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In vitro and *in vivo* evaluation of novel chromeno[2,3-*d*]pyrimidinones as therapeutic agents for triple negative breast cancer†

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Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer, and the limited therapeutic options show poor efficacy in patients, associated to severe side effects and development of resistance. Considering that chromene-based scaffolds proved to be attractive candidates for cancer therapy, herein we prepared new chromeno[2,3-*d*]pyrimidinone derivatives by a simple two step procedure, starting from the reaction of cyanoacetamide and a salicylaldehyde. A cell viability screening in several breast cancer cell lines allowed to identify two promising compounds with IC_{50} values in the low micromolar range for TNBC cells. These chromenes inhibited cell proliferation, induced cell cycle arrest and triggered cell death through apoptosis. *In vivo* studies revealed a safe profile in invertebrate and vertebrate animal models and confirmed their capacity to inhibit tumor growth in the CAM model, inducing significant tumor regression after 4 days of treatment. The two compounds identified in this study are promising drug candidates for TNBC treatment and valuable hits for future optimization, using the versatile synthetic platform that was developed.

1. Introduction

Breast cancer (BC) is the most diagnosed type of cancer worldwide and the main cause of cancer-related death in women.[1] In 2020, more than 2 million new BC cases were diagnosed and nearly 700 000 people died from this disease.[1] BC is considered a heterogeneous disease, comprising several molecular subtypes (luminal A and B, HER2-enriched and triple-negative) that present distinct prognostic, risk factors and, consequently, different responses to the available treatment.[2–4] Triple-negative breast cancer (TNBC) subtype represents 15–20% of BC cases, presenting a phenotype characterized by negative expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu), hindering the development of effective molecular-based targeted treatments.[5] Fast tumor progression, low response to therapy and high patient relapse rates (50% of early-stage patients) are the main concerns associated to this aggressive subtype. The 5 year mortality rate after surgery is approximately 40%, which rises to 90% in late-stage diagnosis cases. Chemotherapy, surgery, and radiation are the main therapeutic approaches used for TNBC patients, although with very limited results and accompanied by severe side effects.[6] TNBC represents a high burden for society and the increasing number of cases (+33.8%) and related deaths (+51.5%) projected for 2040, urges the need for new effective therapeutic options.[1] Consequently, the interest of the scientific community has been focusing on the discovery of new and safer anticancer drugs with specific mechanisms of action, to overcome problems associated with current therapies.[7]

Considering the effort to find effective and transversal therapies to TNBC, medicinal chemists often search for inspiration in naturally occurring compounds presenting important bio-activities, as is the case of the chromene scaffold.[8,9] Regarding the biological activity of natural or

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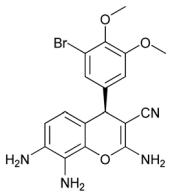


Fig. 1 Chemical structure of EPC2407 (Crolibulin™).

synthetic chromenes, several studies have been carried out to exploit their potential as anticancer drugs. Quercetin is an example of a natural chromene widely distributed in plants and fruits, which exhibits important biological properties, including anticancer activity.[10] The synthetic chromene EPC2407 (Crolibulin™, Fig. 1) is an example with promising anticancer activity.[11] This chromene progressed to Phase I/II of clinical trials for anaplastic thyroid cancer treatment and is classified as a microtubule inhibitor.

Other examples of 4*H*-chromene derivatives were previously prepared in our research group[12] and tested for their anticancer activity on breast cancer cell lines (Hs578t and MCF-7), leading to the identification of several drug candidates.

The specific preparation of chromeno[2,3-*d*]pyrimidinone derivatives was first reported in 1970's[13] and 80's.[14] In 1999, Hadfield *et al.* synthesized a series of analogues and identified several compounds with promising activity against various cancer types (*e.g.* chronic myelogenous leukemia and chronic myelogenous leukemia, ovarian cancer).[15]

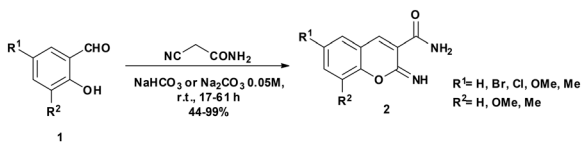
Inspired by the potential of this family of compounds, we pursued on the design and synthesis of novel chromene-based molecules as anticancer agents, especially for aggressive and poor prognosis cancers, thus addressing currently unmet medical needs.[12,16] In this study, a selection of novel chromeno[2,3-*d*]pyrimidinones were synthesized and their anticancer potential was assessed using *in vitro* and *in vivo* breast cancer models.

2. Results and discussion

2.1 Chemistry

The synthesis of 2-imino-2*H*-chromene-3-carboxamides **2** was performed by the reaction of a salicylaldehyde **1** with cyanoacetamide in 0.05 M aqueous solution of $NaHCO_3$, at room temperature, following a methodology described in the literature (Scheme 1).[17]

Chromenes **3** and **4** were prepared by the combination of 2-imino-2*H*-chromenes **2** with aromatic or aliphatic aldehydes, in ethanol and in the presence of piperidine, at 100 °C for several hours. Using this procedure, 6 derivatives of **3** and 30 derivatives of **4** were prepared and isolated in 17–81% yield (Scheme 2).



Scheme 1 Synthesis of 2-imino-chromenes **2**.

