7 and 0.18 μg/mL for ZR-75-1. In a different work, the same research group investigated a new set of neo-tanshinlactone analogues **1.41**, varying the substitution pattern in different ring positions [124]. Once again several chromenes were identified as promising anticancer agents with low ED_{50} values (lower than 1 μg/mL). The methyl group in the R^1 position and alkyl groups in R^3 led once again to the best results.

Dong *et al.* prepared analogous chromenes **1.42** (Figure 15), bearing an amine moiety in C_4 instead of a furan ring. The cytotoxicity of these compounds was assessed for several tumor cell lines [125]. In terms of substitution pattern, the most advantageous in the tested lines showed to be the aromatic, due to the low ED_{50} values (0.01-5.8 µM). Later, the same authors synthetized similar chromenes **1.43** (Figure 15) [126]. These molecules involved a five or six-membered ring appended to the aromatic chromene unit. On the other hand, chromenes **1.42** had a second aromatic ring. Compounds combining a six-membered ring and a 4-methoxyphenyl group in the amine unit, with R^3 = H or Me, presented great cytotoxic effect against different human tumor cell lines from diverse tissues, with ED_{50} values from 0.008 to 0.32 µM.

Compound **1.44** (Figure 15) described by Chandra and co-workers was studied as an inhibitory of hyperplasia formation agent using estradiol-induced rat uterine hyperplasia [127]. Atypical hyperplasia and consequent cancer formation in the endometrium was induced by estrogen hormones which primarily change the structure of the tissue in the uterus. The molecule was injected in the ovariectomized rats at 100 and 200 µg/kg concentrations. The results suggest that this chromene induces apoptosis via the intrinsic pathway and inhibits the formation estradiol-induced hyperplasia in rat uterus.

Xing *et al.* evaluated the cytotoxic effect of 4*H*-chromene **1.45** and derivatives in various tumor cell lines (Figure 15). These authors were mostly interested in compounds that selectively target drug-resistant cancer cells to overcome this problem. Cancer cells overexpressing the antiapoptotic Bcl-2 family proteins were treated with compound **1.45**, with R^1 = R^2 = Et and R^3 = H, and they perform an antagonist role for malignant cells indicating selective cytotoxicity and overcome drug resistance [106,107]. This compound induced cell death through the endoplasmatic reticulum (ER) pathway, causing stress and ER Ca^{2+} release. Compound **1.45** with

$R^1 = R^2$ = Et and R^3 = 3,5-OMe led to more promising results and demonstrated lower micromolar cytotoxicity against a series of hematologic and solid tumor cells (11 different cancer cell lines) [108,109]. The performance of this chromene was also assessed in two drug-resistant cancer cells for camptothecin (CCRF-CEM/C2) and mitoxantrone (HL-60/MX2) and selectively killed these resilient cells over parent cancer cells. A structure-activity relationship study resulted in the synthesis of other analogues of compound **1.45**, bearing similar substitution pattern in R^3 as the former compound, but with $R^1 = R^2 = CH_2C{\equiv}CH$ [131]. This compound presented improved activity in the drug resistant HL60/MX2 human leukemia cell line with an IC_{50} value of 640 nM.

The activation of HIF pathway aids tumor cells to adapt to low levels of oxygen (hypoxia), leads to therapeutic resistance and has become a vital target for several cancer therapies. 2*H*-Chromenes **1.46** (Figure 15) demonstrated to inhibit HIF-1, when assessed against LN229-HRE-Lux (human glioma cell line) using hypoxia conditions [110,111]. The substitution pattern was studied and when R^2 = phenyl group and the R^1 substituent was varied between a 3,4-dimethoxyphenyl, 4-methoxyphenyl or 3,5-dimethylphenyl moiety, IC_{50} values were lower (nM range). It was demonstrated that when compound **1.46** bare a sulfone group a 3,4-dimethoxyphenyl and an aromatic ring in the amine moiety (IC_{50} value of 650 nM) the levels of HIF-1 were not modified, though the ability of HIF-1α/HIF-1β to interact with cofactors p300/CBP was affected interfering with the creation of an active transcriptional complex.

2*H*-Chromene **1.47** (Figure 15) were also evaluated as possible anticancer agents, using four human cell lines: chronic myelogenous leukemia (K562), alveolar basal epithelial adenocarcinoma (A549), acute lymphoblastic leukemia (MOLT-4) and breast adenocarcinoma (MCF-7) [134]. The chromene with R^1 = 6-Br and a hydantoin moiety in the 3-position (X = O, Y = NH and R^2 = H) led to the best results, with similar IC_{50} values to a common anticancer agent, cisplatin.

A series of homoisoflavonoid derivatives **1.48** (Figure 15) were investigated against three human breast cancer cell lines including MCF-7, T47D and MDA-MB-231 by a MTT assay and acridine orange/ethidium bromide double staining technique [135]. From the results of the MTT assay results, researchers concluded that these derivatives had high activity against MCF-7, T47D and MDA-MB-231 cell lines with IC_{50} values of 6.2±0.1, 4.6±0.1, and 9.3±2.1 μg/ml, respectively. However, the cytotoxic power of the compound seems to be influenced by the presence of benzo[*d*][1,3]dioxole in the benzylidene moiety, decreasing it. According to the results of the double staining technique with acridine orange and ethidium bromide, these compounds may induce apoptosis in cancer cell lines by nuclear DNA fragmentation.

Hematoxylin is a natural compound that demonstrated to have an inhibitory power against tyrosine kinases and *in vitro* antitumor activity. Hematoxylin analogue DW534 (compound **1.49** in Figure 15) was synthetized and tested in different cell lines such as HT-29 (human colorectal adenocarcinoma), K562 (human erythromyeloblastoid leukemia), T47D (human breast cancer), MCF-7 (human breast adenocarcionoma), PC-3 (prostate cancer), HCT-116 (human colon carcinoma), A549 (human lung carcinoma) and MDAMB-231 (human breast cancer), by ELISA assay and Western-Blot. According to the results obtained, DW532 inhibits cell growth, arrests cell cycle and leads to apoptosis selectively in cancer cells. It was also demonstrated that compound **1.49** suppressed angiogenesis *in vitro* and *in vivo*.

Some derivatives of compound **1.32** (Figure 14) were recently synthetized and these included compound **1.50** (Figure 15) [136]. As it happened with **1.32**, this novel compound presented activity through cellular arrest in G_2/M phase and also a pro-apoptotic effect. The major advantage achieved with this synthesis was the reduction of the IC_{50} value from 2.86 µM, in the initial compound **1.32**, to 18 nM in **1.50**.

Due to the increasing cancer incidence, research for novel and effective anticancer drugs has been growing at the same rate. Thus, chromenes have proven their anticancer properties as natural product and the enhanced abilities when synthetized in laboratory.

Aims

The skills developed over the years by the organic chemistry group at the Chemistry Research Center of the University of Minho in the area of chromene chemistry resulted in the development of a variety of novel and potentially bioactive molecules, including chromene derivatives [82,133–136]. This project was conducted with the general aim to develop synthetic methodologies to generate new chromene derivatives with anticancer properties.

Inspired by the known anticancer potential of chromenes as well as chalcones and also on the results of an *in silico* screening of the library of chromene-based compounds prepared by the research group, a sequential synthetic method will be thought and optimized to allow the isolation of these molecules and test them in breast cancer cell lines. The chalcones and chromene scaffolds were identified as promising core units by preliminary results and a number of derivatives will be prepared by varying the substitution pattern.

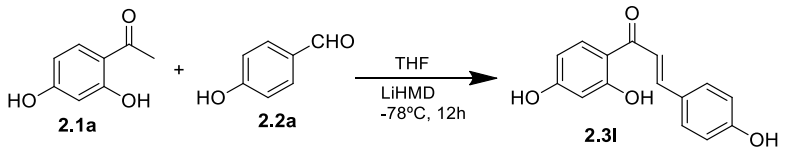
The synthesized chalcones and chromenes will be first-screened for their anticancer potential inhibiting cell survival using luminal and basal-like breast cancer models, MCF-7 and HS578t cell lines, respectively [141]. The effects of the most interesting and promising compounds will be further characterized by assessing cell viability, cell proliferation, cell migration and expression levels of several proteins. Evaluation of the toxicity for the most active compounds to normal cells will be also performed using a non-neoplasctic breast MCF-10 cell line.

CHAPTER II – CHEMICAL SYNTHESIS:
RESULTS AND DISCUSSION

1. Chalcone derivatives

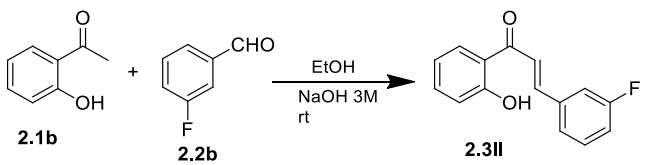
Our synthetic approach to prepare chromenes requires the synthesis of substituted α,β-unsaturated carbonyl compounds and this section emphasizes the different procedures that have been used to generate these molecules from aromatic aldehydes and acetophenones. Several synthetic approaches have been reported, based on the aldol condensation catalyzed by base or acid. The aldol condensation catalyzed by base is still the most commonly used method for the formation of these structures. This procedure is simple and environmentally benign (water and ethanol are the most commonly used solvents) and the product is usually isolated in good yield. The basic catalysis is often, due to NaOH, KOH or $Ba(OH)_2$. However, for a wider perspective of the extent and importance of the synthesis of these derivatives, some relevant studies mediated by catalysts of a different nature will be mentioned.

In 1999, Daskiewicz *et al.* synthetized 2-hydroxychalcones by a LiHMDS-mediated (LiHMD) aldol condensation combining appropriate acetophenones and benzaldehydes [142]. The reaction with the aldehyde occurred after metalation of the ketone, in dry THF. As an example, Scheme 1 shows one of these synthesis, carried out during 12 hours at -78ºC.



Scheme 1. 2-Hydroxychalcone synthesis by a LiHMDS-mediated aldol condensation

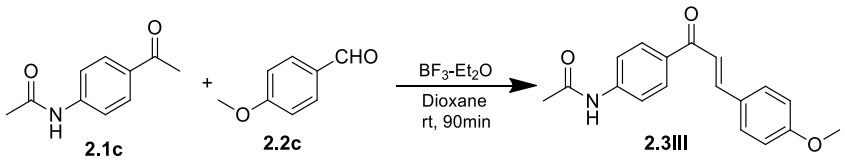
In 2013, Dias *et al*. obtained a wide range of 2'-hydroxychalcones from the combination of 2-hydroxyacetophenone and appropriate aromatic aldehydes [138]. The reaction proceeded under the typical conditions for the aldol condensation with aqueous base (NaOH), at room temperature and an example is presented in Scheme 2. (*E*)-3-(3-Fluorophenyl)-1-(2-hydroxyphenyl)prop-2-en-1-one (**2.3ll**) was isolated with a 99.2% yield. This synthetic method was very effective and eco-friendly and, as consequence, this will be the main method optimized in this thesis.



Scheme 2. Synthesis of 2'-hydroxychalcones under basic catalysis.

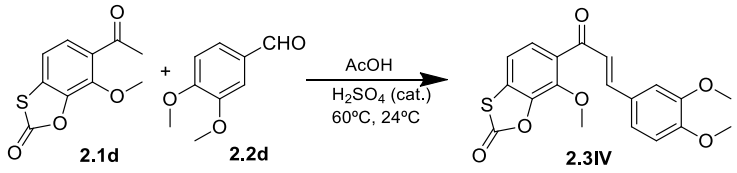
One the other hand, acid catalysis for chalcone synthesis is not as common in the literature as reactions catalyzed by base. Nevertheless, two reported examples will be presented, using Lewis acids (Scheme 3) or protic acids (Scheme 4).

In 2007, Narender *et al.* reported the synthesis of numerous chalcones through the reaction of different acetophenones and substituted benzaldehyde in dioxane using 0.5 eq of BF_3-Et_2O. The final products (**2.3III**) were isolated in 75 to 96% yield [143]. Scheme 3 shows the synthetic procedure for one of the analogs, synthetized at room temperature.



Scheme 3. Chalcone synthesis under borontrifluoride-etherate catalysis.

In the same year, Konieczny *et al.* described the synthesis of *α*,*β*-unsaturated carbonyl compounds with an oxathiolone ring, under acetic acid and sulfuric acid catalysis [144]. As the oxathiolone ring was alkali-sensitive in both the starting material and final product, this approach was preferable instead of the traditional base-catalyzed Claisen-Schmidt condensation. A substituted acetophenone was combined with a substituted benzaldehyde and led to the isolation of (*E*)-6-(3-(3,4-dimethoxyphenyl)acryloyl)-7-methoxybenzo[d][1,3]oxathiol-2-one (**2.3IV**) in 50% yield (Scheme 4).



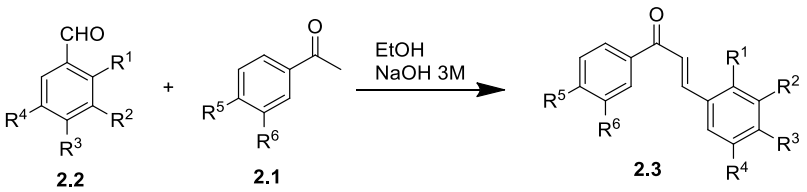
Scheme 4. Acidic catalysis of acetophenone with 3,4-dimethoxybenzaldehyde reaction.

1.1. Synthesis and mechanistic discussion

In this work, the synthesis of *α*,*β*-unsaturated carbonyl compounds was performed under base-catalyzed aldol condensation conditions. Thus, the reaction of aromatic benzaldehydes with acetophenones occurred in EtOH and aqueous NaOH (3M) with magnetic stirring, generally, at room temperature (**2.3c**, **2.3d**, **2.3e**, **2.3f**, **2.3g**, **2.3h**, **2.3i**, **2.3j**, **2.3k**, **2.3l**,

2.3m, **2.3n**, **2.3o**, **2.3p**, **2.3r**, **2.3s**, **2.3t**, **2.3u**, **2.3v**, **2.3w**, **2.3x** and **2.3z**). However, some reactions required heating at 40ºC (**2.3a**, **2.3b** and **2.3q**) or 60ºC (**2.3y**) (Table 2).

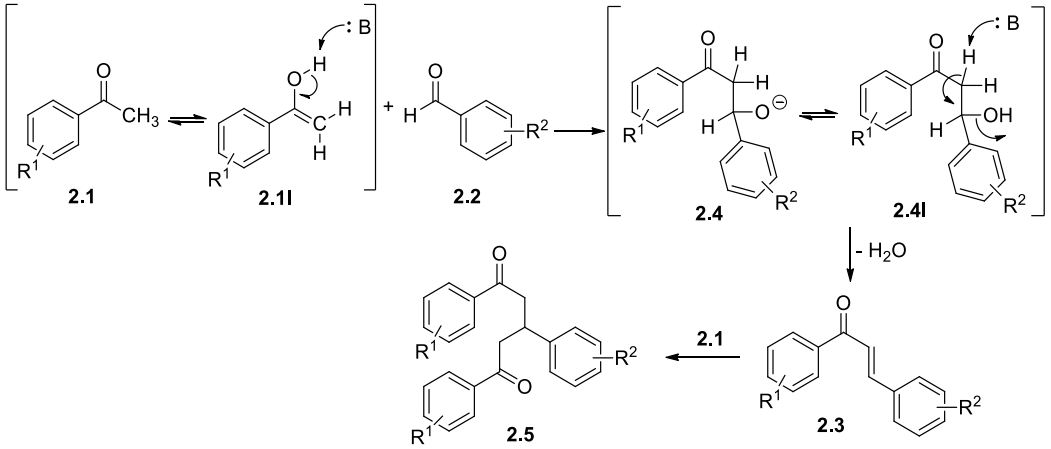
Table 2. *General substitution pattern of chalcones **2.3a-z**.*



Comp	R^1	R^2	R^3	R^4	R^5	R^6
2.3a	OH	OCH_3	H	Br	H	H
2.3b	OH	OCH_3	H	Br	Cl	H
2.3c	OH	OCH_3	H	Br	H	OCH_3
2.3d	OH	H	H	Cl	H	OCH_3
2.3e	OH	H	H	Br	H	OCH_3
2.3f	OH	F	H	F	H	H
2.3g	OH	H	H	Cl	CH_3	H
2.3h	OH	OCH_3	H	Br	CH_3	H
2.3i	OH	H	H	Br	Br	H
2.3j	OH	OCH_3	H	H	Cl	H
2.3k	OH	H	H	Br	CH_3	H
2.3l	OH	F	H	F	CH_3	H
2.3m	OH	OCH_3	H	Br	Br	H
2.3n	H	H	H	Br	CH_3	H
2.3o	OH	H	H	H	H	H
2.3p	OH	OCH_3	H	H	H	H
2.3q	OH	H	H	Br	H	H
2.3r	OH	H	H	Cl	Cl	H
2.3s	OH	F	H	F	Cl	H
2.3t	OH	H	H	Cl	H	H
2.3u	OH	H	H	CH_3	Cl	H
2.3v	OH	H	H	Br	Cl	H
2.3w	OH	F	H	F	H	OCH_3
2.3x	OH	F	H	F	H	Br
2.3y	OH	H	OCH_3	H	Cl	H
2.3z	OH	H	H	Br	F	H

The synthetic mechanism proposed to obtain these compounds was already extensively studied and reported in the literature. It involves nucleophilic attack of the enol form from the ketone **2.1l** to an electrodeficient carbon from the carbonyl of the aldehyde **2.2**. This attack leads to an intermediate β-hydroxycarbonilic compound **2.4**. Finally, dehydration produces the respective conjugated enone **2.3** (Scheme 5). As will be described later, some reactions led to

the isolation of structure **2.5**, where the *α,β*-unsaturated carbonyl compound further incorporated another unit of acetophenone **2.1** by Michael addition to the β-carbon atom.



Scheme 5. *Synthesis of chalcones **2.3** from substituted acetophenones and benzaldehydes*

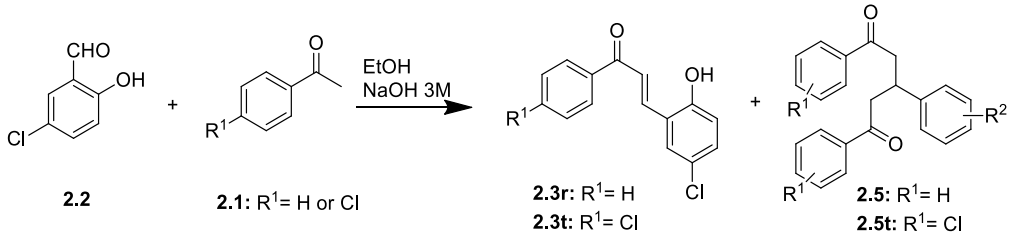
Table 3. *Physical and analytical data for chalcones **2.3a-z**.*

Comp	R^1	R^2	η (%)	m.p. (ºC)	Molecular Formula/Weight
2.3a	H	2-OH;3-OCH_3;5-Br	99.7	175-177	$C_{16}H_{15}BrO_3$/335.2gmol^{-1}
2.3b	4'-Cl	2-OH;3-OCH_3;5-Br	65.6	163-165	$C_{16}H_{15}BrClO_3$/369.6gmol^{-1}
2.3c	3'-OCH_3	2-OH;3-OCH_3;5-Br	99.7	148-150	$C_{17}H_{17}BrO_4$/365.2gmol^{-1}
2.3d	3'-OCH_3	2-OH;5-Cl	86.0	158-159.5	$C_{16}H_{15}ClO_3$/290.1gmol^{-1}
2.3e	3'-OCH_3	2-OH;5-Br	81.8	162-164	$C_{16}H_{15}BrO_3$/335.2gmol^{-1}
2.3f	H	2-OH;3,5-F	75.0	180-181.5	$C_{15}H_{14}F_2O_2$/262.1gmol^{-1}
2.3g	4'-CH_3	2-OH;5-Cl	87.3	179-181	$C_{16}H_{15}ClO_2$/274.1gmol^{-1}
2.3h	4'-CH_3	2-OH;3-OCH_3;5-Br	76.6	192.6-195	$C_{17}H_{17}BrO_3$/349.2gmol^{-1}
2.3i	4'-Br	2-OH;5-Br	70.0	171-173	$C_{15}H_{12}Br_2O_2$/384.1gmol^{-1}
2.3j	4'-Cl	2-OH,3-OCH_3	83.8	141.5-143	$C_{16}H_{15}ClO_3$/290.7gmol^{-1}
2.3k	4'-CH_3	2-OH;5-Br	77.8	191-193	$C_{16}H_{15}BrO_2$/319.2gmol^{-1}
2.3l	4'-CH_3	2-OH;3,5-F	78.8	202-204	$C_{16}H_{14}F_2O_2$/276.3gmol^{-1}
2.3m	4'-Br	2-OH;3-OCH_3;5-Br	80.3	189-191	$C_{16}H_{14}Br_2O_3$/414.1gmol^{-1}
2.3n	4'-CH_3	5-Br	96.8	127-128.5	$C_{16}H_{15}BrO$/302.2gmol^{-1}
2.3o	H	2-OH	48.4	169-171	$C_{15}H_{14}O_2$/226.3gmol^{-1}
2.3p	H	2-OH,3-OCH_3	94.0	262-264	$C_{16}H_{16}O_3$/256.3gmol^{-1}
2.3q	H	2-OH;5-Br	85.2*	-	$C_{15}H_{13}BrO_2$/304.2gmol^{-1}
2.3r	4'-Cl	2-OH;5-Cl		165-167	$C_{15}H_{12}Cl_2O_2$/295.2gmol^{-1}
2.3s	4'-Cl	2-OH;3,5-F	63.4*	-	$C_{15}H_{14}ClF_2O_2$/296.7gmol^{-1}
2.3t	H	2-OH;5-Cl	87.3*	-	$C_{15}H_{13}ClO_2$/260.7gmol^{-1}
2.3u	4'-Cl	2-OH;5-CH_3	43.0*	-	$C_{16}H_{15}ClO_2$/304.2gmol^{-1}
2.3v	4'-Cl	2-OH;5-Br	75.2*	-	$C_{15}H_{12}BrClO_2$/339.6gmol^{-1}
2.3w	3'-OCH_3	2-OH;3,5-F	39.2*	-	$C_{16}H_{14}F_2O_3$/292.3gmol^{-1}
2.3x	4'-Br	2-OH;3,5-F	38.6*	-	$C_{15}H_{11}BrF_2O_2$/340.1gmol^{-1}
2.3y	4'-Cl	2-OH;3-OCH_3	10.4	156-158	$C_{16}H_{15}ClO_3$/290.1gmol^{-1}
2.3z	4'-F	2-OH;5-Br	96.7*	-	$C_{15}H_{12}BrFO_2$/323.2gmol^{-1}

* the compound did not present a satisfactory purity degree, according to ^{1}H NMR data.

One of the main aims was to vary the substitution pattern in both aromatic rings. However, many limitations in the optimization of the reaction conditions were observed. Particularly, the reaction of 5-chloro-2-hydroxybenzaldehyde with substituted and non-substituted acetophenone (Table 4) led usually to complex product mixtures in aqueous base and ethanol. Table 4 summarizes the reaction conditions that were used and the corresponding product mixtures identified by ^{1}H NMR.