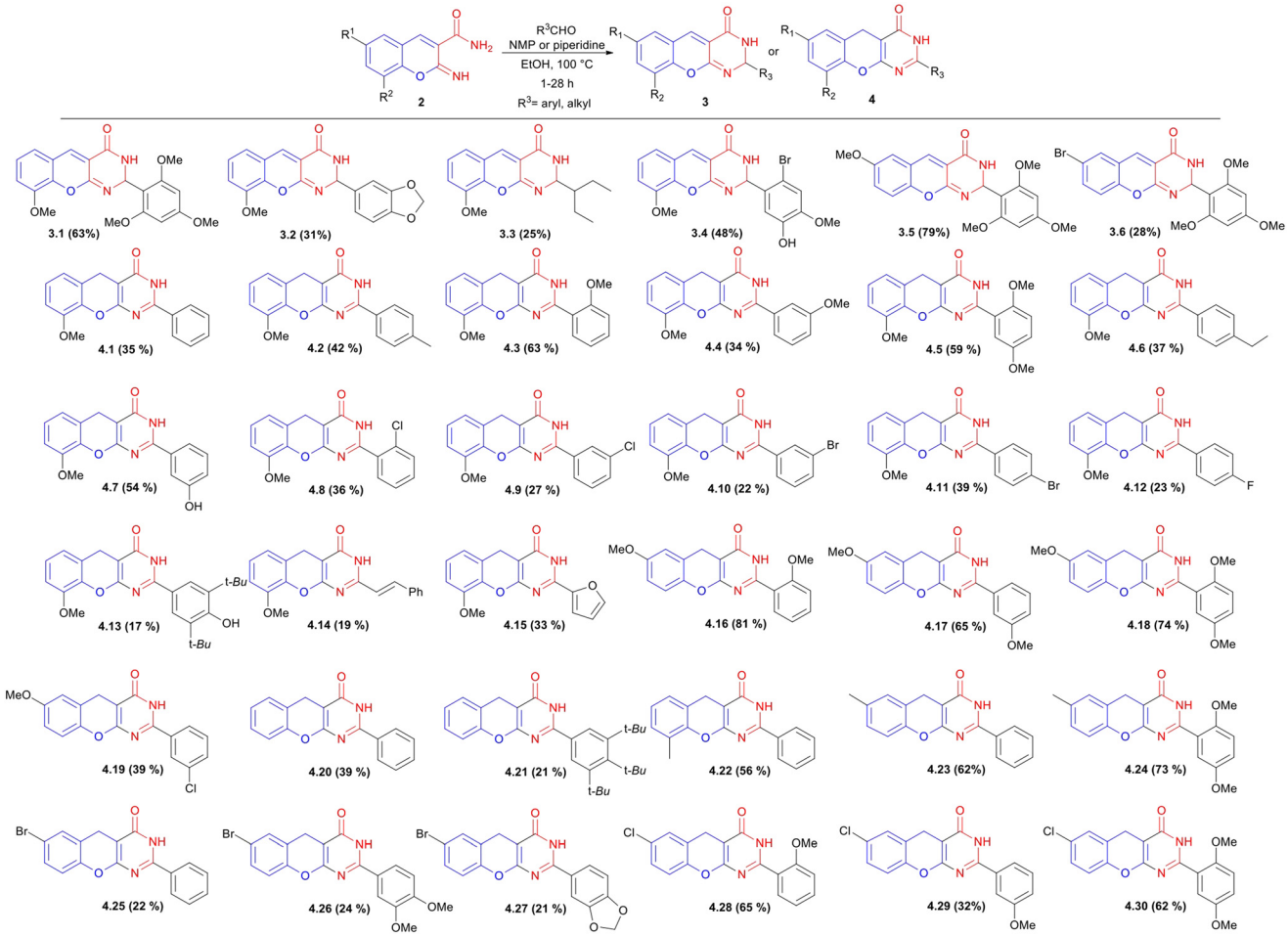
Compound **8** was also prepared and the synthetic pathway is summarized in Scheme 3. Compound **5**, used as starting material, was prepared from the reaction of 5-bromosalicylaldehyde and acetophenone, in aqueous NaOH 3 M/EtOH, at room temperature, following a synthetic procedure developed in-house and previously reported.[12] The reaction of chromene **5** with triethylorthoformate (TEOF) and acetic anhydride was carried out under reflux for 30 minutes. Addition of *n*-hexane and cooling the mixture in an ice bath led to the isolation of compound **6** (91% yield). Imidate **6** was combined with aniline in a mixture of EtOH/H_2O, in the presence of a catalytic amount of acetic acid, at 80 °C, leading to amidine **7** (85% yield) after 5 h.

Synthesis of the tetracyclic compound **8** was performed from a suspension of amidine **7** in EtOH. Stirring at 80 °C for 4 h followed by addition of 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU), led to the isolation of product **8** (42% isolated yield) after 2.5 h, and no further purification was required.

2.2 Biological evaluation of the chromene derivatives

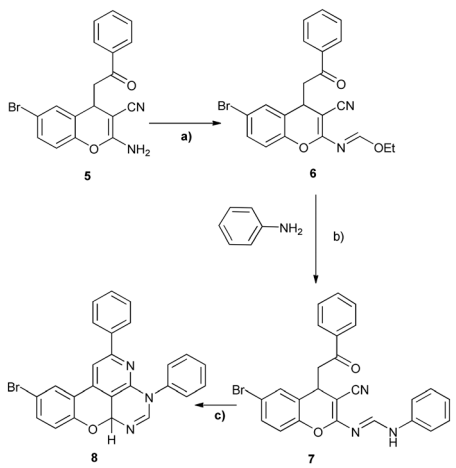
2.2.1 Anticancer activity. The anticancer potential of compounds **3**, **4**, **7** and **8** was assessed in a first cell viability screening in the breast cancer cell lines Hs578t (TNBC), MDA-MB-231 (TNBC) and MCF-7 (luminal A), using the sulforhodamine B (SRB) assay (Table S1†). Cells were treated with 10 and 30 μM concentrations of each compound, for 72 h. These concentrations were selected according to previous results.[12,16] This screening demonstrated that, in general, compounds showed higher capacity to reduce cell viability in the TNBC cell line Hs578t over TNBC cells MDA-MB-231. For some compounds, results in the luminal A subtype MCF-7 cells were also satisfactory, however Hs578t was selected for further characterization of its anticancer activity. TNBC represents un unmet medical need, justifying efforts for pursuing new drug candidates that could reach the clinics.

The cell viability assays demonstrated that chromenes **3** did not shown significant anticancer activity. However, chromene **4.13**, bearing a methoxy group on C9 of the chromene unit and two *tert*-butyl substituents on the aromatic ring of the pyrimidinone unit, presented a promising anticancer profile. Compounds **4.25** and **4.29**, bearing halogenated substituents (–Cl and –Br) on C7 of the chromene moiety and methoxy groups in the aromatic moiety linked to the pyrimidinone unit, were also able to significantly reduce breast cancer cells viability at the tested concentrations. Compound **8** demonstrated a potent capacity to decrease cell viability, which may be related to its planar and rigid tetracyclic structure, with two phenyl groups in the pyridine and pyrimidine rings, and a C7 halogenated substituent in the chromene moiety.



Scheme 2 Synthesis of chromeno[2,3-*d*]pyrimidinone derivatives **3** and **4**.

Since chromenes **4.13**, **4.25**, **4.29** and **8** demonstrated the most promising ability to decrease Hs578t cell viability (reduction of at least 50% at 10 μM), their anticancer potential was further analyzed by half-maximal inhibitory concentration (IC_{50}) determination in this cell line and also in non-neoplastic breast cell line MCF-10A (Table 1). Doxorubicin, a well-known cytotoxic drug used in the clinics for TNBC treatment,[18] and quercetin,[19] a naturally occurring chromene with anticancer activity, were used in this assay as reference compounds.