Table 4. *Attempted reactions, experimental conditions and obtained products.*



2.2

2.1: R^1= H or Cl

2.3r: R^1= H
2.3t: R^1= Cl

2.5: R^1= H
2.5t: R^1= Cl

Experiment	Reagent 2.2	Reagent 2.1	Reactional conditions	Product by ^{1}H NMR
1	3.24mmol	R^1=Cl (2.95mmol, 0.9eq)	EtOH (10mL) + 3M NaOH (10mL) 1: 40ºC, 4h 2: 60ºC, 12h30	Dark yellow solid **2.3t** : **2.5** : **2.2** (1:0.34:0.33)
2	3.52mmol	R^1=Cl (3.52mmol, 1.0eq)	EtOH (10mL) + 3M NaOH (10mL) IKA, 300rpm, 50ºC, 4 days	Dark oil – complex mixture
3	3.33mmol	R^1=Cl (3.67mmol, 1.1eq)	EtOH (12mL) + 3M NaOH (10mL) rt, 19h	Yellow solid **2.3t** : **2.5** : **2.2** (1:0.41:0.06)
4 (as 2.3t in Section V)	1.88mmol	R^1=Cl (2.07mmol, 1.1eq)	EtOH (6mL) + 3M NaOH (6mL) rt, 6h	Yellow solid (η=88.3%) **2.3t** and traces of **2.5**
5	1.68mmol	R^1=Cl (1.84mmol, 1.1eq)	EtOH (10mL) + 3M NaOH (10mL) rt, 19h	Dark yellow solid **2.3t** : **2.5** : **2.2** (1:0.70:0.06)
6	1.37mmol	R^1=H (1.50mmol, 1.1eq)	EtOH (5mL) + 3M NaOH (5mL) rt, 19h	Yellow solid **2.3r** : **2.5** : **2.2** (1:0.15:0.06)
7	2.05mmol	R^1=H (2.25mmol, 1.1eq)	EtOH (12mL) + 3M NaOH (12mL) rt, 19h	Dark yellow solid **2.3r** : **2.5** (1:0.43)
8	2.05mmol	R^1=H (2.05mmol, 1.0eq)	EtOH (10mL) + 3M NaOH (10mL) rt, 19h	Dark yellow solid **2.3r** : **2.5** (1:0.39)
9	2.05mmol	R^1=H (2.05mmol, 1.0eq)	EtOH (12mL) + 0.5M NaOH (12mL) rt, 16h	Light yellow solid **2.3r** : **2.2** (1:0.02)
10 (as 2.3r in Section V)	3.17mmol	R^1=H (3.49mmol, 1.1eq)	EtOH (10mL) + 3M NaOH (10mL) rt, 24h	Yellow solid (η=71.0%) **2.3r** and traces of **2.5**
11	2.05mmol	R^1=H (2.25mmol, 1.1eq)	EtOH (14mL) + 3M NaOH (14mL) rt, 5h	Yellow solid **2.3r** : **2.5** : **2.1** (1:0.06:0.10)

Comparing experiments 3 and 5, in which the experimental conditions were identical in terms of time (19h) and temperature (rt) however, the concentration of the reagents in experiment 5 was approximately half of the concentration used in experiment 3. Regarding the final products, the molar ratio shows that in experiment 5 the proportion of **2.5** is almost

doubled in comparison to experiment 3, when compared to the desired product **2.3t**. This observation indicated that in dilute solution, the *α*,*β*-unsaturated carbonyl compound **2.3t** is more prone to nucleophilic attack than the salicylic aldehyde used as starting material. This leads to a higher yield of adduct **2.5** in experiment 5. Experiments 6 and 7 were performed under the same reaction conditions (time and temperature) using unsubstituted acetophenone. The concentration of the reagents in experiment 6 is almost twice the concentration used in experiment 7. Once again, dilution seems to favor the formation of the adduct **2.5** as was also previously observed for experiments 3 and 5.

Regarding experiments 3 and 4 where the concentration of reagents is similar, the only factor that varies, is the time of reaction (19h and 6h, respectively). The results indicate that the desired product **2.3t** is isolated with only traces of **2.5** after 6 hours of reaction while in experiment 3, after 19 hours, this product appears to be contaminated with almost 41% of the adduct **2.5**.

Reaction time was also an important factor in the synthesis of **2.3r**, as after 16h at rt the product was isolated with only 2% of the adduct **2.5**. When the reaction was extended for a further 3h (Experiment 7) the reaction mixture contained 43% of **2.5**, together with the desired product **2.3r**.

This study allowed to select the best experimental conditions to prepare compounds **2.3t** and **2.3r** (experiments 4 and 10 respectively).

1.2. Spectroscopic characterization

The structure of compounds **2.3** was assessed by ^{1}H, ^{13}C NMR and IR spectroscopic data, summarized in Tables 10 to 14.

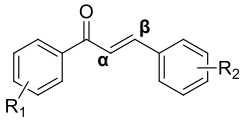
Infrared spectroscopy

Regarding the IR spectroscopic data (Table 5), the stretching vibration of the carbonyl group (C=O) varies from 1615 to 1676 cm^{-1} a relatively low value compared with the standard value for isolated carbonyl groups [145]. This low wavenumber might be due to the conjugation with the double bond and the aromatic ring.

For all the compounds, except **2.3n**, a peak was identified around 3085-3383 cm^{-1} assigned to the stretching vibration of the OH bond, confirming the presence of the hydroxyl

group. As reported in Table 5, compound **2.3n** does not present any hydroxyl group and this peak is absent on the IR spectrum

Table 5. *Infrared spectroscopic data (Nujol, cm^{-1}) for chalcones **2.3a-p** and **2.3y***



Comp	R^1	R^2	4000-3000	C=O	1700-1500
2.3a	H	2-OH;3-OCH_3;5-Br	3359m	1657s	1585s; 1574s
2.3b	4'-Cl	2-OH;3-OCH_3;5-Br	3343m	1657m	1597s
2.3c	3'-OCH_3	2-OH;3-OCH_3;5-Br	3292m	1649s	1581s
2.3d	3'-OCH_3	2-OH;5-Cl	3281w	1643s	1573s
2.3e	3'-OCH_3	2-OH;5-Br	3270m	1676s	1644s; 1599s; 1572w
2.3f	H	2-OH;3,5-F	3210m	1650s	1595s; 1572s
2.3g	4'-CH_3	2-OH;5-Cl	3085m	1644s	1606m; 1557s
2.3h	4'-CH_3	2-OH;3-OCH_3;5-Br	3299w	1654s	1607m; 1588m
2.3i	4'-Br	2-OH;5-Br	3285m	1645s	1593s; 1568s; 1554w
2.3j	4'-Cl	2-OH,3-OCH_3	3274m	1652m	1584m
2.3k	4'-CH_3	2-OH;5-Br	3375w	1644s	1606m; 1559s
2.3l	4'-CH_3	2-OH;3,5-F	3151m	1649s	1607m; 1587s; 1565w
2.3m	4'-Br	2-OH;3-OCH_3;5-Br	3324m	1656s	1596s; 1578s
2.3n	4'-CH_3	5-Br	-	1665m	1602m
2.3o	H	2-OH	3364m	1655s	1598m; 1586s; 1569w
2.3p	H	2-OH,3-OCH_3	3383w	1632s	1598m; 1580m; 1560m
2.3y	4'-Cl	2-OH;3-OCH_3	3278w	1615s	1588s; 1573s; 1539s

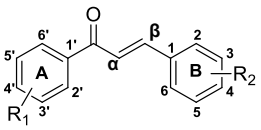
^{1}H NMR Spectroscopy

In the ^{1}H NMR spectrum of *α,β*-unsaturated carbonyl compounds **2.3**, the proton of the hydroxyl group (-OH) appears as a singlet at δ 9.45–10.60 ppm reflecting the acidity of the phenolic proton. As can be seen in Table 6, compound **2.3n** does not present this signal confirming the absence of this proton in the structure.

Protons H_α and H_β, both appear as doublets between δ 7.81-8.03 ppm and δ 7.88-8.09 ppm, respectively, with a coupling constant between 15.6 and 16.0Hz, confirming the *trans* configuration of the alkene.

The detailed data for all the compounds is combined in Table 6.

Table 6. *^{1}H NMR (400 MHz, DMSO-d_6, δ ppm) spectroscopy data for chalcones* ***2.3***



Compound	R^1	R^2	H_α	H_β	Ar^1-H (Ring A)	Ar-H (Ring B)
2.3a	H	2-OH;3-OCH_3;5-Br	8.03 (d, J=15.6Hz)	7.88 (d, J=15.6Hz)	7.65 (tt, J=1.3Hz, 1H, $H_{4'}$), 8.13 (d, J=6.8Hz, 1H, $H_{6'}$)	3.85 (s, 3H, -OCH_3), 7.17 (d, J=2.0Hz,1H, H_4), 7.77 (d, J=2.4Hz, 1H, H_6), 9.74 (s, 1H, -OH)
2.3b	4′-Cl	2-OH;3-OCH_3;5-Br	8.00 (d, J=16.0Hz)	7.91 (d, J=16.0Hz)	7.63 (dt, J=8.4Hz, 1H, $H_{5'}$), 8.18 (d, J=6.0Hz, 1H, $H_{6'}$)	3.85 (s, 3H, -OCH_3), 7.17 (d, J=2.4Hz, 1H, H_4), 7.77 (d, J= 2.0Hz, 1H, H_6), 9.77 (s, 1H, -OH)
2.3c	3′-OCH_3	2-OH;3-OCH_3;5-Br	7.88 (d, J=15.6Hz)	7.98 (d, J=15.6Hz)	3.84 (s, 3H, -OCH_3), 7.59 (dd, J=2.8, 1.6Hz, 1H, $H_{2'}$), 7.23 (dd, J=8.4, 2.8Hz, 1H, $H_{4'}$), 7.47 (t, J=8.0Hz, 1H, $H_{5'}$), 7.75 (d, J=1.2Hz, 1H, $H_{6'}$)	3.84 (s, 3H, -OCH_3), 7.17 (d, J=2.4Hz, 1H, H_4), 7.77 (d, J=2.4Hz, 1H, H_5), 9.75 (s, 1H, -OH)
2.3d	3′-OCH_3	2-OH;5-Cl	7.92 (d, J=16.0Hz)	7.96 (d, J=16.0Hz)	3.84 (s, 3H, -OCH_3), 7.60 (dd, J=1.6, 1.2Hz, 1H, $H_{2'}$), 7.37 (d, J=7.6Hz, 1H, $H_{4'}$), 7.48 (t, J=8.0Hz, 1H, $H_{5'}$), 7.24 (dd, J=5.6, 2.8Hz, 1H, $H_{6'}$)	6.95 (d, J=8.8Hz, 1H, H_3), 7.30 (dd, J=8.8, 2.4Hz, 1H, H_4), 8.03 (d, J=2.8Hz, 1H, H_5), 10.55 (s, 1H, -OH)
2.3e	3′-OCH_3	2-OH;5-Br	7.92 (d, J=15.6Hz)	7.96 (d, J=15.6Hz)	3.84 (s, 3H, -OCH_3), 7.60 (q, J=1.6Hz, 1H, $H_{2'}$), 7.37 (dt, J=7.6, 1.6Hz, 1H, $H_{4'}$), 7.48 (t, J=, 8.0Hz, 1H, $H_{5'}$), 7.23 (dd, J=5.6, 2.8Hz, 1H, $H_{6'}$)	6.90 (d, J=8.8Hz, 1H, H_3), 7.42 (dd, J=8.8, 2.4Hz, 1H, H_4), 8.13 (d, J=2.4Hz, 1H, H_5), 10.60 (s, 1H, -OH)
2.3f	H	2-OH;3,5-F	8.03 (dd, J=15.6, 1.2Hz)	7.97 (d, J=15.6Hz)	7.65 (m, 1H, $H_{4'}$), 7.57 (t, J=7.2Hz, 2H, $H_{3'}$+$H_{5'}$), 8.14 (d, J=7.2Hz, 2H, $H_{2'}$+$H_{6'}$)	7.33 (m, 1H, H_4), 7.77 (m, 1H, H_6), 10.33 (s, 1H, -OH)
2.3g	4′-CH_3	2-OH;5-Cl	7.94 (d, J=16.0Hz)	7.93 (d, J=16.0Hz)	8.06 (q, J=6.4, 1.6Hz, 2H, $H_{2'}$+$H_{6'}$), 7.37 (d, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$),	2.39 (s, 3H, -CH_3), 6.97 (d, J=8.8Hz, 1H, H_3), 7.28 (dd, J=8.8, 2.8Hz, 1H, H_4), 10.58 (s, 1H, -OH)
2.3h	4′-CH_3	2-OH;3-OCH_3;5-Br	7.97 (d, J=16.0Hz)	7.90 (d, 16.0Hz)	2.39 (s, 3H, -CH_3), 7.37 (d, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 8.07 (d, J=8.0Hz, 2H, $H_{2'}$+$H_{6'}$)	3.85 (s, 3H, -OCH_3), 7.17 (d, J=2.4Hz, 1H, H_4), 7.76 (d, J= 2.0Hz, 1H, H_6), 9.71 (s, 1H, -OH)
2.3i	4′-Br	2-OH;5-Br	7.83 (d, J=15.6Hz)	7.86 (d, J=15.6Hz)	7.67 (d, J=6.8Hz, 2H, $H_{3'}$+$H_{5'}$), 7.98 (d, J=6.4Hz, 2H, $H_{2'}$+$H_{6'}$)	6.79 (d, J=8.8Hz, 1H, H_3), 7.31 (dd, J=8.8, 2.4Hz, 1H, H_4), 8.04 (d, J= 2.4Hz, 1H, H_6), 10.49 (s, 1H, -OH)
2.3j	4′-Cl	2-OH,3-OCH_3	7.81 (d, J=16.0Hz)	8.09 (d, J=16.0Hz)	8.12 (d, J=6.4Hz, 2H, $H_{3'}$+$H_{5'}$), 7.63 (d, J=8.8Hz, 2H, $H_{2'}$+$H_{6'}$)	3.83 (s, 3H, -OCH_3), 6.89 (d, J=8.8Hz, 1H, H_4), 7.05 (dd, J=8.0, 0.8Hz, 1H, H_5), 6.83 (t, J= 8.0Hz, 1H, H_6), 9.47 (s, 1H, -OH)
2.3k	4′-CH_3	2-OH;5-Br	7.93 (d, J=16.0Hz)	7.94 (d, J=16.0Hz)	2.40 (s, 3H, -CH_3), 7.37 (d, J=8.0Hz, 2H,	6.89 (d, J=8.8Hz, 1H, H_3), 7.40 (dd, J=8.8, 2.4Hz, 1H, H_4), 8.13

					$H_{3'}$+$H_{5'}$), 8.06 (d, J=8.0Hz, 2H, $H_{2'}$+$H_{6'}$)	(d, J= 2.4Hz, 1H, H_6), 10.55 (s, 1H, -OH)
2.3l	4′-CH_3	2-OH;3,5-F	8.01 (dd, J=15.6, 1.6Hz)	7.95 (d, J=15.6Hz)	2.40 (s, 3H, -CH_3), 7.38 (d, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 8.07 (d, J=8.4Hz, 2H, $H_{2'}$+$H_{6'}$)	7.32 (m, 1H, H_4), 7.75 (m, 1H, H_6), 10.30 (s, 1H, -OH)
2.3m	4′-Br	2-OH;3-OCH_3;5-Br	7.91 (d, J=16.0Hz)	8.05 (d, J=16.0Hz)	7.79 (d, J=8.8Hz, 2H, $H_{3'}$+$H_{5'}$), 8.11 (d, J=8.4Hz, 2H, $H_{2'}$+$H_{6'}$)	3.86 (s, 3H, -OCH_3), 7.19 (d, J=2.4Hz, 1H, H_4), 7.78 (d, J= 3.2Hz, 1H, H_6), 9.79 (s, 1H, -OH)
2.3n	4′-CH_3	5-Br	8.00 (d, J=15.6Hz)	7.69 (d, J=15.6Hz)	2.40 (s, 3H, -CH_3), 7.38 (d, J=7.2Hz, 2H, $H_{3'}$+$H_{5'}$), 8.10 (d, J=8.4Hz, 2H, $H_{2'}$+$H_{6'}$)	7.85 (d, J=7.6Hz, 1H, H_2), 7.42 (d, J=8.0Hz, 1H, H_3), 7.63 (m, 1H, H_4), 8.04 (t, J= 1.6Hz, 1H, H_6)
2.3o	H	2-OH	7.86 (d, J=16.0Hz)	8.02 (d, J=16.0Hz)	7.79 (d, J=8.8Hz, 2H, $H_{3'}$+$H_{5'}$), 7.56 (t, J=7.2Hz, 2H, $H_{2'}$+$H_{6'}$)	6.95 (dd, J=8.0, 0.8Hz, 1H, H_3), 7.27 (td, J=7.2, 1.6, 1H, H_4), 6.87 (t, J= 7.6Hz, 1H, H_5), 7.84 (dd, J= 8.0, 1.6Hz, 1H, H_6), 10.29 (s, 1H, -OH)
2.3p	H	2-OH,3-OCH_3	7.83 (d, J=16.0Hz)	7.90 (d, J=16.0Hz)	7.92 (d, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 7.50 (t, J=7.2Hz, 2H, $H_{2'}$+$H_{6'}$)	3.60 (s, 3H, -OCH_3), 6.43 (dd, J=7.6, 1.6Hz, 1H, H_4), 6.87 (dd, J=8.0, 1.6Hz, 1H, H_5), 6.83 (t, J=1.6Hz, 1H, H_6), 10.03(s, 1H, -OH)
2.3q [(a)]	H	2-OH;5-Br	7.97 (d, J=16.0Hz)	7.95 (d, J=16.0Hz)	7.40 (dd, J=8.8, 2.4Hz, 1H, $H_{4'}$), 7.92 (d, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 8.13 (d, J=4.8Hz, 2H, $H_{2'}$+$H_{6'}$)	6.90 (d, J=8.8Hz, 1H, H_3), 7.42 (dd, J=8.8, 2.4Hz, 1H, H_4), 7.56 (t, J= 6.4Hz, 1H, H_5), 8.15 (d, J= 4.8Hz, 1H, H_6), 10.56 (s, 1H, -OH)
2.3r[(a)]	4′-Cl	2-OH;5-Cl	7.97 (d, J=16.0Hz)	7.94 (d, J=16.0Hz)	7.57 (d, J=8.4Hz, 2H, $H_{3'}$+$H_{5'}$), 8.17 (d, J=8.8Hz, 2H, $H_{2'}$+$H_{6'}$)	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), 10.64 (s, 1H, -OH)
2.3s[(b)]	4′-Cl	2-OH;3,5-F				
2.3t[(a)]	H	2-OH;5-Cl	7.94 (d, J=16.0Hz)	7.97 (d, J=16.0Hz)	8.14 (d, J=8.8Hz, 2H, $H_{3'}$+$H_{5'}$), 7.56 (t, J=7.6Hz, 2H, $H_{2'}$+$H_{6'}$)	6.96 (d, J=8.0Hz, 1H, H_3), 7.65 (t, J=7.2Hz 1H, H_4), 7.30 (dd, J= 8.8, 2.8Hz, 1H, H_6), 10.57 (s, 1H, -OH)
2.3u[(a)]	4′-Cl	2-OH;5-CH_3	8.01 (d, J=15.6Hz)	7.82 (d, J=15.6Hz)	7.56 (d, J=8.4Hz, 2H, $H_{3'}$+$H_{5'}$), 8.12 (d, J=8.4Hz, 2H, $H_{2'}$+$H_{6'}$)	2.24 (s, 3H, CH_3), 6.84 (d, J=8.4Hz, 1H, H_3), 7.27 (dd, J=8.4, 2.0, 1H, H_4), 7.63 (dd, J= 8.4, 2.0Hz, 1H, H_6), 10.05 (s, 1H, -OH)
2.3v[(a)]	4′-Cl	2-OH;5-Br	7.97 (d, J=15.6Hz)	7.93 (d, J=15.6Hz)	8.18 (d, J=8.8Hz, 2H, $H_{3'}$+$H_{5'}$), 7.95 (d, J=7.6Hz, 2H, $H_{2'}$+$H_{6'}$)	6.84 (d, J=8.4Hz, 1H, H_3), 7.27 (dd, J=8.4, 2.0, 1H, H_4), 7.63 (dd, J= 8.4, 2.0Hz, 1H, H_6), 10.05 (s, 1H, -OH)
2.3w[(a)]	3′-OCH_3	2-OH;3,5-F	8.03 (dd, J=16.0, 1.6Hz)	7.94 (d, J=16.0Hz)	3.80 (s, 3H, OCH_3), 7.60 (t, J=2.0Hz, 1H, $H_{2'}$), 7.37 (d, J=7.6Hz, 1H, $H_{4'}$), 7.48 (t, J=, 8.0Hz, 1H, $H_{5'}$), 7.25 (dd, J=5.6, 2.8Hz, 1H, $H_{6'}$)	7.33 (ddd, J=11.2, 2.8Hz, 1H, H_4), 7.77 (m, 1H, H_6), 10.32 (s, 1H, -OH)
2.3x[(a)]	4′-Br	2-OH;3,5-F	8.00 (dd, J=15.6, 1.6Hz)	7.95 (d, J=15.6Hz)	7.77 (d, J=8.8Hz, 2H, $H_{3'}$+$H_{5'}$), 8.10 (d, J=8.8Hz, 2H, $H_{2'}$+$H_{6'}$)	7.34 (ddd, J=11.2, 2.8Hz, 1H, H_4), 7.76 (m, 1H, H_6), 10.35 (s, 1H, -OH)

2.3y[(a)]	4′-Cl	2-OH;3-OCH_3	7.72 (d, J=15.6Hz)	8.02 (d, J=15.6Hz)	7.62 (dd, J=8.8, 2.0Hz, 2H, $H_{3'}$+$H_{5'}$), 8.09 (dd, J=8.8, 2.0Hz, 2H, $H_{2'}$+$H_{6'}$)	3.75 (s, 3H, -OCH_3), 6.50 (d, J=2.4Hz, 1H, H_4), 6.48 (d, J=2.4Hz, 1H, H_5), 7.82 (d, J= 9.6Hz, 1H, H_6), 10.43 (s, 1H, -OH)
2.3z[(b)]	4′-F	2-OH;5-Br				

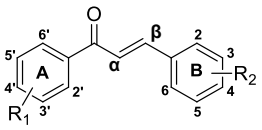
(a) Bands were identified from a mixture of products.

(b) The ^{1}H NMR presented a complex mixture of signals, thus it was not possible to identify with accuracy the signals corresponding to the pure product.

^{13}C NMR Spectroscopy

Compounds **2.3** were characterized by ^{13}C spectroscopy an analytical chemistry technique used to confirm the structure and purity of a sample. This method showed that the carbon from the carbonyl group (C=O) varied between δ 188.01–189.50 ppm (Table 7). The chemical shift for C_α and C_β varies in the range δ 120.73-123.73 ppm and δ 136.27-141.83 ppm respectively. Finally, C_2 generally associated to the hydroxyl group (-OH), presents a chemical shift between δ 141.10 ppm and 157.25 ppm. On the other hand, for compound **2.3n**, the same carbon is observed at δ 128.22 ppm.