Scheme 3 Synthesis of tetracyclic chromene **8**. Reagents and conditions: (a) TEOF (8 equiv.) and acetic anhydride (3 equiv.), reflux, 40 min., 91%; (b) EtOH/H_2O 1 : 1, acetic acid cat., 80 °C, 5 h, 85%; (c) EtOH, 80 °C, 4 h followed by DBU (1 equiv.), 80 °C, 2.5 h, 42%.

Compounds **4.13**, **4.25** and **8** presented the lowest IC_{50} values against Hs578t cells. The presence of a bromine substituent in C7 of the chromene moiety seemed to enhance the anticancer properties of chromenes **4.25** and **8**, with IC_{50} values of 4.50 ± 0.29 and 8.63 ± 0.04 μM, respectively. Chromene derivative **4.13** (IC_{50} = 9.08 ± 0.10 μM), with a methoxyl group in C9 of the chromene unit, also presented an interesting anticancer potential. Furthermore, the effect of all compounds on cell viability in a non-neoplastic breast cell line, MCF-10A, was evaluated to assess the *in vitro* cytotoxicity of these compounds for normal cells and selectivity towards cancer cells. The selectivity index for compounds **4.13**, **4.25** and **8**, was found to be higher than the SI value for doxorubicin, a favourable indication for a save profile. This is an important parameter to consider in a potential drug candidate, which is indicative of a good safety profile.[20–23]

Table 1 IC_{50} values and selectivity index (SI) of chromenes **4.13**, **4.25**, **4.29** and **8** and reference compounds, for Hs578t and MCF-10A cell lines, after 72 h of treatment

Compound[a]	Breast cell lines		
	Hs578t IC_{50} (μM) ± SD[b]	MCF-10A IC_{50} (μM) ± SD[b]	SI[c]
4.13	9.08 ± 0.10	>60	>5.61
4.25	4.50 ± 0.29	>60	>12.33
4.29	13.10 ± 0.05	13.98 ± 0.11	0.06
8	8.63 ± 0.04	36.77 ± 0.09	3.26
Doxorubicin[d]	0.52 ± 0.01	1.54 ± 0.08	1.96
Quercetin	50.54 ± 0.12	9.32 ± 0.04	0.82

[a] Each compound was tested at least in triplicate and data are presented as mean values. [b] Standard deviation. [c] Selectivity index for Hs578t *vs.* MCF-10A. [d] Previously determined by the research group in ref. 12.

Considering the biological profile of compounds **4.25** and **8**, these chromenes were selected for subsequent studies to further characterize their anticancer activity and mechanism of action.

2.2.2 Inhibition of cell proliferation. Chromene-based compounds previously demonstrated an antiproliferative effect against several cancer cell lines, including Hs578t cells.[12,24] In 2020, a newly synthetized chromene-based compound demonstrated a potent capacity to inhibit cell proliferation (up to 80% compared to control) in MCF7 cell line (luminal breast cancer subtype).[16] In this work, synthetized chromenes **4.25** and **8** were evaluated for their capacity to inhibit cell proliferation *via* bromodeoxyuridine (BrdU) incorporation during DNA replication. Hs578t cells were treated with $1/2IC_{50}$ and IC_{50} concentrations of each compound, for 24 and 48 h (Fig. 2).

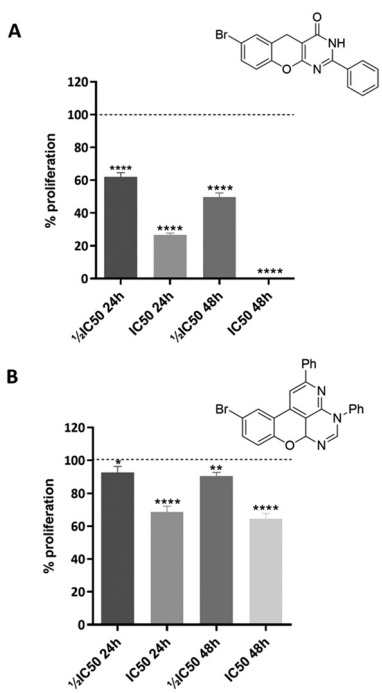


Fig. 2 Effect of chromenes **4.25** (A) and **8** (B) on Hs578t cell proliferation, after 24 and 48 h treatment. Results are presented as mean ± SEM of at least three independent experiments. ****$p < 0.001$, **$p < 0.01$, *$p < 0.05$ when compared to control (0.5% DMSO) by Student's *t*-test.

The selected chromenes showed evident inhibitory effect on Hs578t cell proliferation. Compound **4.25** exhibited the highest inhibitory activity at both 24 and 48 h of treatment, displaying a dose- and time-dependent inhibitory profile (Fig. 2A). After 24 h, cells treated with $1/2IC_{50}$ and IC_{50} of compound **4.25** suffered a reduction in proliferation of about 40% and 70%, respectively. When treated for 48 h proliferation decreased up to 50% and 99%, with $1/2IC_{50}$ and IC_{50} concentrations, respectively. Comparably, chromene **8** also inhibited cell proliferation in a dose- and time-dependent manner, being the proliferation rate reduced in approximately 8% and 30% (at 24 h), and in 10% and 35% (at 48 h), for cells treated with $1/2IC_{50}$ and IC_{50}, respectively (Fig. 2B). Overall, both variables, time and dose, impact the compound's effect on cell proliferation.

2.2.3 Induction of cell death *via* apoptosis. Natural and synthetic chromene derivatives exert their anticancer activity through diverse mechanisms of action[25–28] and have been shown to induce cell cycle arrest and cell death through apoptosis.[29,30] To evaluate the capacity of chromenes to trigger apoptosis on Hs578t cells, the expression levels of proteins associated with cell death were assessed by Western blot. In this study, compounds **4.13**, **4.29**, **4.25** and **8** were included for comparison, namely, to infer about structure-specific features (chromenes **4** *vs.* **8**). Hs578t cells were treated for 24 and 48 h, using the respective IC_{50} concentrations. The results presented in Fig. 3 show that chromene **4.25** effectively induced the expression of cleaved (poly (ADP-ribose) polymerase) (PARP) (c-PARP) at both timepoints, while the effect of chromene **4.13** and **8** was more noticeable after 48 h of treatment.

This protein plays a key role in DNA repair single strand breaks and represents an important death marker in apoptosis.[31,32] Initiators 8 and 9 are two proteins belonging to the caspase cascade and are responsible for triggering the extrinsic and intrinsic apoptotic pathways, respectively.[3.3] As displayed in Fig. 3A, the 24 h treatment of the cells with the compounds induced the expression of caspase 8. Nevertheless, the expression of this protein decreased for the 48 h timepoint for all compounds, more evident for cells

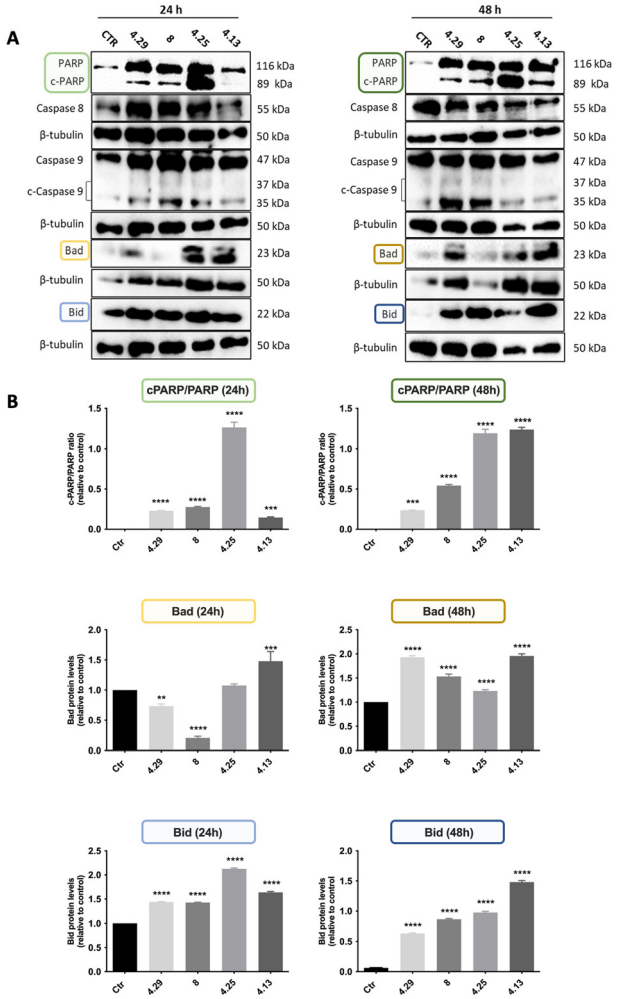


Fig. 3 A) Representative immunoblots of Hs578t cells treated for 24 h and 48 h with chromenes **4.13**, **4.29**, **4.25** and **8**. Similar blots were obtained from at least 3 independent experiments. B) Quantification of the c-PARP/PARP ratio and the protein levels of Bad and BID, after 24 and 48 h of incubation of Hs578t cells with compounds **4.13**, **4.29**, **4.25** and **8**. Values are mean ± SEM of 3 independent experiments. ****$p < 0.001$, ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$ when compared with control by one-way ANOVA.

treated with chromenes **4.13** and **8** (Fig. S1†). Regarding caspase 9, the tested chromenes triggered a significant increase in the protein expression levels, being chromenes **4.29** and **8** the most effective (Fig. S1†). The B-cell lymphoma 2 (Bcl-2) family proteins Bid and Bad are involved in the regulation of the intrinsic apoptotic pathway through the balance of the pro- (Bid and Bad) and anti-apoptotic proteins within the mitochondria. Once these proteins are activated, their interaction promotes the formation of the mitochondrial outer membrane permeabilization complex, triggering a cascade of events that culminates in cell death.[3,4] The levels of pro-apoptotic proteins Bid and Bad were increased for all treated cells after 48 h of incubation. Overall, all chromenes presented a good pro-apoptotic profile since they promoted the increase of the expression of several pro-apoptotic proteins and no significant difference was detected between chromenes **4** *vs.* **8** regarding cell death induction capacity.

To further validate the hypothesis that the studied chromenes were capable to induce apoptosis, flow cytometry was performed using annexin V and propidium iodide (V/PI) staining, using compounds **4.25** and **8**, based on their promising apoptosis inducing capacity, studied by Western blot (Fig. 4). This staining is widely used to determine if cells are viable, in apoptotic or necrotic stages.[35,36]

Hs578t cells treated with chromenes **4.25** and **8** (at IC_{50} concentration) revealed a significant decrease in viable cells after 24 and 48 h (Fig. 4). Treatment with chromene **8** led to an increase in the percentage of early and late apoptotic cells, in comparison to control cells for both tested timepoints. This effect was remarkable at the 48 h timepoint, with a percentage of early apoptotic cells of approximately 50% (Fig. 4D). The viability of this cell line was also affected by the action of chromene **4.25**, leading to the accumulation of early apoptotic cells at both timepoints and increase in late apoptotic cells at 24 h (Fig. 4C).

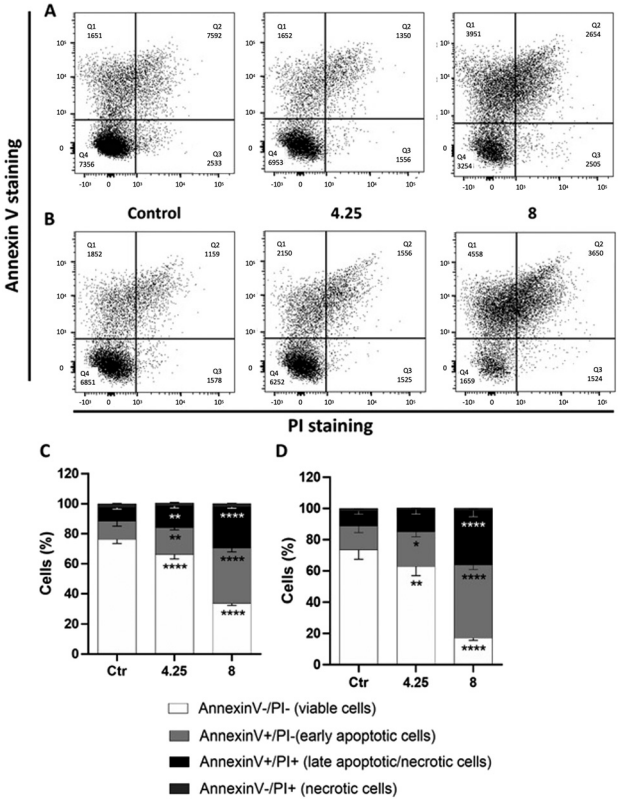


Fig. 4 Flow cytometry analysis of the viability of Hs578t cells assessed by annexin V/PI assay. Representative dot plots of Hs578t cells untreated (control) or treated for (A) 24 h and (B) 48 h with IC_{50} concentration of chromenes **4.25** and **8**. Graphical representation after quantification of the percentage of cells in each quadrant of the dot plot for (C) 24 h and (D) 48 h. The results were obtained using the non-treated cells as control and mean ± SEM. Annexin V/PI data was analysed by two-way ANOVA and Bonferroni *post hoc* test, ****$p < 0.001$, **$p < 0.005$, *$p < 0.05$.