Table 7. *^{13}C NMR (100 MHz, DMSO-d_6, δ ppm) spectroscopy data for chalcones **2.3**.*



Comp	R^1	R^2	C_α	C_β	C=O	Ar^1-H (Ring A)	Ar-H (Ring B)
2.3a	H	2-OH;3-OCH_3;5-Br	122.09	137.34	189.17	128.51 ($C_{2'}$+$C_{6'}$), 128.72 ($C_{3'}$+$C_{5'}$), 133.08 ($C_{4'}$), 137.60 ($C_{1'}$)	56.36 (-OCH_3), 123.20 (C_1), 149.04 (C_2), 145.93 (C_3), 116.14 (C_4), 110.50 (C_5), 121.49 (C_6)
2.3b	4′-Cl	2-OH;3-OCH_3;5-Br	121.67	137.75	188.01	128.84 ($C_{2'}$+$C_{6'}$), 130.45 ($C_{3'}$+$C_{5'}$), 136.24 ($C_{4'}$) 138.06 ($C_{1'}$)	56.37 (-OCH_3), 123.09 (C_1), 146.03 (C_2), 149.05 (C_3), 116.26 (C_4), 110.59 (C_5), 121.47 (C_6)
2.3c	3′-OCH_3	2-OH;3-OCH_3;5-Br	122.12	137.43	188.92	55.36 (-OCH_3), 139.05 ($C_{1'}$), 113.06 ($C_{2'}$), 159.52 ($C_{3'}$), 121.07 ($C_{4'}$), 129.87 ($C_{5'}$), 119.06 ($C_{6'}$)	56.36 (-OCH_3), 123.19 (C_1), 145.94 (C_2), 149.03 (C_3), 116.16 (C_4), 110.57 (C_5), 121.51 (C_6)
2.3d	3′-OCH_3	2-OH;5-Cl	122.05	137.67	188.95	55.28 (-OCH_3), 139.08 ($C_{1'}$), 113.05 ($C_{2'}$), 159.53 ($C_{3'}$), 121.04 ($C_{4'}$), 129.88 ($C_{5'}$), 119.05 ($C_{6'}$)	123.05 (C_1), 155.95 (C_2), 117.85 (C_3), 131.40 (C_4), 123.23 (C_5), 127.47 (C_6)

2.3e	3′-OCH_3	2-OH;5-Br	122.03	137.60	188.95	55.37 (-OCH_3), 139.09 ($C_{1'}$), 113.06 ($C_{2'}$), 159.54 ($C_{3'}$), 121.07 ($C_{4'}$), 129.89 ($C_{5'}$), 119.05 ($C_{6'}$)	123.13 (C_1), 155.92 (C_2), 117.86 (C_3), 131.24 (C_4), 123.19 (C_5), 127.40 (C_6)
2.3f	H	2-OH;3,5-F	123.29	136.67 (t, J=3.0Hz)	188.17	128.54 ($C_{2'}$+$C_{6'}$), 128.80 ($C_{3'}$+$C_{5'}$), 133.34 ($C_{4'}$), 137.44 ($C_{1'}$)	124.94 (dd, J=9.0, 4.0Hz, C_1), 141.19 (dd, J=11.0, 3.0Hz, C_2), 151.59 (dd, J=241.0, 13.0Hz, C_3), 106.28 (dd, J=23.0, 28.0Hz, C_4), 154.33 (dd, J=235.7, 11.6Hz, C_5), 109.08 (dd, J=23.0, 4.0Hz, C_6)
2.3g	4′-CH_3	2-OH;5-Cl	122.04	137.24	188.63	21.17 (-CH_3), 128.65 ($C_{2'}$+$C_{6'}$), 129.31 ($C_{3'}$+$C_{5'}$), 143.50 ($C_{4'}$), 135.12 ($C_{1'}$)	123.13 (C_1), 155.92 (C_2), 117.86 (C_3), 131.24 (C_4), 123.19 (C_5), 127.40 (C_6)
2.3h	4′-CH_3	2-OH;3-OCH_3;5-Br	122.11	136.96	188.58	21.18 (-CH_3), 128.67 ($C_{2'}$+$C_{6'}$), 129.30 ($C_{3'}$+$C_{5'}$), 143.52 ($C_{4'}$), 135.08 ($C_{1'}$)	53.36 (-OCH_3), 123.28 (C_1), 145.85 (C_2), 149.03 (C_3), 116.06 (C_4), 110.57 (C_5), 121.45 (C_6)
2.3i	4′-Br	2-OH;5-Br	121.56	137.94	188.24	130.55 ($C_{2'}$+$C_{6'}$), 131.80 ($C_{3'}$+$C_{5'}$), 127.25 ($C_{4'}$), 136.59 ($C_{1'}$)	123.56 (C_1), 156.43 (C_2), 118.33 (C_3), 134.38 (C_4), 110.85 (C_5), 130.34 (C_6)
2.3j	4′-Cl	2-OH,3-OCH_3	120.73	139.70	188.28	128.85 ($C_{2'}$+$C_{6'}$), 130.25 ($C_{3'}$+$C_{5'}$), 136.49 ($C_{4'}$), 137.82 ($C_{1'}$)	55.95 (-OCH_3), 121.51 (C_1), 146.72 (C_2), 148.01 (C_3), 113.76 (C_4), 119.05 (C_5), 119.82 (C_6)
2.3k	4′-CH_3	2-OH;5-Br	122.01	137.11	188.59	21.17 (-CH_3), 128.66 ($C_{2'}$+$C_{6'}$), 129.30 ($C_{3'}$+$C_{5'}$), 143.51 ($C_{4'}$), 134.09 ($C_{1'}$)	123.75 (C_1), 156.28 (C_2), 118.28 (C_3), 134.09 (C_4), 110.83 (C_5), 130.26 (C_6)
2.3l	4′-CH_3	2-OH;3,5-F	123.30	136.27 (t, J=4.0Hz)	188.55	21.17 (-CH_3), 128.69 ($C_{2'}$+$C_{6'}$), 129.36 ($C_{3'}$+$C_{5'}$), 143.72 ($C_{4'}$), 134.93 ($C_{1'}$)	125.02 (dd, J=10.0, 4.0Hz, C_1), 141.10 (dd, J=15.0, 3.0Hz, C_2), 151.53 (dd, J=240.0, 13.0Hz, C_3), 106.16 (dd, J=23.0, 28.0Hz, C_4), 154.34 (dd, J=236.0, 12.0Hz, C_5), 109.02 (dd, J=23.0, 4.0Hz, C_6)
2.3m	4′-Br	2-OH;3-OCH_3;5-Br	121.65	137.74	188.23	130.56 ($C_{2'}$+$C_{6'}$), 131.80 ($C_{3'}$+$C_{5'}$), 127.27 ($C_{4'}$), 136.57 ($C_{1'}$)	56.37 (-OCH_3), 123.09 (C_1), 149.06 (C_2), 146.04 (C_3), 116.27 (C_4), 110.59 (C_5), 121.48 (C_6)
2.3n	4′-CH_3	5-Br	123.51	141.83	188.40	21.18 (-CH_3), 128.78 ($C_{2'}$+$C_{6'}$), 129.34 ($C_{3'}$+$C_{5'}$), 143.79 ($C_{4'}$), 134.82 ($C_{1'}$)	137.23 (C_1), 128.22 (C_2), 130.89 (C_3), 132.93 (C_4), 122.36 (C_5), 130.76 (C_6)
2.3o	H	2-OH	120.92	139.54	189.50	128.31 ($C_{2'}$+$C_{6'}$), 128.75 ($C_{3'}$+$C_{5'}$), 132.86 ($C_{4'}$), 137.90 ($C_{1'}$)	121.33 (C_1), 157.25 (C_2), 116.22 (C_3), 132.03 (C_4), 119.38 (C_5), 128.70 (C_6)

The assignments were confirmed by the correlation spectra between ^{1}H and ^{13}C NMR for all the compounds. It was possible to identify the 3-bond interactions for proton H_α with carbon C_1 and the weak interaction at two bonds with the carbonyl group and C_β. It was also identified the 4-bonds interaction with carbons $C_{2'}$ and C_5. For proton H_β, interactions with the carbon of the carbonyl group were confirmed as well as with C_2. At two bonds, a weak interaction was observed with carbon C_α and a weak 4-bonds interaction with carbon $C_{1'}$ (Figure 16).

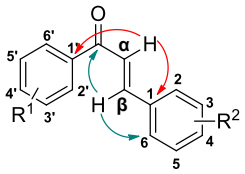
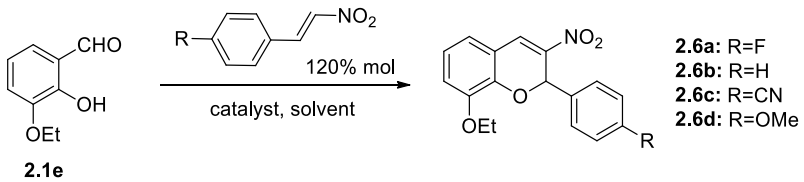


Figure 16. *Interaction by bidimensional correlation of H_α and H_β with the vicinity, for compounds* ***2.3****.*

2. 4*H*-chromene derivatives

The chromene nucleus is widely present in animals and plants and has also been intensely studied by the scientific community. Different synthetic approaches were described in the literature and the example selected involves the use of salicylaldehyde as starting material in the reaction with an electron-deficient alkene.

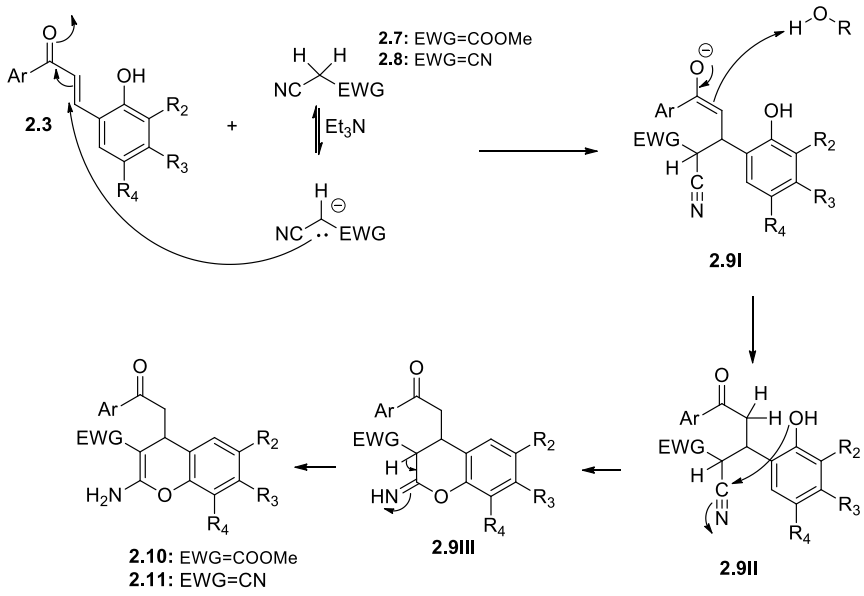
In 2013, Yin and co-workers synthetized a series of chromenes from salicylaldehyde **2.1e** and an excess of β-nitrostyrene via the oxa-Michael-Henry reaction (Scheme 6) [146]. This reaction was catalyzed by L-proline and triethylamine and products **2.6** were obtained in 21 to 53% yield. A specific compound, 8-methoxy-2-(4-fluorophenyl)-3-nitro-2*H*-chromene **2.6a** presented biological activity as a PI3K inhibitor.



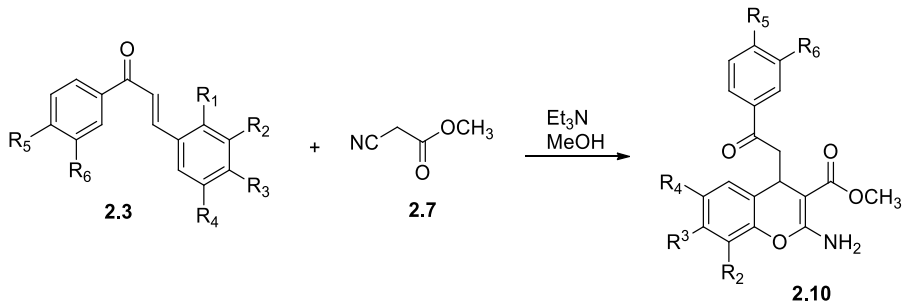
Scheme 6. *Preparation of 8-methoxy-2-(4-fluorophenyl)-3-nitro-2H-chromene and analogues.*

2.1. Synthesis and mechanistic discussion

In order to synthetize 4*H*-chromenes from *α*,*β*-unsaturated carbonyl compounds **2.3**, these chalcones were combined with active methylene compounds (methyl cyanoacetate **2.6** or malononitrile **2.7**) (Scheme 7). Reactions were carried out in the presence of triethylamine, with methanol (MeOH) for chromenes **2.9** (Scheme 8) or ethanol (EtOH) for chromenes **2.10** (Scheme 9) as solvent and stirring at room temperature for a variable number of hours. The reactions were followed by TLC and, after the consumption of the limiting reagent, were cooled to -20ºC and the solid product was isolated by filtration.



Scheme 7. *Proposed mechanism for the synthesis of 4H-chromenes from chalcones **2.3** and the active methylene compounds.*

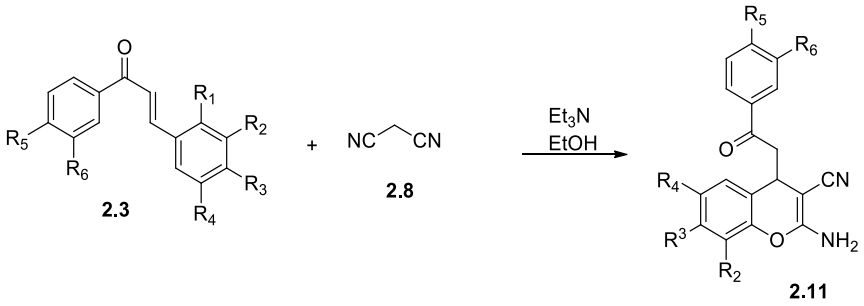


Scheme 8. *Simplified reaction of chalcones **2.3** with methyl cyanoactetate **2.7**.*

Table 8. *Physical and analytical data for 4H-chromenes* ***2.10****.*

Comp	R^2	R^3	R^4	R^5	R^6	η (%)	m.p. (ºC)	Molecular Formula/Weight
2.10a	OCH_3	H	H	Cl	H	71.8	141-143	$C_{20}H_{18}ClNO_5$/387.8gmol^{-1}
2.10b	H	H	Br	H	H	58.5	145-147	$C_{19}H_{16}BrNO_4$/402.2gmol^{-1}
2.10c	OCH_3	H	Br	H	OCH_3	45.2	159-161	$C_{21}H_{20}BrNO_6$/462.3gmol^{-1}
2.10d	H	H	Br	Cl	H	57.1	115-117	$C_{19}H_{15}BrClNO_4$/436.7gmol^{-1}
2.10e	OCH_3	H	Br	H	H	69.3	183-184.5	$C_{20}H_{18}BrNO_5$/432.3gmol^{-1}
2.10f	H	H	Cl	H	H	56.5	116-118	$C_{19}H_{16}ClNO_4$/357.8gmol^{-1}
2.10g	H	H	Br	Br	H	62.3	140-141	$C_{19}H_{15}Br_2NO_4$/481.1gmol^{-1}
2.10h	OCH_3	H	Br	Br	H	57.1	164-165.5	$C_{20}H_{17}Br_2NO_5$/511.2gmol^{-1}

Almost every experiment in the synthesis of compounds **2.10** resulted in the isolation of pure chromenes and with good yield. However, by TLC, in some reactions, it was possible to prove that the initial products were consumed but when the reaction mixture was cooled to -20ºC, even then, the chromene did not precipitate possibly due to the absence of a crystallization core.



Scheme 9. *Simplified reaction of chalcones* ***2.3*** *with malononitrile* ***2.8****.*

Table 9. *Physical and analytical data for 4H-chromenes* ***2.11.***

Comp	R^2	R^3	R^4	R^5	R^6	η (%)	m.p. (ºC)	Molecular Formula/Weight
2.11a	H	H	H	H	H	27.0	136.5-138	$C_{18}H_{14}N_2O_2$/290.3gmol^{-1}
2.11b	H	H	Br	CH3	H	61.8	183-184.5	$C_{19}H_{15}BrN_2O_2$/383.2gmol^{-1}
2.11c	OCH_3	H	Br	H	H	41.8	177-179	$C_{19}H_{15}BrN_2O_3$/399.2gmol^{-1}
2.11d	OCH_3	H	Br	Cl	H	68.1	169-171	$C_{19}H_{14}BrClN_2O_3$/433.7gmol^{-1}
2.11e	OCH_3	H	Br	H	OCH_3	63.8	157-159.5	$C_{20}H_{17}BrN_2O_4$/429.3gmol^{-1}
2.11f	H	H	Cl	CH_3	H	39.6	175-176.5	$C_{19}H_{15}ClN_2O_2$/338.8gmol^{-1}
2.11g	H	H	Br	H	OCH_3	24.7	134-135	$C_{19}H_{15}BrN_2O_3$/399.2gmol^{-1}
2.11h	F	H	F	H	H	21.4	149-151	$C_{18}H_{12}F_2N_2O_2$/326.3gmol^{-1}
2.11i	OCH_3	H	Br	CH_3	H	12.1	129-131	$C_{20}H_{17}BrN_2O_3$/413.3gmol^{-1}
2.11j	H	H	Br	Br	H	50.3	186-188	$C_{18}H_{12}Br_2N_2O_2$/448.1gmol^{-1}
2.11k	OCH_3	H	Br	Br	H	56.6	161-163	$C_{19}H_{14}Br_2N_2O_3$/478.1gmol^{-1}

Compared with compounds **2.10**, chromenes **2.11** were much easier to precipitate from the reaction mixture. Even at room temperature, and when the reaction was not yet concluded, the product started to precipitate. However, cooling to -20ºC resulted in an increased yield of this product.

2.2. Spectroscopic characterization

The structure of compounds **2.10** and **2.11** was assessed by ^{1}H, ^{13}C NMR and IR spectroscopic analysis and the data is combined in Tables 10 to 14.

Infrared Spectroscopy

The infrared spectra of compounds **2.10** and **2.11** show two absorption bands in the region from υ=3489 to 3204 cm^{-1} assigned to the NH_2 stretching vibration of the amino group (Table 10). For the region between 1689 and 1512 cm^{-1}, bands with variable intensity are observed, probably due to the stretching vibrations of C=C and C=O. Finally, between 2176 and 2195 cm^{-1} we can see the characteristic bands for the stretching vibration of nitrile group (-CN), only present in compounds **2.11**.

Table 10. *Infrared spectroscopic data (Nujol, cm^{-1}) for 4H-chromenes **2.10** and **2.11**.*

Comp	R^2	R^3	R^4	R^5	R^6	4000-3000	C≡N	1700-1500
2.10a	OCH_3	H	H	Cl	H	3428m; 3316m		1681s; 1664s; 1635m; 1613s; 1583w
2.10b	H	H	Br	H	H	3420m; 3300w		1672s; 1656s; 1614m; 1599s; 1581w
2.10c	OCH_3	H	Br	H	OCH_3	3458i; 3312w		1676s; 1609s; 1580m; 1521s
2.10d	H	H	Br	Cl	H	3405m; 3280w		1674s; 1605s; 1584m; 1522s
2.10e	OCH_3	H	Br	H	H	3405m; 3281w		1673s; 1605s; 1584m; 1522s
2.10f	H	H	Cl	H	H	3416m		1659s; 1603s; 1514m
2.10g	H	H	Br	Br	H	3466m		1680s; 1600m; 1516m
2.10h	OCH_3	H	Br	Br	H	3404w; 3280w		1674s; 1605s; 1584m; 1522s
2.11a	H	H	H	H	H	3409m; 3325w; 3204w	2182m	1689s; 1649m; 1609m; 1579s
2.11b	H	H	Br	CH_3	H	3461w; 3320w	2193m	1668s; 1649m; 1602m; 1568s
2.11c	OCH_3	H	Br	H	H	3466w; 3354w	2176m	1669s; 1643m; 1599m; 1575m
2.11d	OCH_3	H	Br	Cl	H	3453w; 3320w	2175w	1657s; 1599s
2.11e	OCH_3	H	Br	H	OCH_3	3489; 3359	2180m	1678s; 1649s; 1607m; 1595m; 1576w
2.11f	H	H	Cl	CH_3	H	3467m; 3319w	2193m	1669s; 1649s; 1603m; 1572s
2.11g	H	H	Br	H	OCH_3	3463w; 3321w	2193m	1671s; 1652s; 1599m; 1580m

2.11h	F	H	F	H	H	3473w; 3325w	2195m	1686s; 1656s; 1619m; 1596s
2.11i	OCH_3	H	Br	CH_3	H	3355w	2191s	1675s; 1605s; 1574m
2.11j	H	H	Br	Br	H	3463w; 3320w	2193m	1677s; 1650s; 1600m; 1568s
2.11k	OCH_3	H	Br	Br	H	3485m; 3355w	2176s	1679s; 1646s; 1607m; 1579m

^{1}H NMR Spectroscopy

Regarding to Tables 11 and 13, we note the presence of the amino group (-NH_2) by a singlet in the spectra of both compounds varying from δ 7.47 to 7.63 ppm and δ 6.82 to 7.03 ppm for chromenes **2.10** and **2.11**, respectively.

The -CH_2 hydrogens appear as a multiplet between δ 3.13 to 3.20 ppm and δ 3.35 to 3.49 ppm for chromenes **2.10** and **2.11**, respectively. The hydrogen of C_4 appears always as a doublet of doublets and varies from δ 4.24 to 4.29 ppm for compounds **2.10** and as a triplet and varies from δ 4.04 to 4.10 ppm for compounds **2.11**. This difference in the multiplicity and chemical shift of the signal seems to be due to the presence of a carbonyl group or a cyano group in C_3, affecting the dihedral angle with the neighboring methylene group.

As we can see, the chemical shift of the many substituent groups do not significantly vary in the same group of compounds or within the two-different group of synthetized chromenes. All **2.10** and **2.11** compounds present chemical shift variations respecting the standard.

^{13}C NMR Spectroscopy

According to Tables 12 and 14, the chemical shift of the carbonyl group (C=O) varies from δ 197.46 to 198.43 ppm for compounds **2.10** and from δ 196.78 to 197.94 ppm for compounds **2.11**, as is expected for the carbonyl group of ketones.

The signal for carbons CH_2 and C_4 vary between δ 47.34 to 48.09 ppm and δ 30.51 to 38.18 ppm for compounds **2.10**, and from δ 45.61 to 46.90 ppm and from δ 30.74 to 31.09 ppm for compounds **2.11**.

The assignments were confirmed by the correlation spectra between ^{1}H and ^{13}C NMR for all the compounds. From the fixed and characteristic chemical shifts of CH_2 and C_4H, it was possible to confirm the identity of many other carbons on the purposed molecules. As it can be

seen by Figure 17, by CH_2, it was possible to identify the carbon of the carbonyl group and, even C_4H. It was also possible to recognize the 3-bond interactions for the two protons of CH_2 with carbon C_{4a} and C_3. For proton C_4H, many interactions were found to be preponderant to the correct identification of the empiric molecular structure. For 3-bond interactions were identified the carbon of the carbonyl group, C_5, C_{8a}, C_2 and the carbon of EWG. At two bonds, a weak interaction was observed with carbon C_3 as well as the carbon of CH_2 and C_{4a}.