2.2.4 Arrest of cell cycle. The duplication of the genetic material and cell division events are ensured by the cell cycle, a highly regulated process that involves four phases, G1, S, G2 and M.[37] In cancer cells, the cell cycle activity is characterized as aberrant, with unscheduled cell division leading to uncontrolled cell growth. This over activity results from upstream mutations in signalling pathways or genetic lesions within genes encoding cell cycle proteins.[38] To evaluate the effect of chromenes **4.25** and **8** on cell cycle, Hs578t cells were stained with PI, and analysed by flow cytometry after 24 h and 48 h of treatment (with the respective IC_{50} concentration), for determination of the DNA content. As presented in Fig. 5, both chromenes were able to promote cell cycle arrest in Hs578t cells.

Chromene **4.25** induced a slight reduction in the G_0/G_1 cell population, while exhibiting no significant effect in S and G_2/M cell fractions. A significant reduction of G_0/G_1 population was registered at 48 h, accompanied by a high accumulation of cells in the S phase. Chromene **8** initially caused an increase of the G_2/M cell population compared to control cells, followed by a decrease of cells in phases S and G_0/G_1. Cell accumulation at S phase was noticed at 48 h.

2.2.5 *In vivo* toxicity evaluation using *Caenorhabditis elegans*. The *Caenorhabditis elegans* (*C. elegans*) gathers several features that make it an appealing model in early drug discovery projects.[39,40] The most striking advantages in its use are the ability to be handled using *in vitro* techniques and the high degree of conservation of several biological processes between *C. elegans* and mammalian species.[39–41] Several studies using drug candidates supported a positive correlation between the toxicity in *C. elegans* and median lethal dose (LD_{50}) in rodents, indicating it as an intermediate between *in vitro* and mammalian studies.[4.2]

The *in vivo* toxicity of chromenes **4.25** and **8** was evaluated though a food clearance-based assay, using *C. elegans* wild-type animals. In this assay, the rate of bacteria consumption (OD_{50}) over-time represents a good prediction factor of this nematodes development, health, and fertility. The compounds were tested at several concentrations, diluted in DMSO, up to 50 μM for both chromenes **4.25** and **8**, comparing to the treatment vehicle of 1% DMSO and to the toxic dose of 5% DMSO (Fig. 6). The study showed no statistical differences in the rate of food consumption for the nematodes treated with the new synthetized chromenes and vehicle. In fact, the chromenes seemed to be well tolerated, even at significantly higher concentrations than those used for the above-described *in vitro* assays, representing a good indicator for non-toxicity in mammalian models.

2.2.6 *In vivo* efficacy evaluation using the chick chorioallantoic membrane assay. The chick chorioallantoic membrane (CAM) model is a useful technique for early pre-clinical *in vivo* efficacy studies for oncology drug development projects, due to the rapid development cycle of the chick embryo and easy accessibility to the CAM.[4.3] The nourishing nature of CAM is one of the advantages of this model since it allows the stimulation of grafted cells. The immunodeficiency of the host during the first 18 days of development also allows the implantation of tumor cells,

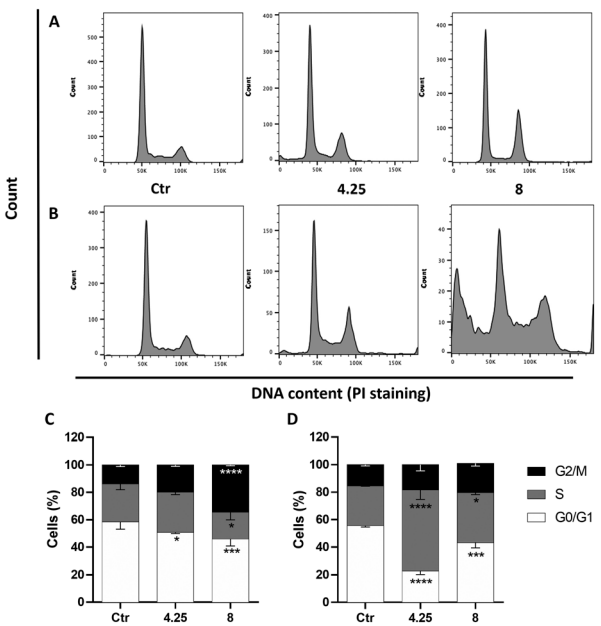


Fig. 5 Flow cytometry analysis of the DNA content of Hs578t cells. Cell cycle profile of Hs578t cells untreated (control) or treated for (A) 24 h and (B) 48 h with the IC_{50} values of chromenes 4.25 and 8. Graphical representation after quantification of the cells in different phases of cell cycle (C) after 24 h and (D) 48 h of treatment. Data was analysed by two-way ANOVA and Bonferroni *post hoc* test. ****$p < 0.001$, ***$p < 0.005$, *$p < 0.05$ compared to control.

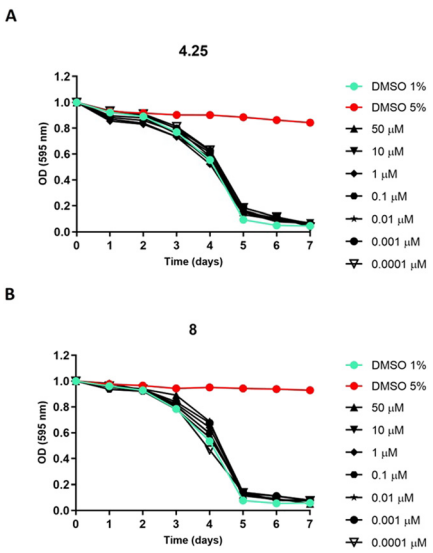


Fig. 6 Analysis of the *in vivo* toxicity of chromenes **4.25** and **8**. Toxicity was assessed in wild-type *C. elegans* through daily evaluation of food consumption (optical density 595 nm) of animals treated with different concentrations of each compound. Animals treated with 1% DMSO and 5% DMSO were used as non-toxic and toxic controls, respectively. Curves in the graphs represent daily optical density (OD) values, normalized for the OD at day 0, being concentrations of each compound measured in quintuple. (A) **4.25**; B) **8**. Data was analyzed using non-linear regression for sigmoidal curves.

without triggering an immune response.[43–45] At last, this model holds an additional ethical advantage in comparison to several mammalian *in vivo* models, since the CAM does not possess nervous terminations and the assays terminate before the development of pain perception by the chick's brain, being an attractive option for *in vivo* experiments in early pre-clinical studies.[43,44] Chromenes **4.25** and **8** were selected for further evaluation of their capacity to inhibit tumor growth using this model. Hs578t cells were seeded on the CAM and the resulting tumors were treated with 2× IC_{50} concentrations of each compound or 0.5% DMSO (control group), to evaluate tumor regression. The results presented in Fig. 7 demonstrate a significant regression of tumor growth upon treatment with chromenes **4.25** and **8**, comparing to control. Chromene **8** proved to be the most effective compound, since treatment caused a regression in the tumors' perimeter of 12.4%, while treatment with chromene **4.25** caused a reduction of 6.3%. Nevertheless, both compounds displayed a promising *in vivo* efficacy profile, when using a single-dose modality treatment.

2.2.7 *In vivo* toxicity evaluation of chromene 8 in mice. Considering that in the CAM assay the chromene 8 showed more efficacy and taking into account the fact that this compound displayed better solubility than chromene **4.25**, a toxicity assay was performed in mice. Toxicity studies in rodents are key to determine the balance between beneficial and toxic effects of novel compounds because of their complexity, which cannot be replicated in cell culture or evolutionarily more distant organisms. For these studies, two dosages of this compound were selected: 10 and 50 mg kg^{-1}. Wild-type mice were daily injected intraperitoneally (i.p.) with either vehicle or one of the dosages of the compound, for 7 days. We chose this route of administration because it delivers the drug to the bloodstream almost immediately, ensuring that the mouse. The body weight and welfare of mice were evaluated daily, starting before treatment initiation, while colonic temperature was measured before treatment and at the end of the 7 days. To assess mouse welfare, we adapted a protocol for phenotypic analysis derived from the SHIRPA protocol.[4,6] Finally, food and water intake were also monitored (Fig. 8A). No differences were found on body weight variation between animals treated with vehicle and animals treated with any of the dosages of chromene **8** (Fig. 8B). In general, all animals have a mildly negative variation of body weight because of the increased physical activity the daily welfare tests demand. Also, no differences were found in animals body temperature between the three groups, before treatment initiation and at the end (Fig. 8C). The slight increase in animals body temperature on day 7 (measurement made after injection of the drug) may be caused by the stress associated with the process and with the daily i.p. treatment, as previously shown.[4,7] Although no statistical analysis can be performed regarding the water and food intake measurements, because the animals of each group were housed in the same cage, we can observe that the animals from all groups showed a mean water intake per day that is slightly above the normal mean value (~4 mL per day per mice) (Fig. 8D). The same was observed for the mean food intake per day, with the animals treated with vehicle and 10 mg kg^{-1} of chromene **8** showing a food consumption above the normal mean value (~3 g per day per mice) (Fig. 8E). Although animals receiving 50 mg kg^{-1} of chromene **8** showed a slightly decreased mean food intake per day (2.94 g per day per mice), this difference is not meaningful. These results suggest that treatment with chromene **8** did not show a negative impact on water and food consumption. Finally, no differences were found between vehicle- and chromene **8**-treated animals in any of the welfare tests performed

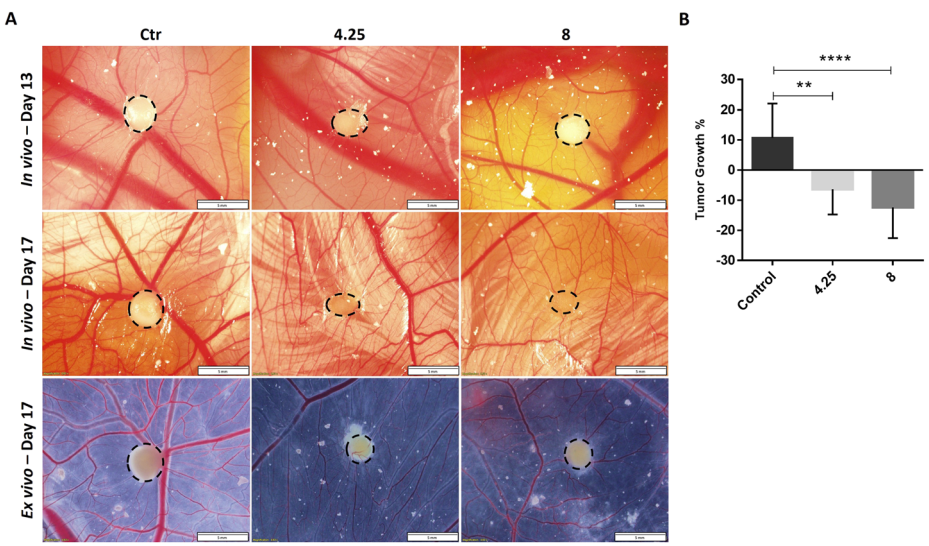
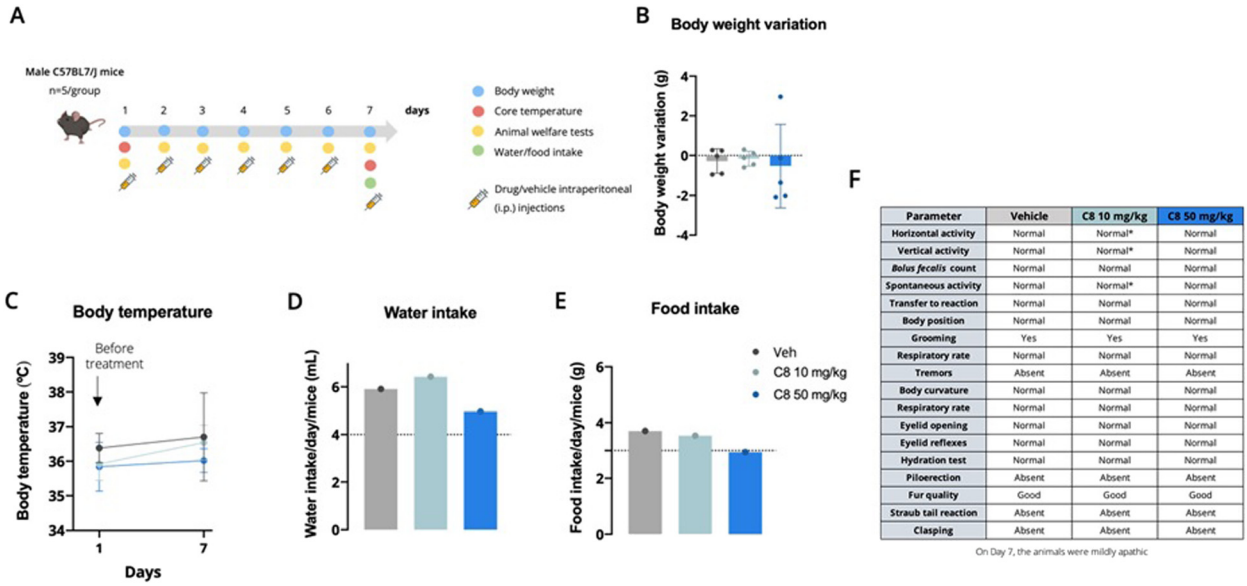