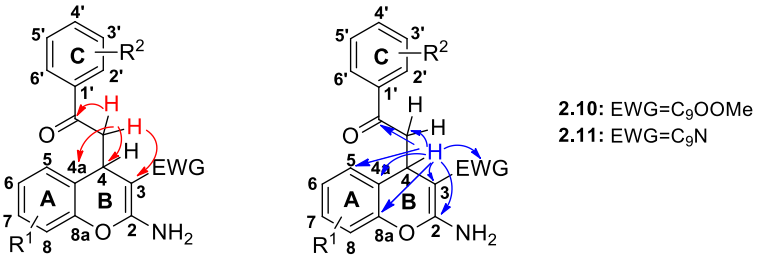
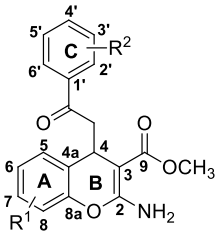


Figure 17. *Interaction by bidimensional correlation of CH_2 and C_4H with the vicinity, for compounds* ***2.10*** *and* ***2.11****.*

Table 11. *Spectroscopic data of ^{1}H NMR (400 MHz, DMSO-d_6) for 4H-chromenes* ***2.10****.*



2.10

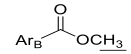
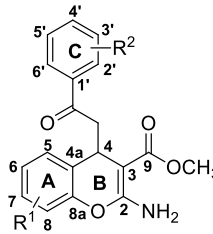
Comp	R^1	R^2		NH_2	CH_2	C_4H	Ar^1-H (Ring A)	Ar'-H (Ring C)
2.10a	8-OCH_3	4'-Cl	3.50 (s, 3H, -OCH_3)	7.62 (s, 2H)	3.13 (m, 2H)	4.27 (dd, J=4.8, 2.4Hz, 1H)	3.78 (s, 3H, -OCH_3), 6.80 (dd, J=7.6, 1.2Hz, 1H, H_5), 6.97 (t, J= 8.0Hz, 1H, H_6), 6.90 (dd, J=8.0, 1.6Hz, 1H, H_7)	7.59 (m, 1H, $H_{4'}$), 7.53 (dd, J=6.4, 2.0Hz, 2H, $H_{2'}$+$H_{6'}$), 7.86 (dd, J=6.8, 2.0Hz, 2H, $H_{3'}$+ $H_{5'}$)
2.10b	6-Br;	H	3.51 (s, 3H, -OCH_3)	7.47 (s, 2H)	3.17 (m, 2H)	4.29 (dd, J=4.8, 2.4Hz, 1H)	7.47 (dd, J=7.6, 1.2Hz, 1H, H_5), 6.95 (d, J= 8.8Hz, 1H, H_8), 7.35 (dd, J=8.8, 2.4Hz, 1H, H_7)	7.48 (dd, J=6.4, 2.0Hz, 2H, $H_{3'}$+ $H_{5'}$), 7.88 (dd, J=8.0, 0.8Hz, 2H, $H_{2'}$+$H_{6'}$)
2.10c	6-Br; 8-OCH_3	3'-OCH_3	3.52 (s, 3H, -OCH_3)	7.63 (s, 2H)	3.18 (m, 2H)	4.25 (dd, J=6.8, 4.8Hz, 1H)	3.81 (s, 3H, -OCH_3), 7.00 (d, J=2.0, 1H, H_5), 7.08 (d, J= 2.0Hz, 1H, H_7)	3.79 (s, 3H, -OCH_3), 7.37 (t, J=1.6Hz, 1H, $H_{2'}$), 7.16 (dd, J=8.0, 2.0Hz, 1H, $H_{4'}$), 7.38 (t, J=8.0Hz, 1H, $H_{5'}$), 7.44 (d, J=7.6Hz, 2H, $H_{6'}$)
2.10d	6-Br	4'-Cl	3.50 (s, 3H, -OCH_3)	7.59 (s, 2H)	3.16 (m, 2H)	4.27 (dd, J=4.8, 2.4Hz, 1H)	7.49 (d, J=2.4Hz, 1H, H_5), 7.36 (dd, J= 8.8, 2.4Hz, 1H, H_7), 7.35 (d, J=8.4Hz, 1H, H_8)	7.88 (dd, J=11.2, 2.4Hz, 2H, $H_{2'}$+$H_{6'}$), 7.54 (dd, J=6.4, 1.6Hz, 2H, $H_{3'}$+ $H_{5'}$)
2.10e	6-Br; 8-OCH_3	H	3.49 (s, 3H, -OCH_3)	7.62 (s, 2H)	3.13 (m, 2H)	4.24 (dd, J=4.8, 2.0Hz, 1H)	3.81 (s, 3H, -OCH_3), 7.03 (d, J=2.0, 1H, H_5), 7.08 (d, J= 2.0Hz, 1H, H_7)	7.53 (dd, J=8.4, 1.6Hz, 2H, $H_{3'}$+ $H_{5'}$), 7.86 (dd, J=6.8, 2.0Hz, 2H, $H_{2'}$+$H_{6'}$)
2.10f	6-Cl	H	3.51 (s, 3H, -OCH_3)	7.57 (s, 2H)	3.20 (m, 2H)	4.29 (dd, J=6.8, 4.4Hz, 1H)	7.35 (d, J=2.8, 1H, H_5), 7.23 (dd, J=8.8, 2.4Hz, 1H, H_7), 7.00 (d, J= 8.8Hz, 1H, H_8)	7.59 (m, 1H, $H_{4'}$), 7.48 (t, J=7.2Hz, 2H, $H_{3'}$+ $H_{5'}$), 7.88 (dd, J=8.0, 0.8Hz, 2H, $H_{2'}$+$H_{6'}$)
2.10g	6-Br	4'-Br	3.50 (s, 3H, -OCH_3)	7.56 (s, 2H)	3.16 (m, 2H)	4.27 (dd, J=6.8, 5.2Hz, 1H)	7.48 (d, J=2.4, 1H, H_5), 7.36 (dd, J=8.8 2.4Hz, 1H, H_7), 6.96 (d, J= 8.4Hz, 1H, H_8)	7.68 (dd, J=6.8, 2.0Hz, 2H, $H_{3'}$+ $H_{5'}$), 7.79 (dd, J=6.4, 2.0Hz, 2H, $H_{2'}$+$H_{6'}$)
2.10h	6-Br; 8-OCH_3	4'-Br	3.49 (s, 3H, -OCH_3)	7.63 (s, 2H)	3.15 (m, 2H)	4.24 (dd, J=6.4, 4.8Hz, 1H)	3.49 (s, 3H, -OCH_3), 7.03 (d, J=2.0, 1H, H_5), 7.08 (d, J= 2.0Hz, 1H, H_7)	7.67 (dd, J=8.4, 1.6Hz, 2H, $H_{3'}$+ $H_{5'}$), 7.77 (dd, J=5.2, 1.6Hz, 2H, $H_{2'}$+$H_{6'}$)

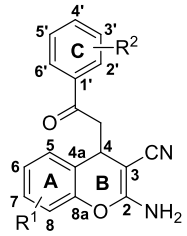
Table 12. *Spectroscopic data of ^{13}C NMR (100 MHz, DMSO-d_6) for 4H-chromenes* ***2.10****.*



2.10

Comp	R^1	R^2	Ar_B-C(=O)-O-CH_3	C_9	C=O	CH_2	C_4H	Ar^1-C (Ring A)	Ar-C (Ring B)	Ar'-H (Ring C)
2.10a	8-OCH_3	4'-Cl	50.31	168.23	197.45	48.09	38.18	55.59 (-OCH_3), 126.88 (C_{4a}), 119.57 (C_5), 124.04 (C_6), 110.61 (C_7), 146.88 (C_8), 138.78 (C_{8a})	161.94 (C_2), 74.30 (C_3)	137.89 ($C_{1'}$), 129.88 ($C_{2'}$+$C_{6'}$), 128.88 ($C_{3'}$+$C_{5'}$), 135.34 ($C_{4'}$)
2.10b	6-Br;	H	50.37	168.08	198.40	47.34	30.51	128.67 (C_{4a}), 130.95 (C_5), 115.58 (C_6), 130.39 (C_7), 117.77 (C_8), 148.93 (C_{8a})	161.62 (C_2), 73.94 (C_3)	136.67 ($C_{1'}$), 127.94 ($C_{2'}$+$C_{6'}$), 128.60 ($C_{3'}$+$C_{5'}$), 133.10 ($C_{4'}$)
2.10c	6-Br; 8-OCH_3	3'-OCH_3	50.41	168.12	198.21	47.94	30.60	56.10 (-OCH_3), 128.71 (C_{4a}), 122.06 (C_5), 115.36 (C_6), 113.67 (C_7), 147.76 (C_8), 138.29 (C_{8a})	161.55 (C_2), 73.98 (C_3)	55.25 (-OCH_3), 138.14 ($C_{1'}$), 112.25 ($C_{2'}$), 120.46 ($C_{6'}$), 129.73 ($C_{5'}$), 119.27 ($C_{4'}$)
2.10d	4-Br	4'-Cl	50.38	168.05	197.48	47.36	30.65	128.70 (C_{4a}), 130.98 (C_5), 128.75 (C_6), 130.46 (C_7), 117.80 (C_8), 148.93 (C_{8a})	161.63 (C_2), 73.82 (C_3)	135.36 ($C_{1'}$), 129.90 ($C_{2'}$+$C_{6'}$), 128.70 ($C_{3'}$+$C_{5'}$), 138.03 ($C_{4'}$)
2.10e	6-Br; 8-OCH_3	H	50.38	168.07	197.46	47.45	30.74	56.19 (-OCH_3), 128.67 (C_{4a}), 122.08 (C_5), 115.44 (C_6), 113.72 (C_7), 147.77 (C_8), 138.28 (C_{8a})	161.56 (C_2), 73.84 (C_3)	137.98 ($C_{1'}$), 129.87 ($C_{2'}$+$C_{6'}$), 128.67 ($C_{3'}$+$C_{5'}$), 135.38 ($C_{4'}$)
2.10f	6-Cl	H	50.39	168.12	198.43	47.33	30.62	128.24 (C_{4a}), 128.07 (C_5), 127.53 (C_6), 127.65 (C_7), 117.40 (C_8), 148.49 (C_{8a})	161.69 (C_2), 73.92 (C_3)	136.68 ($C_{1'}$), 127.96 ($C_{2'}$+$C_{6'}$), 128.63 ($C_{3'}$+$C_{5'}$), 133.13 ($C_{4'}$)
2.10g	6-Br	4'-Br	50.37	168.03	197.68	47.33	30.62	128.52 (C_{4a}), 130.97 (C_5), 115.63 (C_6), 130.45 (C_7), 117.79 (C_8), 148.92 (C_{8a})	161.61 (C_2), 73.80 (C_3)	135.66 ($C_{1'}$), 130.00 ($C_{2'}$+$C_{6'}$), 131.65 ($C_{3'}$+$C_{5'}$), 127.22 ($C_{4'}$)
2.10h	6-Br; 8-OCH_3	4'-Br	50.36	168.05	197.65	47.43	30.71	56.11 (-OCH_3), 128.62 (C_{4a}), 122.06 (C_5), 115.43 (C_6), 113.71 (C_7), 147.76 (C_8), 138.27 (C_{8a})	161.54 (C_2), 73.82 (C_3)	135.69 ($C_{1'}$), 129.97 ($C_{2'}$+$C_{6'}$), 131.61 ($C_{3'}$+$C_{5'}$), 127.16 ($C_{4'}$)

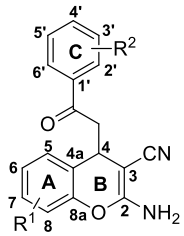
Table 13. *Spectroscopic data of ^{1}H NMR (400 MHz, DMSO-d_6) for 4H-chromenes* ***2.11****.*



2.11

Comp	R^1	R^2	NH_2	CH_2	C_4H	Ar^1-H (Ring A)	Ar'-H (Ring C)
2.11a	H	H	6.82 (s, 2H)	3.36 (m, 2H)	4.10 (t, J=5.6Hz, 1H)	7.23 (m, 2H, H_5+H_6), 7.05 (td, J=7.2, 1.2Hz, 1H, H_7) (6.98 (dd, J= 8.0, 0.8Hz, 1H, H_8)	7.60 (tt, J=14.8, 7.6, 1.2Hz, 1H, $H_{4'}$), 7.47 (t, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 7.90 (dd, J=8.4, 1.2Hz, 2H, $H_{2'}$+$H_{6'}$)
2.11b	6-Br	4'-CH_3	6.89 (s, 2H)	3.43 (m, 2H)	4.08 (t, J=5.6Hz, 1H)	7.23 (dd, J=2.4, 0.4Hz, 1H, H_5), 7.38 (dd, J=8.8, 2.4Hz, 1H, H_7), 6.94 (dd, J= 8.8Hz, 1H, H_8)	2.35 (s, 3H, -CH_3), 7.28 (d, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 7.81 (dd, J=6.4, 1.6Hz, 2H, $H_{2'}$+$H_{6'}$)
2.11c	6-Br; 8-OCH_3	H	6.90 (s, 2H)	3.39 (m, 2H)	4.06 (t, J=5.2Hz, 1H)	3.81 (s, 3H, -OCH_3), 7.10 (d, J=2.0Hz, 1H, H_5), 7.05 (d, J=1.6Hz, 1H, H_7)	7.61 (tt, J=7.6, 1.2Hz, 1H, $H_{4'}$), 7.48 (t, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 7.91 (d, J=4.8Hz, 2H, $H_{2'}$+$H_{6'}$)
2.11d	6-Br; 8-OCH_3	4'-Cl	6.91 (s, 2H)	3.38 (m, 2H)	4.05 (t, J=5.6Hz, 1H)	3.81 (s, 3H, -OCH_3), 7.06 (d, J=2.4Hz, 1H, H_5), 7.10 (d, J=2.4Hz, 1H, H_7)	7.54 (d, J=8.4Hz, 2H, $H_{3'}$+$H_{5'}$), 7.91 (d, J=8.4Hz, 2H, $H_{2'}$+$H_{6'}$)
2.11e	6-Br; 8-OCH_3	3'- OCH_3	6.91 (s, 2H)	3.41 (m, 2H)	4.04 (t, J=5.2Hz, 1H)	3.81 (s, 3H, -OCH_3), 7.05 (d, J=2.0Hz, 1H, H_5), 7.10 (d, J=2.0Hz, 1H, H_7)	3.79 (s, 3H, -OCH_3), 7.37 (t, J=2.4Hz, 1H, $H_{2'}$), 7.49 (dt, J=8.0, 1.2Hz, 1H, $H_{4'}$), 7.39 (d, J=8.0Hz, 1H, $H_{5'}$), 7.18 (dd, J=8.4, 1.2Hz, 1H, $H_{6'}$)
2.11f	6-Cl	4'-CH_3	6.89 (s, 2H)	3.39 (m, 2H)	4.08 (t, J=5.6Hz, 1H)	7.37 (d, J=2.4Hz, 1H, H_5), 7.24 (dd, J=8.4, 2.4Hz, 1H, H_7), 7.00 (d, J= 8.8Hz, 1H, H_8)	2.35 (s, 3H, -CH_3), 7.30 (d, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 7.82 (d, J=8.0Hz, 2H, $H_{2'}$+$H_{6'}$)
2.11g	6-Br	3'- OCH_3	6.89 (s, 2H)	3.44 (m, 2H)	4.08 (t, J=5.2Hz, 1H)	7.49 (m, 1H, H_5), 7.36 (dd, J=7.2, 2.8Hz, 1H, H_7), 6.94 (d, J= 8.8Hz, 1H, H_8)	3.79 (s, 3H, -OCH_3), 7.38 (t, J=2.4Hz, 1H, $H_{2'}$), 7.18 (ddd, J=8.0, 2.4, 0.8Hz, 1H, $H_{4'}$), 7.42 (d, J=8.0Hz, 1H, $H_{5'}$), 7.50 (m, 1H, $H_{6'}$)
2.11h	6,8-F	H	7.03 (s, 2H)	3.49 (m, 2H)	4.11 (t, J=5.2Hz, 1H)	7.07 (dt, J=9.2, 4.4, 2.0Hz, 1H, H_5), 7.25 (m, 1H, H_7)	7.60 (tt, J=8.8, 6.8, 1.6Hz, 1H, $H_{4'}$), 7.48 (t, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 7.92 (dd, J=8.4, 1.2Hz, 2H, $H_{2'}$+$H_{6'}$)
2.11i	6-Br; 8-OCH_3	4'-CH_3	6.89 (s, 2H)	3.35 (m, 2H)	4.04 (t, J=5.2Hz, 1H)	3.81 (s, 3H, -OCH_3), 7.04 (d, J=2.0Hz, 1H, H_5), 7.09 (d, J=2.4Hz, 1H, H_7)	2.35 (s, 3H, -CH_3), 7.28 (d, J=8.0Hz, 2H, $H_{3'}$+$H_{5'}$), 7.81 (d, J=8.0Hz, 2H, $H_{2'}$+$H_{6'}$)
2.11j	6-Br	4'-Br	6.90 (s, 2H)	3.45 (m, 2H)	4.08 (t, J=5.6Hz, 1H)	7.52 (d, J=2.4Hz, 1H, H_5), 7.38 (dd, J=8.8, 2.4Hz, 1H, H_7), 6.92 (d, J= 8.4Hz, 1H, H_8)	7.70 (d, J=8.8Hz, 2H, $H_{3'}$+$H_{5'}$), 7.84 (d, J=8.8Hz, 2H, $H_{2'}$+$H_{6'}$)
2.11k	6-Br; 8-OCH_3	4'-Br	6.91 (s, 2H)	3.37 (m, 2H)	4.05 (t, J=5.2Hz, 1H)	3.81 (s, 3H, -OCH_3), 7.06 (d, J=2.0Hz, 1H, H_5), 7.10 (d, J=2.4Hz, 1H, H_7)	7.70 (dd, J=6.8, 2.0Hz, 2H, $H_{3'}$+$H_{5'}$), 7.82 (dd, J=6.8, 2.0Hz, 2H, $H_{2'}$+$H_{6'}$)

Table 14. *Spectroscopic data of ^{13}C NMR (100 MHz, DMSO-d_6) for 4H-chromenes* ***2.11****.*



2.11

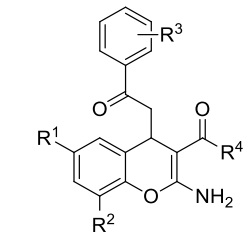
Comp	R^1	R^2	C≡N	C=O	CH_2	C_4H	Ar^1-C (Ring A)	Ar-C (Ring B)	Ar'-H (Ring C)
2.11a	H	H	120.64	197.94	46.90	31.09	123.92 (C_{4a}), 128.11 (C_5), 128.10 (C_6), 124.54 (C_7), 115.94 (C_8), 149.35 (C_{8a})	161.64 (C_2), 54.18 (C_3)	136.74 ($C_{1'}$), 128.08 ($C_{2'}$+$C_{6'}$), 128.76 ($C_{3'}$+$C_{5'}$), 133.34 ($C_{4'}$)
2.11b	6-Br	4'-CH_3	120.25	197.14	46.06	30.74	126.52 (C_{4a}), 130.56 (C_5), 115.82 (C_6), 130.65 (C_7), 118.03 (C_8), 148.67 (C_{8a})	161.23 (C_2), 53.80 (C_3)	21.12 (-CH_3), 134.18 ($C_{1'}$), 128.11 ($C_{2'}$+$C_{6'}$), 129.18 ($C_{3'}$+$C_{5'}$), 143.64 ($C_{4'}$)
2.11c	6-Br; 8-OCH_3	H	120.26	197.65	46.27	30.88	56.15 (-OCH_3), 126.57 (C_{4a}), 113.89 (C_5), 115.62 (C_6), 121.62 (C_7), 147.88 (C_8), 138.18 (C_{8a})	161.19 (C_2), 53.82 (C_3)	136.61 ($C_{1'}$), 127.99 ($C_{2'}$+$C_{6'}$), 128.65 ($C_{3'}$+$C_{5'}$), 133.26 ($C_{4'}$)
2.11d	6-Br; 8-OCH_3	4'-Cl	120.20	196.78	46.31	30.95	56.13 (-OCH_3), 126.40 (C_{4a}), 121.61 (C_5), 115.63 (C_6), 113.89 (C_7), 147.85 (C_8), 138.15 (C_{8a})	161.19 (C_2), 53.65 (C_3)	135.26 ($C_{1'}$), 129.92 ($C_{2'}$+$C_{6'}$), 128.70 ($C_{3'}$+$C_{5'}$), 138.15 ($C_{4'}$)
2.11e	6-Br; 8-OCH_3	3'- OCH_3	120.48	197.48	46.30	30.92	56.14 (-OCH_3), 126.52 (C_{4a}), 121.61 (C_5), 115.59 (C_6), 113.87 (C_7), 147.86 (C_8), 138.18 (C_{8a})	161.18 (C_2), 53.77 (C_3)	55.28 (-OCH_3), 138.04 ($C_{1'}$), 112.23 ($C_{2'}$), 159.34 ($C_{3'}$), 120.48 ($C_{4'}$), 129.78 ($C_{5'}$), 119.53 ($C_{6'}$)
2.11f	6-Cl	4'-CH_3	120.25	197.13	46.03	30.81	126.08 (C_{4a}), 127.68 (C_5), 127.87 (C_6), 127.76 (C_7), 117.64 (C_8), 148.20 (C_{8a})	161.26 (C_2), 53.74 (C_3)	21.11 (-CH_3), 134.64 ($C_{1'}$), 128.11 ($C_{2'}$+$C_{6'}$), 129.18 ($C_{3'}$+$C_{5'}$), 143.64 ($C_{4'}$)
2.11g	6-Br	3'- OCH_3	120.30	197.52	46.18	30.75	126.41 (C_{4a}), 130.58 (C_5), 115.83 (C_6), 130.68 (C_7), 118.03 (C_8), 148.71 (C_{8a})	161.25 (C_2), 53.73 (C_3)	55.31 (-OCH_3), 138.04 ($C_{1'}$), 112.27 ($C_{2'}$), 159.36 ($C_{3'}$), 119.55 ($C_{4'}$), 129.81 ($C_{5'}$), 120.51 ($C_{6'}$)
2.11h	6,8-F	H	120.02	197.58	45.61	30.96	127.63 (d, J=9.0Hz, C_{4a}), 109.70 (dd, J=24.0, 3.0Hz, C_5), 149.25 (dd, J=248.0, 14.0Hz, C_6), 103.04 (dd, J=27.0, 21.0Hz, C_7), 157.13 (dd,	160.84 (C_2), 53.69 (C_3)	136.53 ($C_{1'}$), 127.98 ($C_{2'}$+$C_{6'}$), 128.68 ($C_{3'}$+$C_{5'}$), 133.35 ($C_{4'}$)

							J=240.0, 11.0Hz, C_8), 134.44 (dd, J=11.0, 4.0Hz, C_{8a})		
2.11i	6-Br; 8-OCH_3	4'-CH_3	120.25	197.13	46.19	30.91	56.15 (-OCH_3), 126.65 (C_{4a}), 121.60 (C_5), 115.60 (C_6), 113.87 (C_7), 147.88 (C_8), 138.15 (C_{8a})	161.17 (C_2), 53.85 (C_3)	21.12 (-CH_3), 134.19 ($C_{1'}$), 128.11 ($C_{2'}$+$C_{6'}$), 129.18 ($C_{3'}$+$C_{5'}$), 143.62 ($C_{4'}$)
2.11j	6-Br	4'-Br	120.22	197.01	46.20	30.76	126.30 (C_{4a}), 130.61 (C_5), 115.86 (C_6), 130.72 (C_7), 118.04 (C_8), 148.67 (C_{8a})	161.26 (C_2), 53.62 (C_3)	135.58 ($C_{1'}$), 130.05 ($C_{2'}$+$C_{6'}$), 131.69 ($C_{3'}$+$C_{5'}$), 127.41 ($C_{4'}$)
2.11k	6-Br; 8-OCH_3	4'-Br	120.21	197.02	46.33	30.97	56.16 (-OCH_3), 126.43 (C_{4a}), 121.63 (C_5), 115.65 (C_6), 113.91 (C_7), 147.87 (C_8), 138.17 (C_{8a})	161.21 (C_2), 56.16 (C_3)	135.60 ($C_{1'}$), 130.04 ($C_{2'}$+$C_{6'}$), 131.67 ($C_{3'}$+$C_{5'}$), 127.28 ($C_{4'}$)

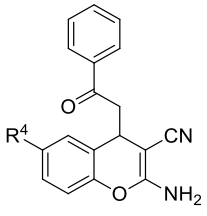
CHAPTER III – BIOLOGICAL ACTIVITY: RESULTS AND DISCUSSION

1. Initial compound viability screening

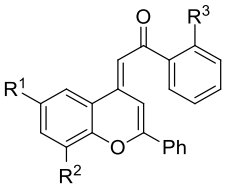
All the pure compounds synthetized and described in Experimental Section (Chapter 6) were tested for their biological activity. However, a series of chromenes, previously prepared in the research group by the PhD student Tatiana Dias [147] (Figure 18), was also included in this screening to assess if besides the substitution pattern, also the molecular scaffold could have any influence in the mode of action of the compounds. Therefore, a range of 51 compounds was evaluated for their anticancer properties by SRB assay. The first screening was performed on the Luminal-A subtype breast cancer cell line, MCF-7.



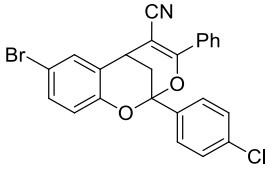
2.10i: R^1=Br; R^2=OCH_3; R^3=H; R^4=OCH_3
2.12a: R^1=Cl; R^2=H; R^3=CH_3; R^4=$NHCH_2Ph$
2.12b: R^1=Br; R^2=H; R^3=OCH_3; R^4=$NHCH_2Ph$



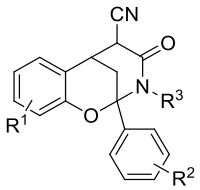
2.11l: R^4=OCH_3
2.11m: R^4=Br



2.13a: R_1=H; R_2=OCH_3; R_3=H
2.13b: R_1=Br; R_2=OCH_3; R_3=H
2.13c: R_1=H; R_2=OCH_3; R_3=OH;
2.13d: R_1=H; R_2=OH; R_3=H



2.14



2.15a: R_1=OCH_3; R_2=H; R_3=CH_3
2.15b: R_1=OCH_3; R_2=Cl; R_3=$(CH_2)_2OCH_3$
2.15c: R_1=OCH_3; R_2=Cl; R_3=CH_3;
2.15d: R_1=OCH_3; R_2=R_3=CH_3
2.15e: R_1=Br; R_2=H; R_3=CH_3
2.15f: R_1=OCH_3; R_2=H; R_3=$(CH_2)_2Ph$
2.15g: R_1=OCH_3; R_2=H; R_3=CH_2CH_3

Figure 18. *Series of previously synthetized chromenes for first screening with MCF-7 cell line.*

Cells were treated with two different concentrations (10 and 30 μM) of all the 51 compounds and evaluated for cell viability using the SRB assay in which total viable protein was quantified through total cell biomass in adherent cells. These concentrations were chosen based on previous results obtain on the research group [138]. After 72 hours of treatment, the SRB incorporated in viable cells was measured and the percentage of cell survival was calculated (Table 15). This first screening aimed to select a range of compounds that presented promising anticancer activity and, hereafter, would be evaluated against other cancer cells, HS578t (basal-

like breast cancer cell line), and assess their cytotoxicity in the non-neoplastic breast MCF-10 cell line (normal breast cell model).

Table 15. *Cell viability for MCF-7 cell line after 72 hours of treatment with two compound concentrations (10 and 30 µM).*

Comp	Cell viability (%)	
	10 µM	30 µM
2.3a	67.0	28.1
2.3b	53.3	28.0
2.3c	77.3	63.1
2.3d	38.2	26.3
2.3e	26.9	28.3
2.3f	109.9	37.6
2.3g	36.8	27.7
2.3h	96.8	61.5
2.3i	28.7	28.3
2.3j	86.9	29.3
2.3k	27.3	31.8
2.3l	33.9	35.3
2.3m	64.6	48.3
2.3n	60.4	34.0
2.3o	71.9	32.1
2.10a	89.8	63.8
2.10b	106.7	41.3
2.10c	142.2	94.9
2.10d	67.6	32.4
2.10e	80.3	33.8
2.10f	87.2	44.2
2.10g	72.5	34.8
2.10h	50.2	31.9
2.10i	68.3	40.3
2.11a	82.7	78.6
2.11b	69.0	32.3

Comp	Cell viability (%)	
	10 µM	30 µM
2.11c	99.1	36.7
2.11d	49.6	33.3
2.11e	55.9	33.9
2.11f	85.5	49.0
2.11g	51.2	34.4
2.11h	99.6	76.9
2.11i	115.8	52.8
2.11j	47.2	34.2
2.11k	39.7	33.1
2.11l	102.0	58.4
2.11m	87.1	75.3
2.12a	111.2	106.3
2.12b	83.7	73.3
2.13a	97.3	81.5
2.13b	119	102.7
2.13c	88.5	52.9
2.13d	91.1	62.7
2.14	56.0	41.5
2.15a	122.2	123.4
2.15b	90.7	65.3
2.15c	109.3	96.3
2.15d	113.9	97.9
2.15e	59.2	48.8
2.15f	81.9	60.94
2.15g	117.5	106.2

As we can see by the analysis of structure-activity, generally 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). The size of the halogen atom decreases in that equal order, but also the polarizability, a measure of the dynamical response of the electron cloud of the atom to the effect of a nearby external fields [138]. It was already reported in the literature that 4'-bromoflavonol presents enhanced activity in a leukemia cell line comparing with other halogen

substituents and even the number of methoxy groups (-OCH_3) introduced in the molecular pattern [148].

In compounds **2.3k** and **2.3n**, assessing the role of hydroxyl group, when present, seems to be determinant in the inhibition of cell viability. Mizuno *et al.* described that chalcones with electron-withdrawing groups in *meta* position of the benzene ring, as halogens and hydroxyl, when tested against colon carcinoma cells (HT-29) had the highest inhibitory activities [149].

Finally, comparing the general activity of chalcones with the respective chromenes, it appears that both type of molecules have promising anticancer activity. In the literature, both molecules have already been described as anticancer agents with diverse ways of action in MCF-7 cell line, as already described in **Section I** (Introduction) [74,101,110].

From this first screening, it was possible to select a range of 22 compounds with more interesting and promising activity from their low cell survival rates for the tested cancer cell line MCF-7. Consequently, treatment of another breast carcinoma cell line (HS578t) was preformed to assess if the subtype of breast cancer could have a different response to the many compounds, probably attending to the differential receptor expression. Cytotoxicity was also evaluated for MCF-10 cell line aiming to determine if the promising molecules for cancer could be also harmful for normal cells. Since chemotherapy present important side effects, screening for cytotoxicity, could allow to manage the molecular scaffold attending to the reduction of the side effects.

As performed for the initial screening, cells were treated with two different concentrations (10 and 30 μM) of the compounds for 72 hours, and cell viability was evaluated by total biomass using the SRB assay. Results for the two cell lines are summarized in Table 16.

In general, HS578t cell line sensitivity to the 22 compounds was inferior than for MCF-7 cells probably explained by the subtype of cancer including differential hormone receptor expression (Table 16). HS578t is a basal-like breast cancer subtype, with no known target, poor prognosis and less options of available treatments that present good response. Consequently, for 30 μM, many were the molecules that presented a significant reduction in cell viability in the range up to 50% with lower cytotoxicity for normal cells, making them interesting molecules to explore in the treatment of this subtype of breast cancer.

Table 16. *Cell viability for HS578t and MCF-10 cell lines after 72 hours of treatment at 10 and 30 μM.*

Comp	Cell Viability (%)			
	10 μM		30 μM	
	HS578t	MCF-10	HS578t	MCF-10
2.3a	46.9	105.8	34.4	40.7
2.3b	53.8	60.3	35.2	47.8
2.3c	74.2	69.8	33.5	47.0
2.3e	38.5	55.8	33.8	66.3
2.3f	96.5	114.1	37.4	41.4
2.3g	66.3	70.5	33.5	51.1
2.3h	84.6	64.2	155.3	147.9
2.3i	63.5	124.1	32.8	47.6
2.3j	105.9	101.9	35.7	48.0
2.3k	55.8	46.7	32.4	49.4
2.10e	63.0	90.2	40.9	47.8
2.10g	90.0	94.8	39.9	43.2
2.11b	88.8	46.3	133.0	33.5
2.11c	100.4	154.9	45.9	45.9
2.11d	90.7	90.7	36.8	41.6
2.11e	46.1	83.3	32.3	46.3
2.11f	92.3	154.9	41.8	53.3
2.11g	71.5	37.9	101.1	43.7
2.11i	92.4	144.3	53.8	128.5
2.11j	67.6	93.8	32.6	43.6
2.13c	73.3	98.9	44.9	78.2
2.15e	67.2	112.1	69.8	106.1

For MCF-10 cells, the results were quite promising. Almost every compound assessed led to a 50% (or more) cell survival for the 30 μM concentration increasing the possibilities of treatment with this type of compounds due to low values of citotoxicity.

Combining all the results from this first screening, we limited the range of compounds to further assess the survival curves and determine the IC_{50} values. Hence, regarding the results of Tables 15 and 16 where we can find the cell viability for the two-tested concentrations (10 and 30 μM) for each of the 22 compounds, it was possible to select chalcones **2.3** (**b**, **e**, **g** and **i**) and chromenes **2.11** (**e**, **g** and **j**).

2. Survival curves and IC_{50} determination

The IC_{50} for each cell line and compound was determined by SRB assay. Cells were treated with 7 different concentrations of compounds **2.3** (**b**, **e**, **g** and **i**) and **2.11** (**e**, **g** and **j**) from 60, 30, 10, 5, 1, 0.1 to 0.01 μM.

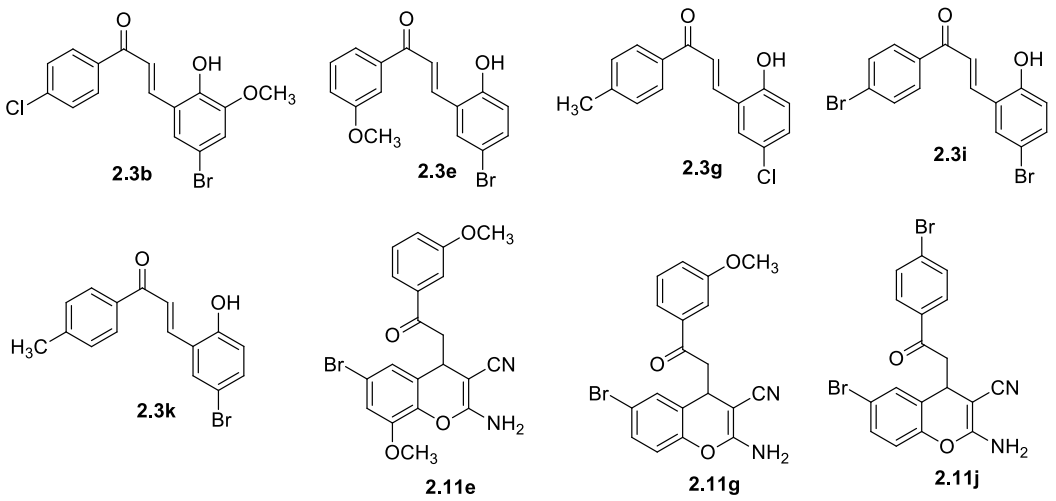


Figure 19. *Selected compound for IC_{50} determination in MCF-7, HS578t and MCF-10 cell lines.*

In general, more molecules with bromine (-Br) in ring A presented enhanced activity when compared to other substituents (Figure 19), as previously reported in the first screening results. These molecules were selected for IC_{50} determination, varying mostly the substituents in ring B. Results from the IC_{50} determination for the 3 cell lines are summarized in Table 17 and the survival curves are presented in Figure 20.

In general, the selected compounds maintained reduced cytotoxicity for MCF-10 cells. However, the minimal inhibitory concentration for some compounds (**2.3b**, **2.3k**, **2.11g** and **2.11j** in Figure 20 and Table 17) when compared with IC_{50} values obtained for normal cells, demonstrated a very low therapeutic index (between 0.9 and 3). Therapeutic index (TI) was defined as the ratio between the concentration of therapeutic agent with desirable effect (IC_{50} for MCF-7 or HS578t) and the amount that causes toxicity (IC_{50} for MCF10). Thus, these four compounds were excluded from the series since the major problem of these compounds is the low TI, turning them less suitable, probably leading to important side effects. However, to confirm these results some other normal models could be used as another non-neoplastic cell line. The low TI is also a problem present in some chemotherapy agents such as 5-FU, still currently used for treatment of basal-like breast cancer subtypes, that has a TI around 2.0-3.0 µg/mL and presents important toxicity to the bone marrow and gastro-intestinal tract [149,150]. In order to increase the therapeutic index of anticancer drugs, cancer research must continue.

On the other hand, compounds **2.3e**, **2.3g**, **2.3i** and **2.11e** (Table 17) presented satisfactory TI as shown by the IC_{50} of the normal cell line MCF-10 comparing to the IC_{50} of both cancer cell lines. Regarding their molecular structure, three of these four compounds contain

bromine and, only one, chlorine. This fact might be justified by the different polarizabilities, as previously discussed.

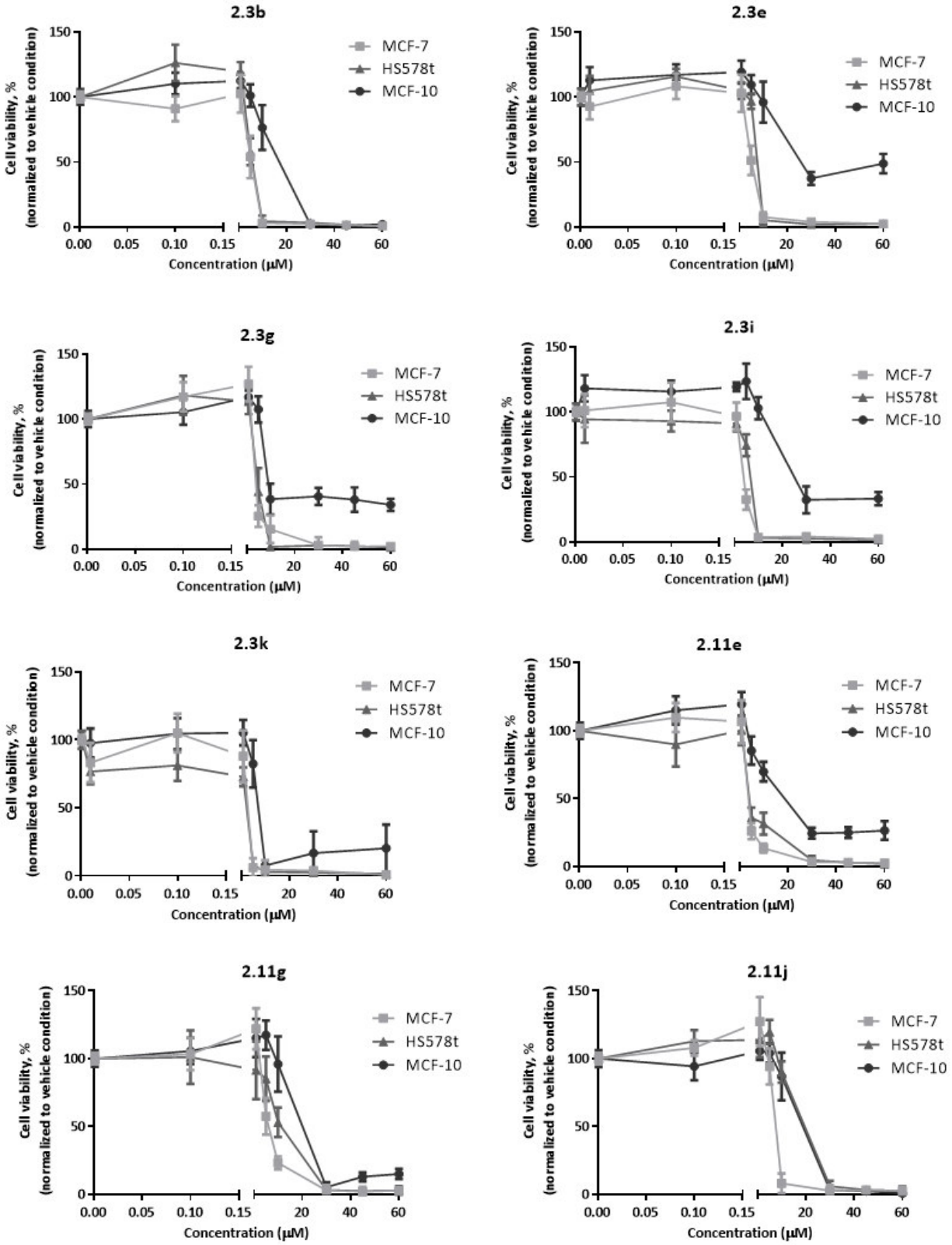


Figure 20. *Effect of chalcones and chromenes on MCF-7, HS578t and MCF-10 cells for total cell biomass (72 hours of treatment). Results are expressed as mean ± SD.*

Table 17. *IC_{50} values of the eight compounds selected for MCF-7, HS578t and MCF-10 cells.*

	IC_{50} (μM)		
Comp	**MCF-7**	**HS578t**	**MCF-10**
2.3b	5.15	5.33	12.11
2.3e	5.07	7.27	40.64
2.3g	3.98	4.80	24.25
2.3i	3.82	5.88	29.26
2.3k	2.00	1.60	6.52
2.11e	3.65	4.52	18.87
2.11g	5.94	10.26	18.93
2.11j	7.24	16.48	14.66

From this assay, only four compounds **2.3e**, **2.3g**, **2.3i** and **2.11e** proceeded to more specific tests such as cell migration capacity (wound healing assay), proliferation (BrdU incorporation), cell membrane integrity (trypan blue assay) and protein expression using Western Blotting.

3. 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. To assess if cell migration was being affected, compounds with more promising anticancer power were tested by treating MCF-7 cells with respective ½IC_{50} and IC_{50} during 48 hours for each compound described in Figure 21 and performing the wound-healing assay. These concentrations were selected once they would kill half of the total cells within 72 hours of treatment however, sometimes, with ½ IC_{50}, the migration is affected which would be advantageous since it would allow an anticancer effect with lower concentration of drug required. At the beginning and regular timepoints (12, 24 and 48 hours), images were captured as it can be seen in Figure 22 and the progression of the scratch closure for each compound is summarized in Figure 23.

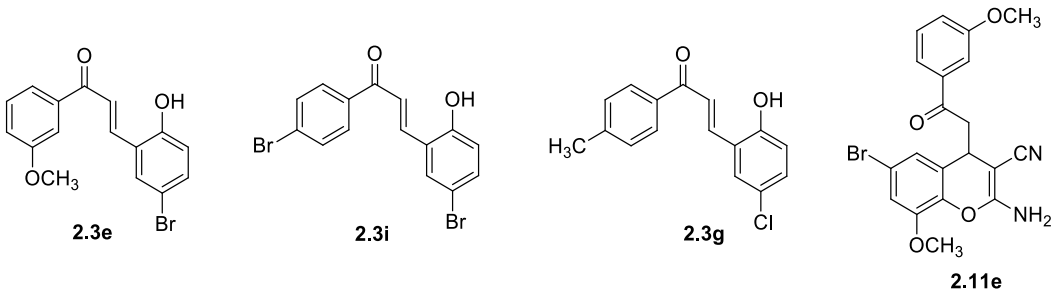


Figure 21. *Selected compounds for cell migration assay in MCF-7 cells.*

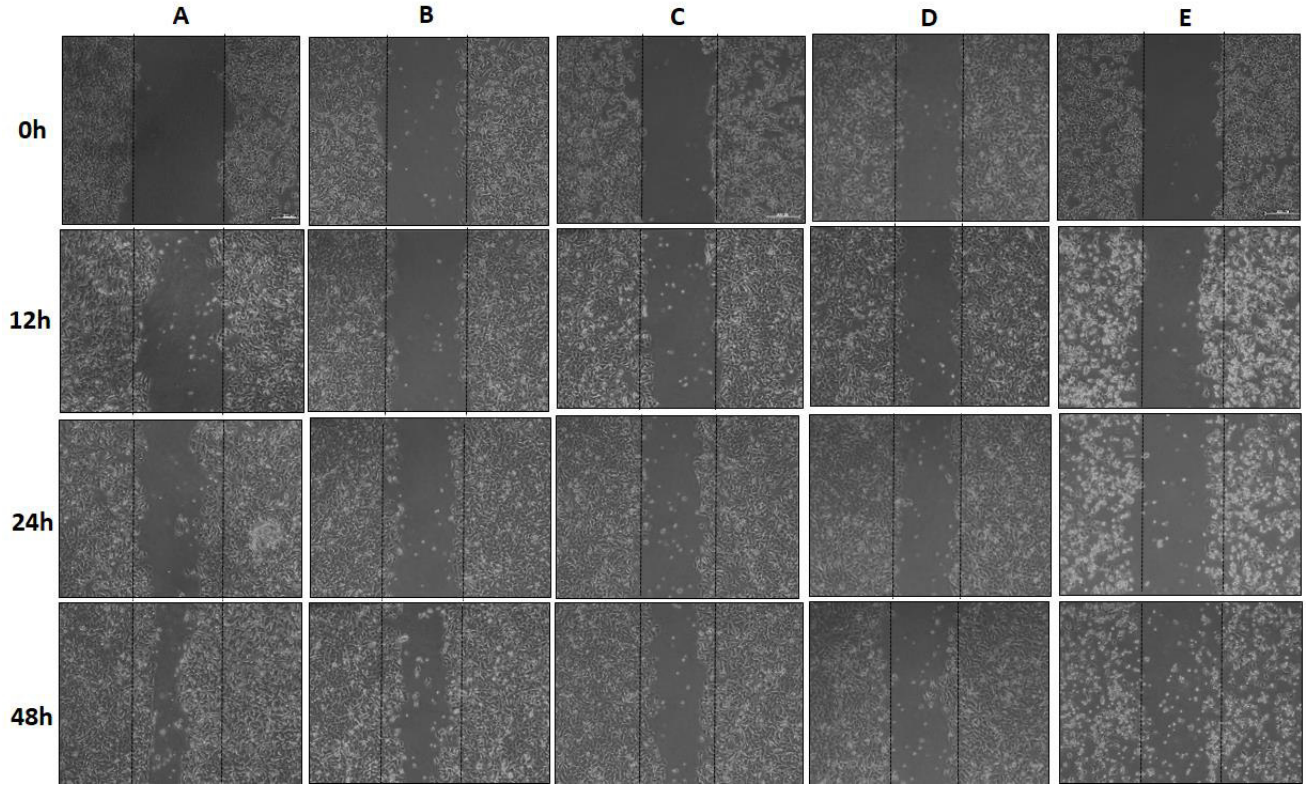


Figure 22. *Representative images of control (A) and effect of chalcones **2.3e** (B), **2.3g** (C) and **2.3i** (D) and chromene **2.11e** (E) on MCF-7 cell migration (12, 24 and 48 hours of treatment with the respective IC_{50} of each compound) by wound healing assay.*

As shown in Figure 22, MCF-7 cells showed a satisfactory migratory capacity since the scratch significantly reduced when cells were not treated. In general, chromene **2.11e** presented a much more promising inhibitory ability than the three chalcones tested (compounds **2.3e**, **2.3g** and **2.3i**). Treatment with this compound for 48 hours was able to decrease the percentage of cell migration relative to control (0.5% DMSO) as shown in Figure 23 (around 50%). Several chromenes were already reported as being good anti-migratory agents against breast cancer cell lines tested through wound-healing assay [144,145]. Both apigenin and luteolin are naturally occurring chromenes with similar and simple structures as compound **2.11e**. 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 as well as adherence junction disassembly [153]. However, as it can be seen in Figure 22, the majority of the cells died by the treatment as it was not able to determine if the inhibitory power was due to loss of migratory ability or by cell death induction. Then, the capacity to induce cell death will be further assessed to examine if cells could be undergoing apoptosis.

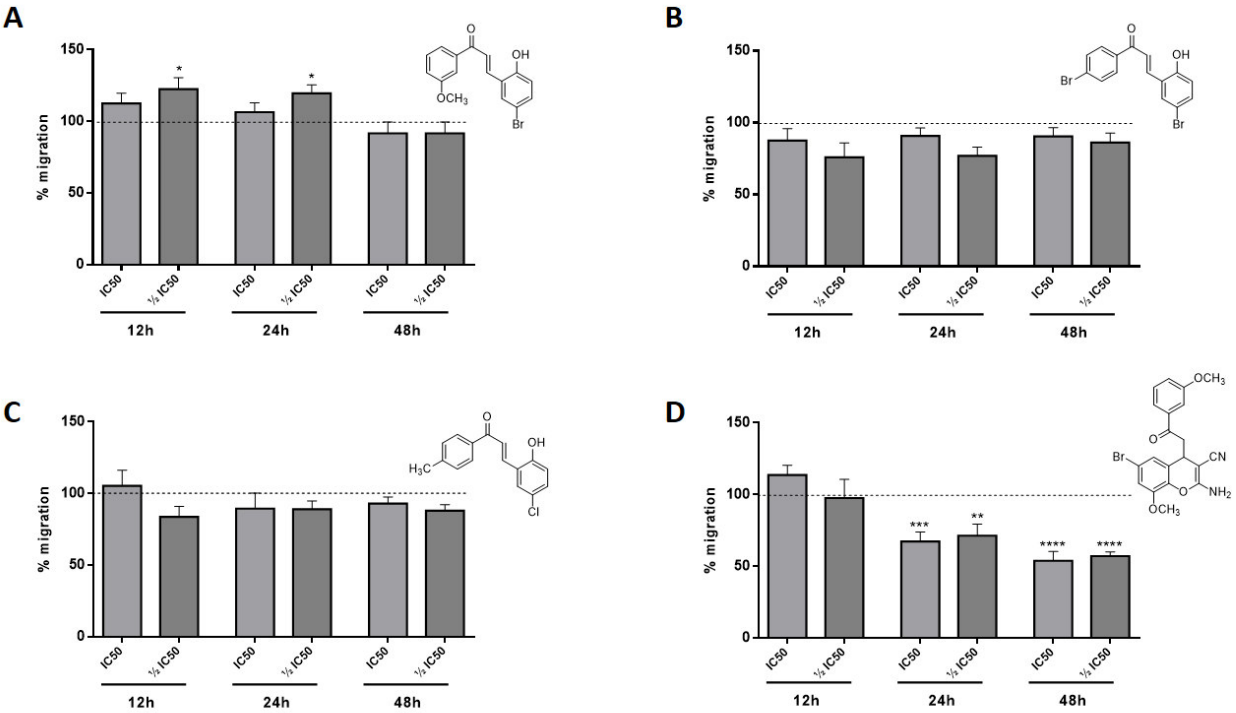


Figure 23. *Effect of chalcones **2.3e** (A), **2.3g** (B) and **2.3i** (C) and chromene **2.11e** (D) on MCF-7 cell migration (12, 24 and 48 hours of treatment) by the wound-healing assay. Results are presented as mean ± SD of three independent experiments. *p=0.01, **p=0.0022 ***p=0.0001, ****p<0.0001 compared to control (DMSO).*

Within chalcones, the cell migration was not significantly affected (Figure 23). In the literature, not many articles are found relating chalcones with inhibition of MCF-7 (or other breast cancer cell line) cell migration. According to results reported by Lin et al. non-substituted chalcones presented anti-migratory effect against AGS (human gastric adenocarcinoma) [154]. This compound, assessed by wound healing assay, inhibited around 67% cell migration after 24 hours of treatment with 2 µg/mL.