Fig. 7 Effect of chromenes **4.25** and **8** in Hs578t tumor progression. A) *In ovo* pictures at day 13 of embryo development and *in ovo* and *ex ovo* pictures at day 17; representative images were taken at 20× in a Zeiss stereomicroscope. B) Graphical representation of tumors' growth. Results are presented as mean ± SD data were analyzed by one-way ANOVA; ***$p < 0.005$, **$p < 0.01$ treated group *vs.* control.



Parameter	Vehicle	C8 10 mg/kg	C8 50 mg/kg
Horizontal activity	Normal	Normal*	Normal
Vertical activity	Normal	Normal*	Normal
Bolus fecalis count	Normal	Normal	Normal
Spontaneous activity	Normal	Normal*	Normal
Transfer to reaction	Normal	Normal	Normal
Body position	Normal	Normal	Normal
Grooming	Yes	Yes	Yes
Respiratory rate	Normal	Normal	Normal
Tremors	Absent	Absent	Absent
Body curvature	Normal	Normal	Normal
Respiratory rate	Normal	Normal	Normal
Eyelid opening	Normal	Normal	Normal
Eyelid reflexes	Normal	Normal	Normal
Hydration test	Normal	Normal	Normal
Piloerection	Absent	Absent	Absent
Fur quality	Good	Good	Good
Straub tail reaction	Absent	Absent	Absent
Clasping	Absent	Absent	Absent

Fig. 8 Evaluation of chromene **8** toxicity in mice. A) Schematic representation of the experimental design used for assessment of toxicity. Wild-type adult mice were treated every day, for 7 days, with vehicle or one of the dosages of chromene **8**. Body weight, welfare and body temperature were always assessed before treatment. Food and water intake were monitored at the end of the experiment. No differences were found on B) body weight variation or C) body temperature between vehicle- and chromene **8**-treated animals. D) Animals' mean water intake per day was always above the mean normal value referred in the literature. E) The same was observed for animals treated with vehicle and 10 mg kg^{-1} of chromene **8** regarding mean food intake per day, while the animals receiving 50 mg kg^{-1} showed slightly lower food and water consumption but within the mean normal value. F) Overall, mice showed no signs of distress in all welfare tests included in the SHIRPA protocol, suggesting that none of the dosages of chromene **8** tested were toxic to the animals. Only the animals receiving 10 mg kg^{-1} of chromene **8** were mildly apathic on the last day of treatment, at a stage when all animals were already habituated to the tests and showed reduced exploration of the environment. Results are presented as mean ± SD. Data were analysed using one-way ANOVA, except for water and food intake, for which no statistics could be computed (n = 1, unit = cae).

throughout the experiment (Fig. 8F). The only exception was found on day 7, where animals receiving 10 mg kg^{-1} of chromene **8** were mildly apathic, in comparison with animals treated with vehicle or 50 mg kg^{-1} of chromene **8**. However, this may be explained also by the lack of interest in the tests, as they were repeated every day since the beginning of the study. Overall, our results suggest that none of the dosages of chromene **8** tested induced toxicity in wild-type animals, demonstrating the safety profile of this compound *in vivo*.

3. Conclusions

In summary, a series of novel chromene-based compounds was synthesized through simple and efficient reactions, from which new chromenopyrimidinones **3** and **4** and dihydrochromene **8** were prepared in good yields. These compounds were studied for their anticancer potential in TNBC cells, and several molecules exhibited a promising anticancer profile. The most potent chromenes exhibited IC_{50} values in the low micromolar range and were evaluated *in vitro* for their capacity to modulate several cancer aggressiveness features. These compounds were able to inhibit cell proliferation, induce cell death through apoptosis and trigger cell cycle arrest. Importantly, *in vivo* toxicity studies using promising compounds **4.25** and **8**, revealed a safe profile in the *C. elegans* model. The capacity of compounds **8** and **4.25** to inhibit tumor growth was assessed using the CAM model, and both compounds were able to induce significant tumor regression after 4 days of treatment, using a single-dose administration. Considering that chromene **8** showed the most promising efficacy profile in the CAM model, a toxicity evaluation was performed in mice, indicating a safety profile of this compound in rodents. These chromenes are therefore attractive drug candidates for TNBC treatment and valuable hits for future optimization. The simple synthetic approach can be used as a versatile platform for the preparation of new derivatives.