4. Effect of chalcones and chromenes on cell proliferation

In this experiment, the inhibitory influence on cell proliferation of the four best chalcones and chromenes was evaluated. MCF-7 cells were treated with IC_{50} and ½ IC_{50} values, for 24 and 48 hours. In this technique, the ability of BrdU incorporation during DNA synthesis is measured. Figure 24 summarizes the obtained results.

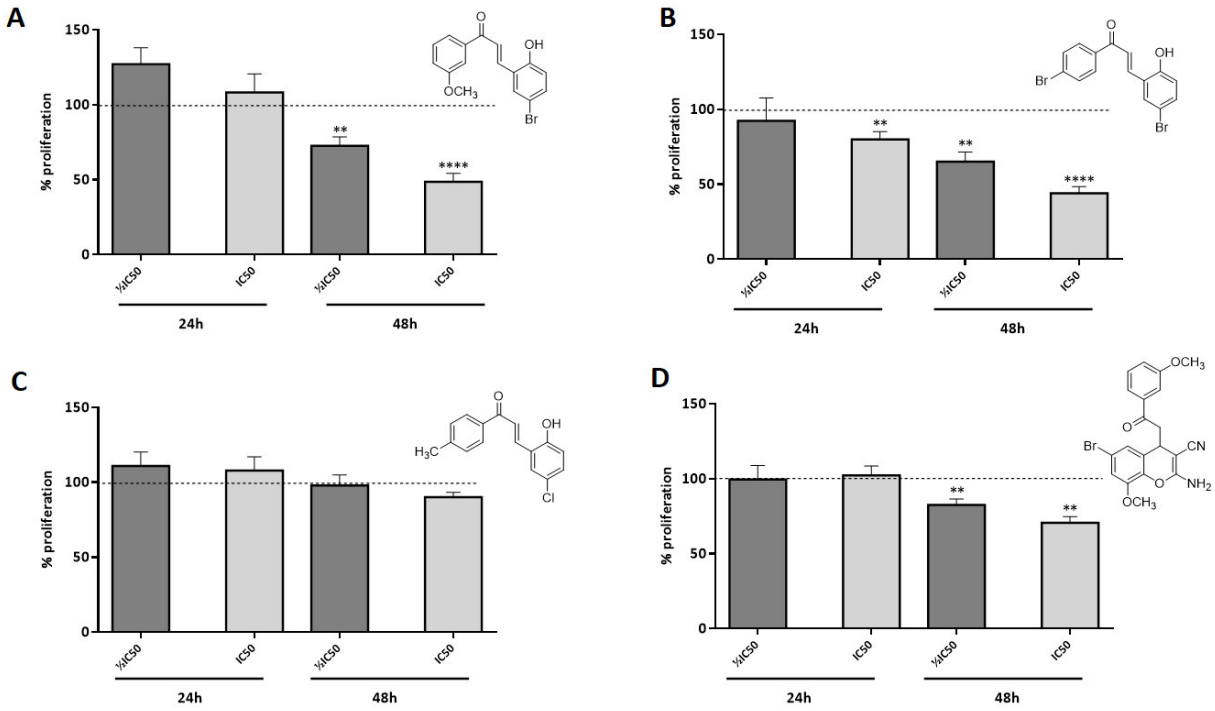


Figure 24. *Effect of chalcones **2.3e** (A), **2.3g** (B) and **2.3i** (C) and chromene **2.11e** (D) on MCF-7 cell proliferation after 24 and 48 hours of treatment. Results are presented as mean ± SD of at least three independent experiments. **p<0.005, ****p<0.0001 compared to control (0.5% DMSO).*

As shown the analysis of Figure 24, chalcones induced a significant decrease in cell proliferation, in a time dependent manner, for MCF-7 cells. In the group of chalcones, the highest effect was observed for compounds **2.3e** and **2.3g** with about 50% reduction in cell proliferation at 48 hours. A smaller effect was observed for compound **2.3i** that only reduced cell proliferation in about 25% at the same timepoint. Recently, a series of synthesized chalcones, very similar to those tested in this assay, presented good activity as anti-proliferative agents against several cell lines, including MCF-7 [155]. One chalcone of this described range, substituted with a methoxy group, was described to inhibit significantly BrdU incorporation and, consequently, cell proliferation around 33% when compared to the control. Additionally, more

assays were performed to analyze the cell cycle profile of NCI-H460 cells treated with this compound. Cellular arrest was significantly observed for the G_0/G_1 phase, from 56.3 to 68.0%, accompanied with decrease of S phase cell arrest.

On the other hand, chromene **2.11e** did not affected cell proliferation maintaining similar rates comparing to the control (0.5% DMSO) (Figure 24). In literature, many studies involve chromenes as anticancer agents affecting cell proliferation [113,115,117,119,120]. In particular, two chromenes with a scaffold similar to compound **2.11e**, were tested against H47D breast carcinoma, these compounds revealed a good inhibitory activity [146]. Attending at the molecular structures, compounds tested by Yin and co-workers were not substituted with an extra aryl group, keeping the structure quite simple and making possible the interaction of the ethoxy oxygen atom, forming an additional hydrogen bond with residues of Try867 and Asp964 in the hydrophobic pocket observed by molecular docking.

5. Effect of selected compounds on membrane integrity

Trypan blue gives a different approach of cell viability and we can assess how the viability of cells can be analyzed through the integrity of their membrane. Trypan blue is an assay used for survival purposes where viable and non-viable cells are counted through the ability to exclude dye. In this case, only adherent and viable cells were analyzed and taken into account. This procedure gave us a final idea of the IC_{50} effect on cell viability after three timepoints (24, 48 and 72 hours. Results are summarized in Figure 25 where cells were exposed to treatment with the respective ¼ IC_{50}, ½ IC_{50} and IC_{50} of each identified compound.

The trypan blue exclusion assay allows a direct identification and enumeration of living (unstained) and dead (blue) cells in a certain population. For many years, trypan blue has been used to assess cell viability and survival, however as every technique, it presents drawbacks. In terms of the operator health, it is considered to be hazardous and must be handled with care and disposed of appropriately. Finally, when compared to fluorescent based detection approaches, multiple publications have observed that trypan blue viability counts presented discrepancies for samples that had lower than 70% viable cells [156].

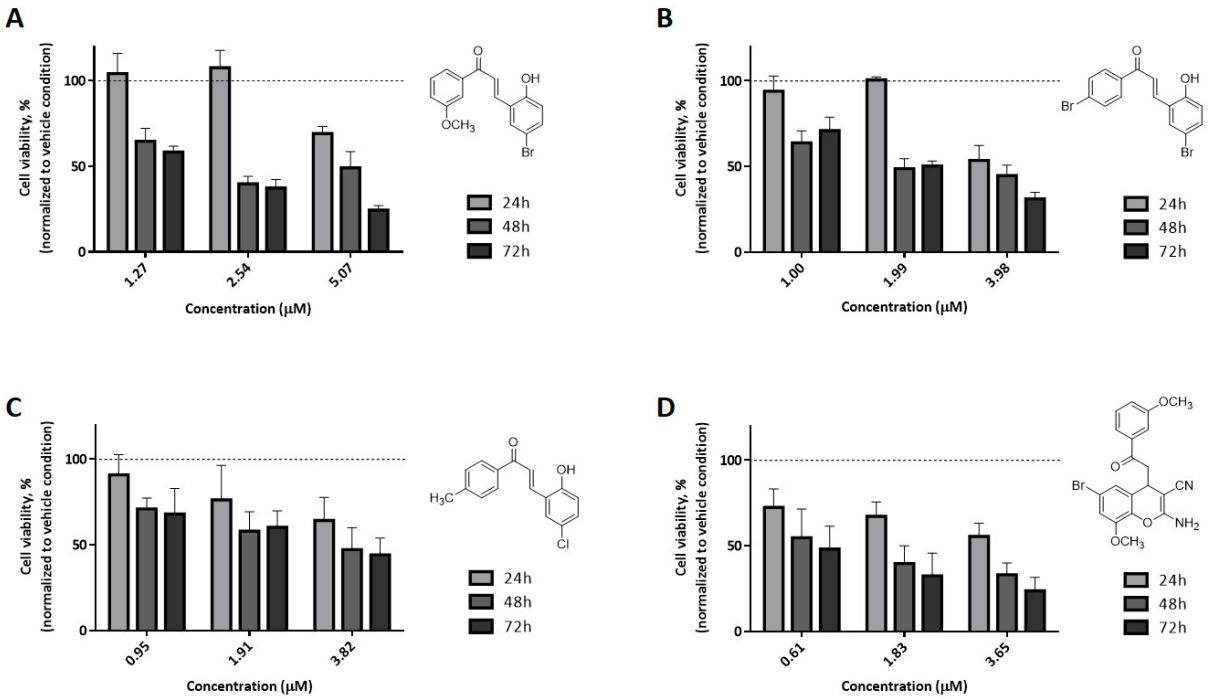


Figure 25. *Membrane integrity of MCF-7 cell line for 24, 48 and 72 hours treated with the respective ¼IC_{50}, ½IC_{50} and IC_{50} of chalcones **2.3e** (A), **2.3g** (B) and **2.3i** (C) and chromene **2.11e** (D). Results are presented as mean ± SD of at least three independent experiments.*

Every tested compound led to a reduction in cell viability and plasmatic membrane integrity of 50% or more after 48 hours of treatment. Chalcone **2.3e** and chromene **2.11e** led to the greatest reduction in viability of the four molecules, with a decrease of about 75% of membrane integrity for the 72 hours treatment with the respective IC_{50} concentration. These two compounds are dissimilar in their molecular scaffold however coincidentally, or not, both molecules have one halogen (-Br) and, at least, a methoxyl group (-OCH_3) in their substitution pattern. On the other hand, compound **2.3g** has chloride (-Cl) and methyl (-CH_3) and **2.3i** has two bromine (-Br).

Overmeyer et al. reported in 2011 that a chalcone-like small molecule induced the accumulation of vacuoles in glioblastoma cells (U251 cell line resistant to temozolomide) and filled the cell, decreasing ATP and mitochondrial membrane potential damaging metabolic function. Ultimately, these alterations led to disruption of membrane integrity and rupture of cells [157]. It was described that it induces cytoplasmic vacuolization and, consequently, a non-apoptotic cell death very similar to methuosis, a way of death triggered by modifications in the trafficking of clathrin-independent endosomes.

Recently, very similar **2.11e** compounds were reported as being apoptotic inducers for diverse cell lines, including MCF-7 cells, through loss of cytoplasmic membrane integrity [158]. The evaluation of apoptosis-inducing ability was made by fluorescent dyes (acridine orange-ethidium bromide) and DNA fragmentation.

On the other hand, MCF-10 cells did not appear to have their membrane integrity affected by exposure to compounds **2.3e**, **2.3g**, **2.3i** and **2.11e** (Figure 26). Only after 72 hours, the aggregated cells seem to begin to respond to the treatment. This fact is important since cancer cells are affected already after 24 hours even at ½IC_{50}, turning the treatment potentially harmless and non-cytotoxic to normal cells and effective for MCF-7 cancer cell line.

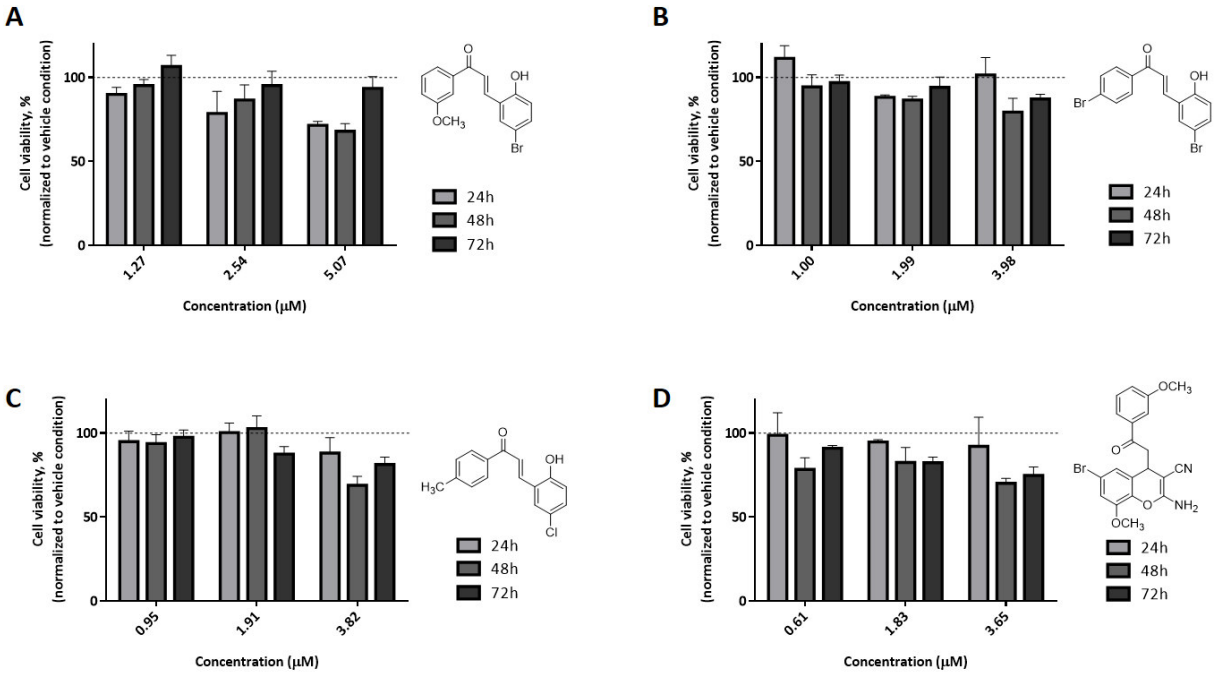


Figure 26. *Membrane integrity of MCF-10 cell line for 24, 48 and 72 hours treated with the respective ¼IC_{50}, ½IC_{50} and IC_{50} of chalcones **2.3e** (A), **2.3g** (B) and **2.3i** (C) and chromene **2.11e** (D). Results are presented as mean ± SD of at least three independent experiments.*

6. Effect on protein expression

As previously described, chalcones and chromenes were reported, by several research groups as anticancer agents through pro-apoptotic mechanisms. Once tumor cells are highly adapted to different conditions due to their high ability of proliferate and escape apoptosis,

protein expression levels was evaluated. To assess which kind of protein expression could be involved in the toxicity induced by these compounds, two proteins involved in apoptotis (PARP and Casp-9) and a protein involved in cancer cell metabolism (CAIX) were tested for their expression. MCF-7 cells were incubated for 24 hours with IC_{50} values of compounds **2.3e**, **2.3g**, **2.3i** and **2.11e**.

Poly (ADP-ribose) polymerase (PARP) is activated by DNA damage as breaks in the DNA strands once it is involved in DNA repair mechanisms and, also, programmed cell death. When, cleaved by caspase family during cellular apoptosis, the peptide strand of 126kD is separated in two segments of 89kD and 24kD. For breast cancer treatment, many PARP inhibitors are currently in trials for the adjuvant, neoadjuvant, and metastatic settings [159].

Caspase-9 (casp-9) is considered an initiator caspase. The stress induction in JNK/SAPK has shown to activate a pro-enzyme of casp-9 through the release of cytochrome C from mitochondria and activation of the apoptosome [160]. In its full length, it has 47kD and, when activated, through cleavage, it can have two different segment lengths, 35kD and/or 37kD. Once activated, it cleaves procaspase-3/7 which will cleave PARP and other several cellular targets.

Carbonic anhydrase IX (CAIX) is a cell membrane-associated protein involved in glycolytic metabolism and hypoxia cellular biomarker [161]. This specific carbonic anhydrase is tumor-associated and overexpressed in many cancer types, including breast cancer. It is believed that it might be involved in cell proliferation. This protein expression was chosen to be evaluated due to a recent emerging hallmark of cancer cells, the ability to alter, or reprogram, cellular metabolism.

As it can be seen in Figure 27, CAIX expression seems to be enhanced in cancer cells treated with the synthetized compounds, as it was predictable by the glycolytic tumor origin of MCF-7 cells. Comparing to control, this expression seems to be increased which may lead to believe that despite the initial adaptive and usual glycolytic capacity of cancer cells, these might be trying to consume more energy possibly to resist treatment. Recently, a docking study was performed to evaluate the possible interactions of the active site of CAIX with newly synthetized chromenes [162]. From the initial series of molecules, two compounds were found to be promising CAIX inhibitors.

One the other hand, cell death associated with apoptosis only seems to be involved in cells treated with compound **2.11e**. The expression of cleaved Casp-9 and cleaved PARP is enhanced with this chromene comparing with the other compounds, chalcones. Many

chromenes, both naturally occurring or synthetized, were previously reported as anticancer agents through apoptosis-inducers [112,139].

It is important to note that besides the importance of this information, all the presented results are preliminary due to the lack of data provided by the housekeeping protein (β-tubulin) and will need to be repeated.

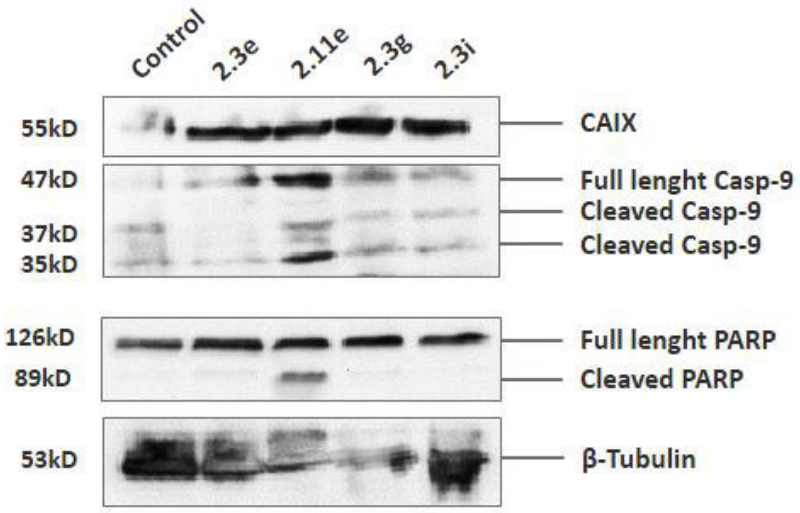


Figure 27. *Representative blot of effect of compounds* ***2.3e***, ***2.3g***, ***2.3i*** *and* ***2.11e*** *on MCF-7 cells - caspase-9, PARP and CAIX (24 hours treatment) by Western blot analysis. Similar blots were obtained in the two independent experiments.*

CHAPTER IV – CONCLUSIONS AND FUTURE WORK

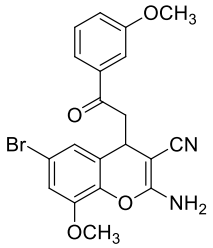
Conclusions

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

In this Master's thesis, a good number of novel compounds (chalcones and chromenes) were synthetized by eco-friendly methodologies, with good yield and high levels of purity.

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

Specifically, a chromene (Figure 28) has demonstrated to be involved in the inhibition of cell migration and, preliminary results, found that apoptotic-inducing could be the cause for cell death when treated with this molecule.



2.11e

Figure 28. *Most promising chromene structure.*

Regarding to chalcones, these compounds also showed to be bioactive molecules by inhibiting cell proliferation and disrupting the membrane integrity.

Future work

As it can be seen by Figure 28, modification of the substitution pattern could be further explored as the nature and position of the substituents on the aromatic rings is crucial for the biological activity one of the further works that can be done. Evaluate the citotoxicity of novel molecules and the possible influence of different substituents in ring C or the variation in the several available positions. Elemental analysis in also one of the many molecular characterization methods that can be performed in future studies to assess, once more, the purity of the synthetized chromenes and chalcones and confirm their empirical formula.

Subsequently, the protein expression assay should be optimized and other several proteins could be assessed for their involvement in the anticancer activity of the tested compounds. It is also intended to understand which pathways might be affected by the treatment with these novel compounds.

Additionally, once compounds were already tested in three different cell lines for *in vitro* assays and they do not mimic all real tumor conditions, for more interesting compounds *in vivo* studies should be performed in order to comprehend the way of action in more complex models.

Finally, some molecular docking studies also could be performed to understand the possible interactions with active sites of different proteins and a way to improve the molecules by increasing the number of interactions with important amino acids.

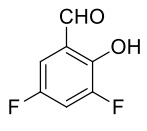
CHAPTER V – EXPERIMENTAL SECTION

1. Experimental procedures

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.

Synthesis of 3,5-difluoro-2-hydroxybenzaldehyde



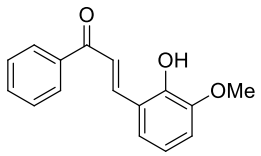
This compound is commercially available but was synthetized in our laboratory according to established procedures. Hexamethylenetetramine (4.35 g; 31.05 mmol) was added to a colorless solution of 2,4-difluorophenol (1.98 mL; 2.109 g; 20.7 mmol) in TFA (14 mL; 21.2 g; 184.76 mmol). The reaction mixture was stirred at 40ºC for 15 minutes, increasing the temperature to 80ºC and, after 1 hour, to 110ºC. After 18 hours, the orange mixture was cooled until to room temperature and a yellow solid precipitated by adding 91.6 mL of H_2O. The product was filtered and washed with H_2O leading to 3,5-difluoro-2-hydroxybenzaldehyde (2.114 g; 18.6 mmol; 89.9%).

1.1. General procedure for the synthesis of chalcone derivatives

Aqueous 3M NaOH was added to a solution of substituted salicylaldehyde and acetophenone in ethanol. The solution was stirred at an appropriate temperature leading to a red solution. The solution was neutralized with concentrated HCl (37%) leading to a yellow suspension, cooled to room

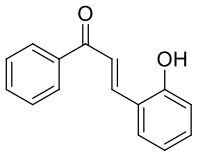
temperature and set in the ultrasound for a few minutes. A yellow solid was filtered and washed with ethanol and water leading to the *α*,*β*-unsaturated carbonyl compound.

*Synthesis of (E)-3-(2-hydroxy-3-methoxyphenyl)-1-phenylprop-2-en-1-one (**2.3p**)*



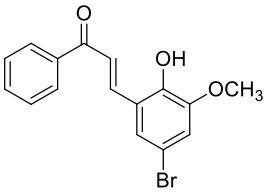
Following the general procedure, aqueous 3M NaOH (10 mL) was added to a solution of 2-hydroxy-3-methoxybenzaldehyde (0.50g, 3.32 mmol) and acetophenone (355µL, 3.01 mmol) in ethanol (10 mL) leading immediately to a red solution that was stirred at room temperature overnight leading to a red suspension. The suspension was filtered under vacuum leading to a red solid, washed with cold ethanol and identified by IR and ^{1}H NMR as (*E*)-3-(2-hydroxy-3-methoxyphenyl)-1-phenylprop-2-en-1-one (0.88g, 3.2 mmol, 94.0%) (Tables 5 and 6).

*Synthesis of (E)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one (**2.3o**)*



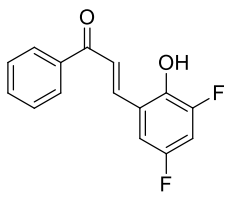
Following the general procedure, aqueous 3M NaOH (7 mL) was added to a solution of salicylaldehyde (260µL, 2.46 mmol) and acetophenone (310µL, 2.11 mmol) in ethanol (7 mL) resulting in a red solution. After being stirred for 31 hours at room temperature, the solution was neutralized with concentrated HCl leading to a light yellow suspension. This suspension was cooled slightly above room temperature and was filtered tepid leading to a pale yellow pure product identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one (0.24g, 1.19mmol, 48.4%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-phenylprop-2-en-1-one (**2.3a**)*



Following the general procedure, aqueous 3M NaOH (10 mL) was added to a solution of 5-bromo-2-hydroxy-3-methoxybenzaldehyde (0.50g, 2.16 mmol) and acetophenone (230µL, 1.96 mmol) in ethanol (10 mL) leading immediately to a red solution that was stirred for 22 hours at 40ºC. Then, the reaction was neutralized at room temperature with concentrated HCl forming a yellow solid suspension that was cooled at 4ºC for 45 minutes. After that, the yellow solid was filtered, washed with water and identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-phenylprop-2-en-1-one (0.72g, 2,16 mmol, 99.7%) (Tables 5, 6 and 7).

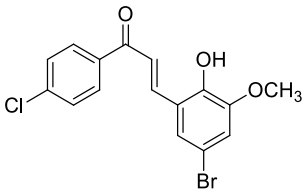
*Synthesis of (E)-3-(3,5-difluoro-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (**2.3f**)*



Following the general procedure, aqueous 3M NaOH (5 mL) was added to a solution of 3,5-difluoro-2-hydroxybenzaldehyde (0.21g, 1.32 mmol) and acetophenone (180µL, 1.45 mmol) in ethanol (5 mL) leading immediately to a red solution that was stirred for 4 hours at room temperature. Then, the reaction was neutralized at room temperature with concentrated HCl leading to a pale yellow suspension that was cooled for 15 hours at -20ºC. After that,

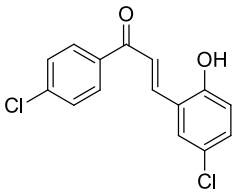
a light yellow solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(3,5-difluoro-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (0.26g, 0.99 mmol, 75.0%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one (**2.3b**)*



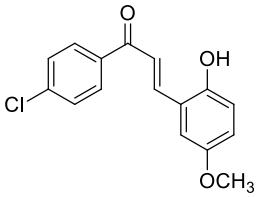
Following the general procedure, aqueous 3M NaOH (10 mL) was added to a solution of 5-bromo-2-hydroxy-3-methoxybenzaldehyde (0.52 g, 2.26 mmol) and 1-(4-chlorophenyl)ethanone (270µL, 2.05 mmol) in ethanol (10 mL) leading to a red solution. This solution was stirred at 40ºC during 18 hours and 30 minutes and was then neutralized with concentrated HCl leading to a light brown suspension that was cooled at 4ºC for 45 minutes. The light brown solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one (0.55g, 1.48 mmol, 65,6%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(5-chloro-2-hydroxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one (**2.3r**)*



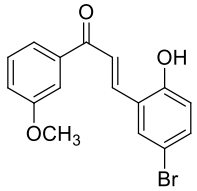
Following the general procedure, aqueous 3M NaOH (6 mL) was added to a solution of 5-chloro-2-hydroxybenzaldehyde (0.29g, 1.88mmol) and 1-(4-chlorophenyl)ethanone (268µL, 2.07mmol) in ethanol (6 mL) leading immediately to a red solution stirred at room temperature for 6 hours. This solution was cooled to -20ºC for 17 hours and, finally, neutralized with concentrated HCl until pH±2 leading to a light yellow suspension that was kept at 4ºC for 45 minutes. The yellow solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(5-chloro-2-hydroxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one (0.49g, 1.66mmol, 88.3%) (Table 6).

*Synthesis of (E)-1-(4-chlorophenyl)-3-(2-hydroxy-5-methoxyphenyl)prop-2-en-1-one (**2.3j**)*



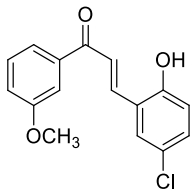
Following the general procedure, aqueous 3M NaOH (12 mL) was added to a solution of 2-hydroxy-4-methoxybenzaldehyde (0.68g, 4.44 mmol) and 1-(4-chlorophenyl)ethanone (765µL, 5.77 mmol) in ethanol (4mL) leading to a dark red solution. The solution was stirred at room temperature for 18 hours and was neutralized with concentrated HCl until pH±2 leading to a colorless solution with an orange precipitate. The solid was filtered and ^{1}H NMR analysis showed that (E)-1-(4-chlorophenyl)-3-(2-hydroxy-5-methoxyphenyl)prop-2-en-1-one was contaminated with 4'-cloroacetophenone contamination in a 1:0.4 ratio. The solid was washed with strong stirring for 4 hours with 10 mL of distilled water and 3 mL of ethanol. The orange solid was filtered and by IR, ^{1}H and ^{13}C NMR confirmed that it was pure (*E*)-1-(4-chlorophenyl)-3-(2-hydroxy-5-methoxyphenyl)prop-2-en-1-one (1.07g, 3.72mmol, 83.8%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (**2.3e**)*



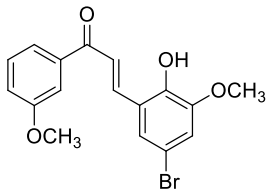
Following the general procedure, aqueous 3M NaOH (10 mL) was added to a solution of 5-bromo-2-hydroxybenzaldehyde (0.46g, 2.31 mmol) and 3'-methoxyacetophenone (350µL, 2.104 mmol) in ethanol (10 mL) leading to a red solution. After stirring for 23 hours at room temperature, the solution was neutralized with concentrated HCl leading to a dark yellow suspension. This suspension was filtered and the yellow solid was identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(5-bromo-2-hydroxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (0.63g, 1.89mmol, 81.8%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(5-chloro-2-hydroxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (**2.3d**)*



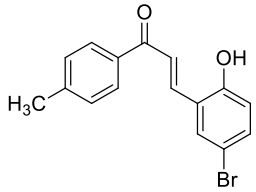
Following the general procedure, aqueous 3M NaOH (10 mL) was added to a solution of 5-chloro-2-hydroxybenzaldehyde (0.39g, 2.100 mmol) and 3'-methoxyacetophenone (378µL, 2.115 mmol) in ethanol (10mL) leading to a dark red solution. After stirring for 16 hours and 30 minutes at room temperature, the solution was neutralized with concentrated HCl until pH±3 resulting in an orange solution with a pale yellow precipitate. Then it was cooled for 2 hours at 4ºC and, finally, a pale yellow product was filtered and identified by, IR, ^{1}H and ^{13}C NMR, as (*E*)-3-(5-chloro-2-hydroxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (0.62g, 2.15mmol, 86.0%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (**2.3c**)*



Following the general procedure, aqueous 3M NaOH (10 mL) was added to a solution of 5-bromo-2-hydroxy-3-methoxybenzaldehyde (0.51g, 2.2 mmol) and 3'-methoxyacetophenone (385µL, 2.119 mmol) in ethanol (10 mL) leading immediately to a red solution that was stirred for 19 hours at room temperature. Then, the reaction mixture was neutralized with concentrated HCl forming a yellow suspension that was cooled at 4ºC for 2 hours. After that, the yellow solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (1.37g, 3.82 mmol, 99.7%) (Tables 5, 6 and 7).

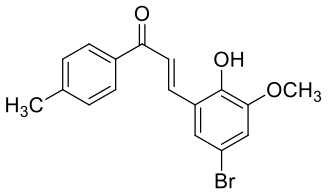
*Synthesis of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one (**2.3k**)*



Following the general procedure, aqueous 3M NaOH (5 mL) was added to a solution of 5-bromo-2-hydroxybenzaldehyde (0.34g, 1.71 mmol) and 1-(p-tolyl)ethanone (320µL, 2.28 mmol) in ethanol (1.6 mL) leading to an orange solution that after 2 hours stirring at room temperature turned into dark red. After stirring for a total of 19 hours, the solution was neutralized with concentrated HCl leading to a yellow suspension kept in the ultrasound bath for a few minutes. A yellow solid was filtered, washed with water and ethanol and identified by IR, ^{1}H and ^{13}C NMR as (*E*)-

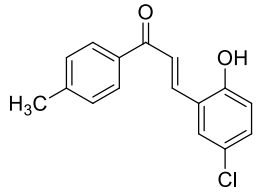
3-(5-bromo-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one (0.42g, 1.33mmol, 77.8%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(p-tolyl)prop-2-en-1-one (**2.3h**)*



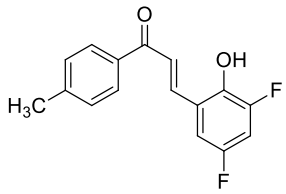
Following the general procedure, aqueous 3M NaOH (8 mL) was added to a solution of 5-bromo-2-hydroxy-3-methoxybenzaldehyde (0.27, 1.17 mmol) and 1-(p-tolyl)ethanone (171µL, 1.284 mmol) in ethanol (8 mL) resulting in a red solution. After being stirred for 24 hours at room temperature, it was neutralized with concentrated HCl until pH±3 and led to a yellow suspension. This suspension was kept in the ultrasound bath for, approximately, half an hour, cooled and filtered. The pale yellow isolated product was identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one (0.31g, 0.90mmol, 76.6%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(5-chloro-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one (**2.3g**)*



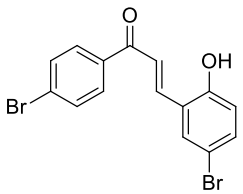
Following the general procedure, aqueous 3M NaOH (10 mL) was added to a solution of 5-chloro-2-hydroxybenzaldehyde (0.38g, 2.44 mmol) and 1-(p-tolyl)ethanone (358µL, 2.108 mmol) in ethanol (10 mL) leading to a red solution. This solution was stirred at room temperature for 18 hours and was then it was neutralized with concentrated HCl. A yellow suspension was formed and cooled for 1 hour at 4ºC. The yellow product was filtered and identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(5-chloro-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one (0.59g, 2.13 mmol, 87.3%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(3,5-difluoro-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one (**2.3l**)*



Following the general procedure, aqueous 3M NaOH (10 mL) was added to a solution of 3,5-difluoro-2-hydroxybenzaldehyde (0.32g, 2.0 mmol) and 1-(p-tolyl)ethanone (297µL, 2.2 mmol) in ethanol (10 mL) leading immediately to a red solution that was stirred for 5 hours at room temperature. Then, the reaction was neutralized at room temperature with concentrated HCl forming a pale yellow suspension which was cooled for 45 minutes at 4ºC. The light yellow solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(3,5-difluoro-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one being pure (0.43g, 1.58 mmol, 78.8%) (Tables 5, 6 and 7).

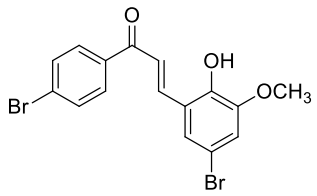
*Synthesis of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one (**2.3i**)*



Following the general procedure, aqueous 3M NaOH (12 mL) was added to a solution of 5-bromo-2-hydroxybenzaldehyde (0.45g, 2.23 mmol) and 1-(4-bromophenyl)ethanone (0.49g, 2.46 mmol) in ethanol (12 mL) leading immediately to a red precipitate in a red solution. After 6 hours at room temperature, the reaction mixture was filtered and a red solid was isolated.

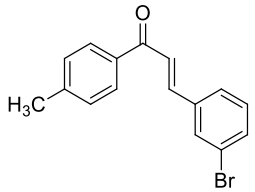
A suspension of this solid in 3mL of water was neutralized with concentrated HCl at room temperature leading to a yellow solid. The neutralized suspension was cooled at 4ºC for 16 hours. Finally, it was isolated a pure yellow product identified by IR, ^{1}H and ^{13}C NMR as (*E*)-3-(5-bromo-2-hydroxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one (0.59g, 1.56mmol, 70.0%) (Tables 5, 6 and 7).

*Synthesis of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one (**2.3m**)*



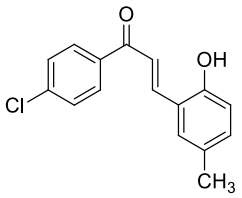
Following the general procedure, aqueous 3M NaOH (8 mL) was added to a solution of 5-bromo-2-hydroxy-3-methoxybenzaldehyde (0.26g, 1.14mmol) and 1-(4-bromophenyl)ethanone (0.25g, 1.26mmol) in ethanol (8 mL) leading to a red solution stirred at room temperature for 22 hours. After that, the solution was kept in an ice bath and concentrated HCl was added to pH±2 turning the red solution into a yellow suspension. The solid was filtered while tepid and the yellow product was identified by ^{1}H NMR, the product was identified as (*E*)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one (0.62g, 1.57mmol, 80.3%) (Table 6).

*Synthesis of (E)-3-(3-bromophenyl)-1-(p-tolyl)prop-2-en-1-one (**2.3n**)*



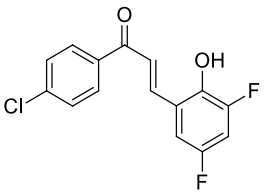
Following the general procedure, aqueous 3M NaOH (7 mL) was added to a solution of 3-bromobenzaldehyde (190µL, 1.62mmol) and 1-(p-tolyl)ethanone (238µL, 1.78mmol) in ethanol (7 mL) leading immediately to a white product in a transparent solution. The reaction mixture was stirred for 4 hours at room temperature. And the solid was filtered resulting in the isolation of a white product from a pale yellow solution. The product was identified by ^{1}H NMR as (*E*)-3-(3-bromophenyl)-1-(p-tolyl)prop-2-en-1-one (0.46g, 1.53mmol, 96.8%) (Tables 5, 6 and 7).

*Synthesis of (E)-1-(4-chlorophenyl)-3-(2-hydroxy-5-methylphenyl)prop-2-en-1-one (**2.3u**)*



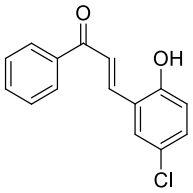
Following the general procedure, aqueous 3M NaOH (5 mL) was added to a solution of 2-hydroxy-5-methylbenzaldehyde (0.25g, 1.84mmol) and 1-(4-chlorophenyl)ethanone (263µL, 2.02mmol) in ethanol (5mL) leading to a red solution. The reaction mixture was stirred for 7 hours at room temperature and, then, neutralized with concentrated HCl to pH±3 resulting in a yellow suspension. As there were some lumps, the suspension was taken to the ultrasound bath, for 30 minutes. Finally, the yellow product was filtered and identified by ^{1}H NMR as (*E*)-1-(4-chlorophenyl)-3-(2-hydroxy-5-methylphenyl)prop-2-en-1-one (0.21g, 0.80mmol, 43%), contaminated with in a 1:0.25 molar ratio. The contaminated product was suspended in water and ethanol (2:1) and the mixture was stirred for 2 hours, the product isolated by filtration maintained the impurity. It was attempted a recrystallization of the contaminated solid (0.19g) under 5 minutes of reflux in 4mL water and 2mL ethanol (2:1). However, after isolation of a yellow solid (0.17g, 0.56g, 30.4%) it was observed that the 1-(4-chlorophenyl)ethanone contamination was preserved in a similar ratio (Table 6).

*Synthesis of (E)-1-(4-chlorophenyl)-3-(3,5-difluoro-2-hydroxyphenyl)prop-2-en-1-one (**2.3s**)*



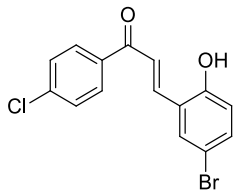
Following the general procedure, aqueous 3M NaOH (10mL) was added to a solution of 3,5-difluoro-2-hydroxybenzaldehyde (0.46g, 3.47mmol) and 1-(4-chlorophenyl)ethanone (486µL, 3.82mmol) in ethanol (10mL) and resulted in a red solution that was stirred at room temperature for 21 hours. After that, addition of concentrated HCl to pH±2.10 and led to a yellow suspension taken to the ultrasound bath for 1 hour. Finally, the suspension was filtered and the solid product was identified by ^{1}H NMR, mostly, as 1-(4-chlorophenyl)ethanone (0.6481g, 2.2mmol, 63.4%), with traces of (*E*)-1-(4-chlorophenyl)-3-(3,5-difluoro-2-hydroxyphenyl)prop-2-en-1-one in a 4.1:1 molar ratio (Table 6).

*Synthesis of (E)-3-(5-chloro-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (**2.3t**)*



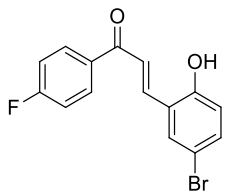
Following the general procedure, aqueous 3M NaOH (10mL) was added to a solution of 5-chloro-2-hydroxybenzaldehyde (0.50g, 3.17mmol) and acetophenone (407µL, 3.49mmol) in ethanol (10mL), leading immediately to a red solution, stirred at room temperature for 24 hours. After that, addition of concentrated HCl to pH±2/3 led to a yellow suspension taken to the ultrasound bath for 30 minutes. The solid was filtered and identified by ^{1}H NMR as (*E*)-3-(5-chloro-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (0.58g, 2.25mmol, 71.0%), with traces of 5-chloro-2-hydroxybenzaldehyde and contaminant **2.5** (Table 6).

*Synthesis of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one (**2.3v**)*



Following the general procedure, aqueous 3M NaOH (10 mL) was added to a solution of 5-bromo-2-hydroxybenzaldehyde (0.61g, 3.06mmol) and 1-(4-chlorophenyl)ethanone (361µL, 2.118mmol) in ethanol (10 mL) leading to a red solution stirred at room temperature. After 19 hours, the red solution was neutralized with concentrated HCl leading to a yellow suspension, cooled at 4ºC during 2 hours. The yellow product was isolated by filtration and identified by ^{1}H NMR as (*E*)-3-(5-bromo-2-hydroxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one (0.71g, 2.09mmol, 75.2%) contaminated with 5-bromo-2-hydroxybenzaldehyde in a 1:0.6 molar ratio. The contaminated product was recrystallized (0.62g) under 3 minutes of reflux in 4mL water and 4mL ethanol (1:1). However, after isolation of a yellow solid (0.60g, 1.77mmol, 57.8%) it was observed that the benzaldehyde contamination was preserved in a similar ratio (Table 6).

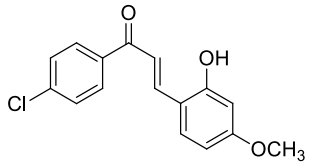
*Synthesis of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(4-fluorophenyl)prop-2-en-1-one (**2.3z**)*



Following the general procedure, aqueous 3M NaOH (7 mL) was added to a solution of 5-bromo-2-hydroxybenzaldehyde (0.42g, 2.09mmol) and 1-(4-fluorophenyl)ethanone (279µL, 2.29mmol) in ethanol (7 mL) leading immediately to a red solution. The reaction mixture was stirred for 50 hours at room temperature. After that, the reaction vessel was cooled, in an ice bath, and concentrated HCl was added to pH±3 leading to a yellow suspension taken to the ultrasound bath for 30 minutes. The reaction mixture was filtered leading to a yellow product, identified by ^{1}H NMR as (*E*)-3-(5-bromo-2-hydroxyphenyl)-1-(4-fluorophenyl)prop-2-en-1-one (0.65g, 2.02mmol, 96.7%) contaminated with 5-bromo-2-hydroxybenzaldehyde and a contaminant

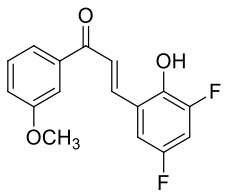
2.5 in a 1:0.57:0.62 molar ratio. It was attempted a recrystallization of the contaminated solid (0.61g) under 3 minutes of reflux in 6mL water and 5mL ethanol. The reaction vessel was cooled at room temperature. However, after filtration of the light yellow solid (0.55g, 1.70mmol, 81.3%) it was observed that both contaminations were preserved in a very similar ratio (Table 6).

*Synthesis of (E)-1-(4-chlorophenyl)-3-(2-hydroxy-4-methoxyphenyl)prop-2-en-1-one (**2.3y**)*



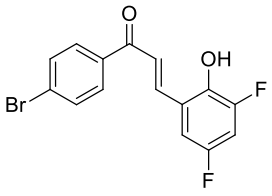
Following the general procedure, aqueous 3M NaOH (10mL) was added to a solution of 2-hydroxy-4-methoxybenzaldehyde (0.25g, 1.65mmol) and 1-(4-chlorophenyl)ethanone (195µL, 1.5mmol) in ethanol (10mL) leading immediately to a red solution, stirred at 60ºC for 27 hours. After that, concentrated HCl was added to pH±2/3 and the red solution turned into a dark orange suspension cooled at 4ºC for 1 hour. The suspension was filtered and the solid was identified by ^{1}H NMR as (*E*)-1-(4-chlorophenyl)-3-(2-hydroxy-4-methoxyphenyl)prop-2-en-1-one (0.05g, 0.17mmol, 10.4%) (Table 6).

*Synthesis of (E)-3-(3,5-difluoro-2-hydroxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (**2.3w**)*



Following the general procedure, aqueous 3M NaOH (10mL) was added to a solution of 3,5-difluoro-2-hydroxybenzaldehyde (0.46g, 2.113mmol) and 1-(3-methoxyphenyl)ethanone (443µL, 3.23mmol) in ethanol (10mL) leading to a red solution, stirred at room temperature for 5 hours. After that, concentrated HCl was added to pH±2/3 and the red solution turned into a yellow suspension cooled at 4ºC for 1 hour. Finally, the suspension was filtered, and the product was identified by ^{1}H NMR as (*E*)-3-(3,5-difluoro-2-hydroxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one (0.33g, 1.15mmol, 39.2%), contaminated with 1-(3-methoxyphenyl)ethanone in a 1:0.16 molar ratio (Table 6).

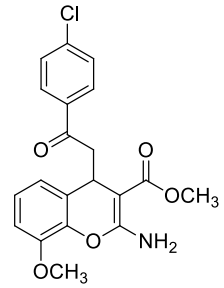
*Synthesis of (E)-1-(4-bromophenyl)-3-(3,5-difluoro-2-hydroxyphenyl)prop-2-en-1-one (**2.3x**)*



Following the general procedure, aqueous 3M NaOH (8mL) was added to a solution of 3,5-difluoro-2-hydroxybenzaldehyde (0.24g, 1.54mmol) and 1-(4-bromophenyl)ethanone (0.34g, 1.7mmol) in ethanol (8mL) leading immediately to a red solution, stirred at room temperature for 22 hours. After that, concentrated HCl was added to pH±2 and the red solution turned into a yellow suspension, taken to the ultrasound bath for 30 minutes due a solid stuck to the magnetic bar. The solid could not be dissociated from the bar so only the suspension was filtered. The solid was identified by ^{1}H NMR as 1-(4-bromophenyl)ethanone with traces of (*E*)-3-(5-chloro-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (0.2015g, 0.594mmol, 38.6%), in a 1.9:1 molar ratio (Table 6).

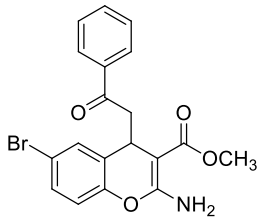
1.2. Synthesis of 4*H*-chromene derivatives

*Synthesis of methyl 2-amino-4-(2-(4-chlorophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carboxylate (**2.10a**)*



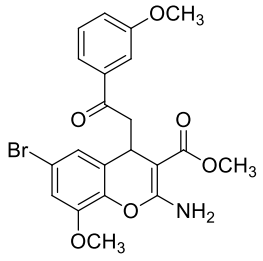
Triethylamine (150µL) was added to a solution of (E)-1-(4-chlorophenyl)-3-(2-hydroxy-3-methoxyphenyl)prop-2-en-1-one **2.3y** (0.07g; 0.25mmol) and methyl 2-cyanoacetate (44µL; 0.5mmol) in methanol (1mL). This orange solution was stirred for 7 hours at room temperature and, then, cooled at -20ºC for 17 hours leading to a beige solid. The solid was filtered, washed with ethanol and identified by IR, ^{1}H and ^{13}C NMR as methyl 2-amino-4-(2-(4-chlorophenyl)-2-oxoethyl)-8-methoxy-4*H*-chromene-3-carboxylate (0.06g; 0.18mmol; 71,8%) (Tables 10, 11 and 12).

*Synthesis of methyl 2-amino-6-bromo-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carboxylate (**2.10b**)*



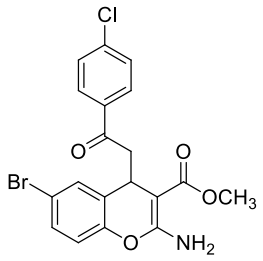
Triethylamine (125µL) was added to a solution of (E)-3-(5-bromo-2-hydroxyphenyl)-1-phenylprop-2-en-1-one **2.3q** (0.05g; 0.171mmol) and methyl 2-cyanoacetate (38µL; 0.343mmol) in methanol (1mL). This dark yellow solution was stirred for 24 hours at room temperature and then cooled at -20ºC for 5 hours leading to a white solid. The solid was filtered, washed with ethanol and identified by IR, ^{1}H and ^{13}C NMR as methyl 2-amino-6-bromo-4-(2-oxo-2-phenylethyl)-4*H*-chromene-3-carboxylate (0.04g; 0.10mmol; 58.5%) (Tables 10, 11 and 12).

*Synthesis of methyl 2-amino-6-bromo-8-methoxy-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4H-chromene-3-carboxylate (**2.10c**)*



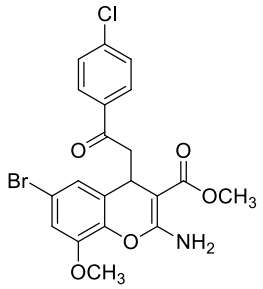
Triethylamine (138µL) was added to a solution of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one **2.3c** (0.08g; 0.24mmol) and methyl 2-cyanoacetate (42µL; 0.48mmol) in methanol (1mL). This light yellow solution was stirred for 17 hours, at room temperature, and cooled at -20ºC for 23 hours. As there was no precipitation, 2 mL of water were added leading to a gummy substance that clung to the magnetic bar. The use of the ultrasound bath to break up the solid was inconsequent. So, we proceed to the addition of 2mL of ethanol and, again, took the solution to the ultrasound bath for 1 hour. A white solid precipitated and was filtered. The solid was identified by IR, ^{1}H and ^{13}C NMR as methyl 2-amino-6-bromo-8-methoxy-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4*H*-chromene-3-carboxylate (0.07g,0.15mmol, 62.10%) (Tables 10, 11 and 12).

*Synthesis of methyl 2-amino-6-bromo-4-(2-(4-chlorophenyl)-2-oxoethyl)-4H-chromene-3-carboxylate (**2.10d**)*



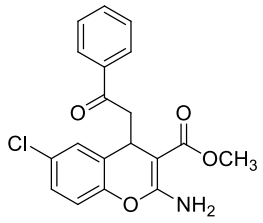
Triethylamine (66µL) was added to a solution of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one **2.3v** (0.07g; 0.21mmol) and methyl 2-cyanoacetate (20µL; 0.23mmol) in methanol (1mL). This yellow solution was stirred for 4 hours at room temperature. The solid was filtered, washed with ethanol and identified by IR, ^{1}H and ^{13}C NMR as methyl 2-amino-6-bromo-4-(2-(4-chlorophenyl)-2-oxoethyl)-4*H*-chromene-3-carboxylate (0.05g, 0.12mmol, 57.1%) (Tables 10, 11 and 12).

*Synthesis of methyl 2-amino-6-bromo-4-(2-(4-chlorophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carboxylate (**2.10e**)*



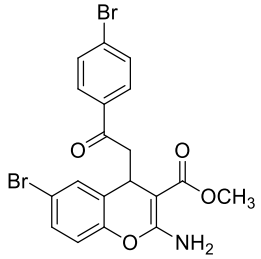
Triethylamine (95µL) was added to a solution of (E)-3-(5-bromo-2-hydroxyphenyl)-1-phenylprop-2-en-1-one **2.3q** (0.11g; 0.3mmol) and methyl 2-cyanoacetate (29µL; 0.33mmol) in methanol (1mL). The mixture was stirred for 21 hours, at room temperature and then cooled at -20ºC during 3 hour, leading to a light pink solid. The solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as methyl 2-amino-6-bromo-4-(2-(4-chlorophenyl)-2-oxoethyl)-8-methoxy-4*H*-chromene-3-carboxylate (0.09g; 0.21mmol; 69.3%) (Tables 10, 11 and 12).

*Synthesis of methyl 2-amino-6-chloro-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carboxylate (**2.10f**)*



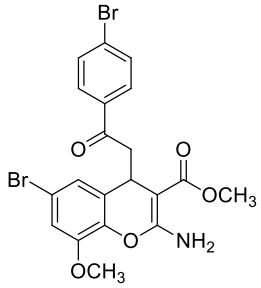
Triethylamine (138µL) was added to a solution of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one **2.3t** (0.08g; 0.24mmol) and methyl 2-cyanoacetate (42µL; 0.48mmol) in methanol (1mL). This light yellow solution was stirred for 17 hours, at room temperature, and cooled at -20ºC for 23 hours. As there was no precipitation, 2 mL of water were added leading to a gummy substance that clung to the magnetic bar. The use of the ultrasound bath to break up the solid was inconsequent. So, we proceed to the addition of 2mL of ethanol and, again, took the solution to the ultrasound bath for 1 hour. A white solid precipitated and was filtered. The solid was identified by IR, ^{1}H and ^{13}C NMR as methyl 2-amino-6-bromo-8-methoxy-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4*H*-chromene-3-carboxylate (0.05g, 0.14mmol, 56.5%) (Tables 10, 11 and 12).

*Synthesis of methyl 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-4H-chromene-3-carboxylate (**2.10g**)*



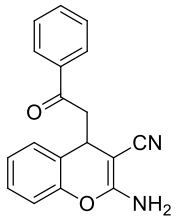
Triethylamine (95µL) was added to a solution of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one **2.3i** (0.04g; 0.11mmol) and methyl 2-cyanoacetate (20µL; 0.22mmol) in methanol (1mL). The mixture was stirred for 18 hours, at room temperature and then cooled at -20ºC during 6 hours, leading to a white solid. The solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as methyl 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-4*H*-chromene-3-carboxylate (0.04g; 0.10mmol; 94.5%) (Tables 10, 11 and 12).

*Synthesis of methyl 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carboxylate (**2.10h**)*



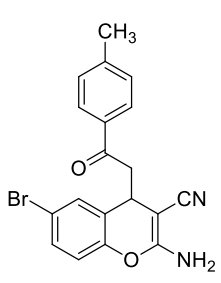
Triethylamine (66µL) was added to a solution of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one **2.3m** (0.05g; 0.14mmol) and methyl 2-cyanoacetate (24µL; 0.27mmol) in methanol (1.2mL). This yellow solution was stirred for 5 hours at room temperature. A with solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as methyl 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-8-methoxy-4*H*-chromene-3-carboxylate (0.0524g, 0.12mmol, 57.1%) (Tables 10, 11 and 12).

*Synthesis of 2-amino-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carbonitrile (**2.11a**)*



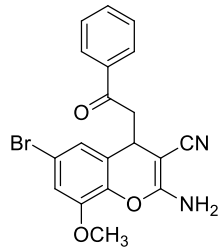
Triethylamine (99µL) was added to a solution of (E)-3-(2-hydroxyphenyl)-1-phenylprop-2-en-1-one **2.3o** (0.05g; 0.19mmol) with malononitrile (0.03g; 0.47mmol) in ethanol (1.2mL). The mixture was stirred for 4 hours, at room temperature and then cooled to -20ºC for 17 hours, leading to a white solid. The solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as 2-amino-4-(2-oxo-2-phenylethyl)-4*H*-chromene-3-carbonitrile (0.02g; 0.06mmol; 27.0%) (Tables 10, 13 and 14).

*Synthesis of 2-amino-6-bromo-4-(2-oxo-2-(p-tolyl)ethyl)-4H-chromene-3-carbonitrile (**2.11b**)*



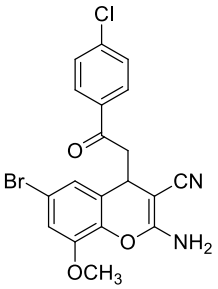
Triethylamine (80µL) was added to a solution of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one **2.3k** (0.05g; 0.19mmol) and malononitrile (0.02g; 0.37mmol) in ethanol (1.5mL). The mixture was stirred for 6 hours at room temperature and then cooled at -20ºC for 19 hours, leading to white crystals. The crystals were filtered and identified by IR, ^{1}H and ^{13}C NMR as 2-amino-6-bromo-4-(2-oxo-2-(p-tolyl)ethyl)-4*H*-chromene-3-carbonitrile (0.04g; 0.12mmol; 61.8%) (Tables 10, 13 and 14).

*Synthesis of 2-amino-6-bromo-8-methoxy-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carbonitrile (**2.11c**)*



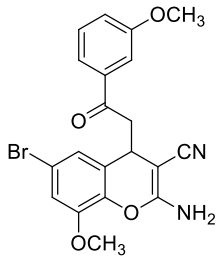
Triethylamine (67µL) was added to a solution of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-phenylprop-2-en-1-one **2.3a** (0.05g; 0.15mmol) and malononitrile (0.02g; 0.31mmol) in ethanol (1.5mL) leading to a light orange solution. The solution was stirred for 5 hours, at room temperature, and cooled to -20ºC for 1 hour, leading to a white solid precipitate in an orange solution. The solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as 2-amino-6-bromo-8-methoxy-4-(2-oxo-2-phenylethyl)-4*H*-chromene-3-carbonitrile (0.03g; 0.06mmol; 41.8%) (Tables 10, 13 and 14).

*Synthesis of 2-amino-6-bromo-4-(2-(4-chlorophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carbonitrile (**2.11d**)*



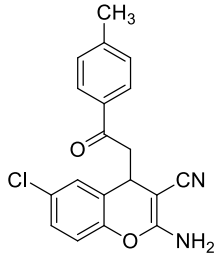
Triethylamine (58µL) was added to a solution of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(4-chlorophenyl)prop-2-en-1-one **2.3b** (0.05g; 0.14mmol) and malononitrile (0.02g; 0.27mmol) in ethanol (1.5mL) leading to a light orange solution. The solution stirred for 5 hours, at room temperature and then cooled to -20ºC for 17 hours, leading to the precipitation a white solid in an orange solution. The solid was filtered and identified by IR, ^{1}H and ^{13}C NMR as 2-amino-6-bromo-4-(2-(4-chlorophenyl)-2-oxoethyl)-8-methoxy-4*H*-chromene-3-carbonitrile (0.04g; 0.10mmol; 68.1%) (Tables 10, 13 and 14).

*Synthesis of 2-amino-6-bromo-8-methoxy-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4H-chromene-3-carbonitrile (**2.11e**)*



Triethylamine (69µL) was added to a solution of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one **2.3c** (0.06g; 0.16mmol) and malononitrile (0.02g; 0.32mmol) in ethanol (1mL) resulting in a dark yellow solution. The solution was stirred for 7 hours, at room temperature, and cooled to -20ºC for 17 hours, leading to a white solid precipitate in an orange solution. The solid was filtered, washed with ethanol and the white product was identified by IR, ^{1}H and ^{13}C NMR as 2-amino-6-bromo-8-methoxy-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4*H*-chromene-3-carbonitrile (0.04g; 0.10mmol; 63.8%) (Tables 10, 13 and 14).

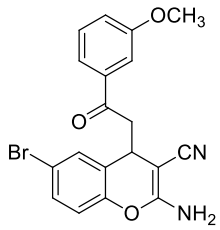
*Synthesis of 2-amino-6-chloro-4-(2-oxo-2-(p-tolyl)ethyl)-4H-chromene-3-carbonitrile (**2.11f**)*



Triethylamine (87µL) was added to a solution of (E)-3-(5-chloro-2-hydroxyphenyl)-1-(p-tolyl)prop-2-en-1-one **2.3g** (0.06g; 0.20mmol) and malononitrile (0.03g; 0.40mmol) in ethanol (1.5mL) resulting in a yellow solution. The solution was stirred for 5 hours, at room temperature, leading to a beige suspension. The product was filtered and the white solid identified by

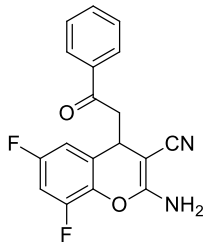
IR, ^{1}H and ^{13}C NMR as 2-amino-6-chloro-4-(2-oxo-2-(p-tolyl)ethyl)-4*H*-chromene-3-carbonitrile (0.03g; 0.08mmol; 39.6%) (Tables 10, 13 and 14).

*Synthesis of 2-amino-6-bromo-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4H-chromene-3-carbonitrile (**2.11g**)*



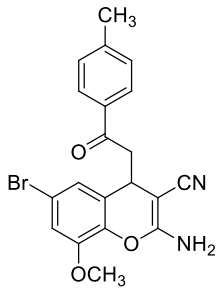
Triethylamine (94µL) was added to a solution of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(3-methoxyphenyl)prop-2-en-1-one **2.3e** (0.07g; 0.22mmol) and malononitrile (0.03g; 0.44mmol) in ethanol (2mL) resulting in a yellow solution. The solution was stirred for 4 hours, at room temperature and cooled to -20ºC for 18 hours leading to an orange solution with a white solid precipitate. The solid was filtered, briefly washed with ethanol and identified by IR, ^{1}H and ^{13}C NMR as being 2-amino-6-bromo-4-(2-(3-methoxyphenyl)-2-oxoethyl)-4*H*-chromene-3-carbonitrile (0.02g; 0.05mmol; 24.7%) (Tables 10, 13 and 14).

*Synthesis of 2-amino-6,8-difluoro-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carbonitrile (**2.11h**)*



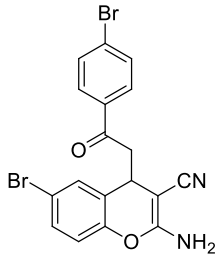
Triethylamine (99µL) was added to a solution of (E)-3-(3,5-difluoro-2-hydroxyphenyl)-1-phenylprop-2-en-1-one **2.3f** (0.06g; 0.23mmol) and malononitrile (0.03g; 0.46mmol) in ethanol (2mL) leading to a yellow solution. The solution was stirred for 17 hours, at room temperature and cooled to -20ºC for 3 hours leading to a light yellow solution with white solid precipitate. The white product was filtered and identified by IR, ^{1}H and ^{13}C NMR as 2-amino-6,8-difluoro-4-(2-oxo-2-phenylethyl)-4*H*-chromene-3-carbonitrile (0.02g; 0.05mmol; 21.4%) (Tables 10, 13 and 14).

*Synthesis of 2-amino-6-bromo-8-methoxy-4-(2-oxo-2-(p-tolyl)ethyl)-4H-chromene-3-carbonitrile (**2.11i**)*



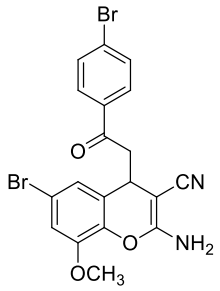
Triethylamine (71µL) was added to a solution of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(p-tolyl)prop-2-en-1-one **2.3h** (0.06g; 0.17mmol) and malononitrile (0.02g; 0.33mmol) in ethanol (2mL) leading to an orange solution. The solution was stirred at room temperature for 23 hours and cooled to -20ºC for 7 days leading to a light yellow solution with white solid precipitate. The white product was filtered and identified by IR, ^{1}H and ^{13}C NMR as 2-amino-6-bromo-8-methoxy-4-(2-oxo-2-(p-tolyl)ethyl)-4*H*-chromene-3-carbonitrile (0.01g, 0.02mmol, 12.1%) (Tables 10, 13 and 14).

*Synthesis of 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-4H-chromene-3-carbonitrile (**2.11j**)*



Triethylamine (48µL) was added to a solution of (E)-3-(5-bromo-2-hydroxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one **2.3i** (0.04g; 0.11mmol) and malononitrile (0.02g; 0.23mmol) in ethanol (2mL) leading to a light orange solution. The solution was stirred for 2 hours, at room temperature and then cooled at -20ºC for 38 hours leading to a white solid precipitate in an orange solution. The white product was filtered and identified by IR, ^{1}H and ^{13}C NMR as 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-4*H*-chromene-3-carbonitrile (0.03g; 0.06mmol; 50.3%) (Tables 10, 13 and 14).

*Synthesis of 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-8-methoxy-4H-chromene-3-carbonitrile (**2.11k**)*



Triethylamine (65µL) was added to a solution of (E)-3-(5-bromo-2-hydroxy-3-methoxyphenyl)-1-(4-bromophenyl)prop-2-en-1-one **2.3m** (0.06g; 0.15mmol) and malononitrile (0.03g; 0.30mmol) in ethanol (1.2mL) leading to a light orange solution. The solution was stirred for 5 hours at room temperature and cooled at -20ºC for 14 hours. After 4 more hours at room temperature, a white solid precipitated in a dark yellow solution. The white product was filtered and identified by IR, ^{1}H and ^{13}C NMR as 2-amino-6-bromo-4-(2-(4-bromophenyl)-2-oxoethyl)-8-methoxy-4*H*-chromene-3-carbonitrile (0.04g; 0.09mmol; 56.6%) (Tables 10, 13 and 14).

2. Biological assays

2.1. Cell lines and culture conditions

Two human breast cancer cell lines Hs578T (basal subtype) and MCF-7 (luminal 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 Fetal 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 Fetal 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°C 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 37°C. To inactivate trypsin DMEM 10% FBS or DMEM/F12 5% FBS were added and cells were collected and centrifuged 5 minutes at 900rpm. Cells were suspended in new medium and 10µl of cell suspension were collected, in which 20µl of trypan blue (Trypan Blue Solution, 0.4%, Gibco) was added. Cells were counted in a Neubauer chamber for posterior density calculation.

2.2. Cell viability assays

2.2.1. Sulforhodamine B assay

Sulforhodamine B assay is based on the quantification of protein from viable cells, allowing cell biomass determination. This method is mainly used to toxicity screening of drugs and chemosensitivity. In this approach, protein basic amino acid residues are electrostatically bound to sulforhodamine B, a protein dye.

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/100µL density per well for all the cell lines (HS578t, MCF-7 and MCF-10). 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% of DMSO (compound vehicle). The compound cytotoxic effect was evaluated by the Sulforhodamine B assay (SRB, TOX-6, Sigma-Aldrich). After cell incubation with the different compound concentrations, culture medium was removed and cells were fixed with cold 10% Trichloroacetic Acid (TCA) for 1h at 4ºC. Plates were rinsed 3 times with distilled water to eliminate TCA excess and then were dried overnight at room temperature. Cells were stained with 0.4% Sulforhodamine B solution during 30 minutes and, after the dying period, plates were again rinsed 3 times with 1% acetic acid until the unincorporated dye was totally removed. Then, plates were dried overnight and after this time, the incorporated Sulforhodamine B was solubilized in a 10mM Tris Base Solution, stirring in the plate shaker for 20 to 30 minutes at room temperature until a homogenous color is obtained. Spectrophotometric measurement of absorbance was read at 490nm, using 690nm 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.

2.2.2. Trypan blue assay

Trypan blue assay is a dye exclusion test that allows the determination of number of viable cells. As live cells possess undamaged cell membranes, they will exclude certain dyes, such as trypan blue, leading to a clear cytoplasm. On the other hand, dead cells will be susceptible to the colorant, presenting a blue cytoplasm.

MCF-10 and MCF-7 cells were plated in 12-well plates at a density of 30000 and 50000 cells, respectively, per 1mL, and grown overnight at 37°C in a 5% CO_2 humidified atmosphere. Adherent cells were treated with compounds **2.3e**, **2.3g**, **2.3i** and **2.11e** at IC_{50}, ½ IC_{50} and ¼ IC_{50} concentrations or 0.5% DMSO (controls). At 24, 48 and 72 hours, cells were reaped by gently rinsing flasks with 200µL of phosphate-buffer saline (PBS 1x) and then detaching with 100 µL trypsin at 37°C. To inactivate trypsin, 200 µL DMEM/F12 5% FBS or DMEM 10% FBS were added and 20µl of cell suspension were collected, in which 20µl of trypan blue (1:1) was added. Cells were counted in a Neubauer chamber for posterior density calculation. For each well, a duplicate count was made. The analysis and IC_{50} values were estimated from three independent experiments (at least), each one in duplicate, using the GraphPad Prism 5 software, applying a sigmoidal dose-response (variable slope) non-linear regression, after logarithmic transformation.

2.3. Proliferation assay

DNA synthesis of cells was measured by BrdU assay (Roche Applied Sciences). BrdU cell proliferation assay is a colorimetric immunoassay based on the quantification of 5-bromo 2´-deoxyuridine (BrdU) incorporation in cells, a pyrimidine analog, during DNA synthesis.

MCF-10 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 **2.3e**, **2.3g**, **2.3i** and **2.11e** at IC_{50} and ½ IC_{50} concentrations or 0.5% DMSO (controls) for 24 and 48 hours. After incubation, cells were labeled by addition of 5µl/well BrdU labeling solution (final concentration: 20 µM BrdU) reincubating for 6 hours to allow BrdU to incorporate into the proliferating cell DNA, replacing thymidine. After labeling, culture medium was removed, cells were fixed and DNA was denatured through incubation with 100µl 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 50µl 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 (100µl/well) and the plate was incubated at room temperature until color development was satisfactory for photometric detection (5-10 minutes). Substrate reaction was stopped by adding 25µl of 1M H_2SO_4 to each well and softly mixed. The reaction product was quantified by measuring absorbance at 450nm (reference wavelength: 690nm) 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.

2.4. Migration assay

Cell migration was assessed by the wound-healing assay that allows to mimic the cell migration during wound-healing *in vivo*. This method is based in making a scratch, simulating a wound in a cell monolayer, capturing the images at the beginning and regular intervals during cell migration to close the wound and comparing the images to quantify the cell migration rate.

MCF-7 were plated in 6-well plates at a density of $9.0x10^5$/2ml and grown overnight at 37°C in a 5% CO_2 humidified atmosphere. Two scratches were made with a 200µl pipette tip in confluent

cells. Cells were gently washed with 500µL 1x PBS and were treated with compounds **2.3e**, **2.3g**, **2.3i** and **2.11e** at the respective IC_{50}, ½ IC_{50} or 0.5% DMSO (control), for 72 hours. At 0, 12, 24, 48 and 72 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.

2.5. Protein extraction and Western Blot

Protein expression levels were determined by Western blot analysis, a technique that involves the transference of proteins that have been separated by gel electrophoresis onto a membrane, followed by immunological detection. In this assay cleaved caspase-3 and PARP (Poly (ADP-ribose) polymerase) were analyzed to confirm the type of cell death, in which caspase-3 is one of the key proteins involved in apoptosis, responsible for proteolytic cleavage of nuclear protein PARP.

Cells (MCF-10 and MCF-7) were grown in T25 flasks and, at 70-80% confluence, cells were treated with compounds **2.3e**, **2.3g**, **2.3i** and **2.11e** at the respective IC_{50} or 0.5% DMSO (controls) for 24 hours. After treatment, cells were collected by scraping and centrifuged at 2000rpm 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°C 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 10-12% polyacrylamide gel (90V for 30 minutes and more 120V for 90 minutes) and transferred to a nitrocellulose membrane (100V for 60 minutes). Membranes were blocked with 5% milk in 1X TBS for 60 minutes before overnight incubation with the specific primary antibodies at 4°C (rabbit anti-CAIX antibody, 1:2000 5% milk, Cell Signaling (#5648); rabbit anti-PARP antibody, 1:1000 5% BSA, Cell Signaling (#5625); rabbit anti-caspase-9 antibody, 1:1000 5% BSA, Cell Signaling (#9502); goat anti-Tubulin antibody, 1:50000 5% BSA, Santa Cruz Biotechnology (sc-1616)). After washing 5 minutes (2 times) and more 15 minutes (1 time) with 0.1% Tween 20, blots were incubated for 1 hour with

the respective secondary antibodies at room temperature (anti-rabbit IgG-HRP (sc-2034) and anti-goat IgG-HRP (sc-2020) secondary antibodies, 1:5000 5% milk, Santa Cruz Biotechnology). 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). The results of two independent protein extractions were quantified using the ImageJ software: the density of each band was measured and the correspondent value was divided by the Actin (loading control) value. Calculation of cleaved protein was performed as follows: Cleaved band/ (Total band + Cleaved band).

2.6. 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 ± SD.

CHAPTER VI – REFERENCES

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