4. Experimental section

4.1 Chemistry

All chemicals, reagents and solvents for synthesis were analytical grade, purchased from commercial sources and used without further purification, unless otherwise specified. The reactions were followed by thin-layer chromatography (TLC), in glass plates coated with silica gel 60 and fluorescent indicator (Macherey-Nagel, DC-Fertigplotten Durasil 25 UV254) or in aluminium plates coated with silica gel 60 and fluorescent indicator (Macherey-Nagel, DC-Fertigfolien ALUGRAM Xtra SIL G/UV254). The spots were observed under UV light using a UV chamber (CN-6 Vilber Lourmat) with a 254 nm lamp and in an iodine chamber. For dry-flash

chromatography, silica gel MN Kieselgel 60 (230 ASTM, particle size <0.063 mm) was used. Some of the reactions were carried out in an IKAMAG RCT equipment with a magnetic stirring of 400 RPM under variable temperatures. The solvent was evaporated in a rotary evaporator Buchi RE III or Buchi R 114. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded in a Bruker Avance III spectrometer, at 400 MHz or 100 MHz, respectively using deuterated dimethyl sulfoxide (DMSO-d_6) as solvent. The chemical shifts (δ) are expressed in parts per million (ppm), and the coupling constants (J) are given in Hertz (Hz), and the multiplicity is represented as follows: s = singlet, d = doublet, t = triplet, dd = doublet of doublet, dt = doublet of triplet, ddd = doublet of doublet of doublet, m = multiplet. The identification of the novel compounds was supported by heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) correlation techniques. For compound **4.29**, the NMR spectra were collected at 70 °C; for compounds **2.5**, **4.6**, **4.9**, **4.11**, **4.22**, **4.26**, the NMR spectra were collected at 80 °C; and for compound **4.27**, the NMR spectra were collected at 100 °C. The remaining spectra were collected at room temperature. Elemental analysis was determined on Thermo Flash EA 1112 (NC Soil Analyzer) apparatus (carrier gas flux (He): 130 mL min^{-1}; reference gas flux (He): 100 mL min^{-1}; oxygen flux: 250 mL min^{-1}; oven temperature: 950 °C; column: multiple analysis 6 × 5 mm, 2.0 m de Cromlab. Column temperature: 75 °C.; standard: sulphanilamide (Thermo Scientific)). Infrared (IR) spectra were recorded on a FT-IR Bomem MB using nujol mulls and NaCl plates. Melting points were determined with a Stuart SMP3 melting point apparatus and are uncorrected. Electrospray (ESI) mass spectra were recorded on a Bruker BIOTOF II mass spectrometer.

4.1.1 General procedure for the synthesis of 2-imino-2*H*-chromenes 2. The synthesis of compounds **2** was performed according to a procedure described in the literature.[17] Cyanoacetamide (2.2–10 mmol) was added to a suspension of the appropriate salicylaldehyde (1 equiv.) in aqueous $NaHCO_3$ (0.05 M, 2.00 ml), and the mixture was stirred at room temperature for 17–61 h. The obtained suspension was then cooled in an ice bath for 1–3 h, filtered and washed with water and a few drops of diethyl ether, affording the desired product **2**. Compounds **2.1** (R^1 = H, R^2 = OMe), **2.2** (R^1 = R^2 = H), **2.3** (R^1 = OMe, R^2 = H), **2.4** (R^1 = Cl, R^2 = H) and **2.5** (R^1 = Br, R^2 = H) were previously prepared and fully characterized in the literature.[48,49]

*2-Imino-6-methyl-2H-chromene-3-carboxamide (**2.6**).* White solid, yield 44%, mp 191–193 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.84 (s, 1H, –NH), 8.35 (d, J = 1.6 Hz, 1H, H4), 7.75 and 9.71 (s, 2H, $CONH_2$), 7.53 (d, J = 7.6 Hz, 1H, H5), 7.40 (d, J = 7.6 Hz, 1H, H7), 7.17 (t, J = 7.6 Hz, 1H, H6), 2.32 (s, 3H, –CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.99 ($CONH_2$), 155.68 (C2), 141.32 (C4), 134.00 (C7), 127.46 (C5), 123.44 (C6), 123.84 (C4a), 120.43 (C3), 118.08 (C8), 14.41 (–CH_3). Anal. calcd for $C_{11}H_{10}N_2O_2$: C, 65.34; H, 4.98; N, 13.95. Found: C, 65.38; H, 4.98; N, 13.99.

*2-Imino-8-methyl-2H-chromene-3-carboxamide (**2.7**).* White solid, yield 73%, mp 222–224 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.64 (s, 1H, –NH), 8.31 (d, J = 1.6 Hz, 1H, H5), 7.75 and 9.48 (s, 2H, $CONH_2$), 7.49 (s, 1H, H5), 7.33 (dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz, 1H, H7), 7.07 (d, J = 8.4 Hz, 1H, H8), 2.31 (s, 3H, –CH_3), ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.67 ($CONH_2$), 155.60 (C2), 140.64 (C4), 133.24 (C7), 129.22 (C5), 127.90 (C6), 120.76 (C3), 117.99 (C4a), 114.32 (C8), 19.75 (–CH_3). Anal. calcd for $C_{11}H_{10}N_2O_2$: C, 65.34; H, 4.98; N, 13.95. Found: C, 65.31; H, 4.93; N, 13.94.

4.1.2 General procedure for the synthesis of chromene[2,3-*d*]pyrimidinone derivatives 3 and 4. Piperidine or *N*-methylpiperazine (for the preparation of **4.14**) (1.2 equiv.) was added to a suspension of the appropriate chromene **2** (0.8–1.1 mmol) and aryl aldehyde (1.2–1.5 equiv.) in ethanol, and the mixture was stirred at 100 °C for 1–28 h. The suspension was cooled to room temperature, diethyl ether (5 mL) was added, and the suspension was allowed to stand in an ice bath for 1 h. The solid was filtered and washed with cold ethanol and diethyl ether. The product was dried and identified as **3** or **4**.

*9-Methoxy-2-(2,4,6-trimethoxyphenyl)-2H-chromeno[2,3-d]pyrimidin-4(3H)-one (**3.1**).* Light-yellow solid, yield 63%, mp 270–271 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.48 (d, J = 1.6 Hz, 1H, –NH), 7.93 (s, 1H, H5), 7.17 (d, J = 8.4 Hz, 1H, H6), 7.16 (d, J = 8.4 Hz, 1H, H8), 7.11 (t, J = 8.0 Hz, 1H, H7), 6.52 (s, 1H, H2), 6.20 (s, 2H, H3′ + H5′), 3.81 (s, 3H, –OCH_3), 3.74 (–OCH_3), 3.73 (br s, 6H, 2× –OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.03 (3C, C2′ + C4′ + C6′), 159.90 (–$CONH_2$), 154.65 (C10a), 146.47 (C9), 142.68 (C9a), 130.99 (C5), 123.95 (C7), 121.03 (C6), 119.08 (C5a), 116.73 (C4a), 115.54 (C8), 109.27 (C1′), 91.46 (C3′ + C5′), 63.02 (C2), 56.22 (–OCH_3), 56.00 (–OCH_3), 55.33 (2C, 2× –OCH_3). Anal. calcd for $C_{2.1}H_{20}N_2O_6$: C, 63.63; H, 5.09; N, 7.07. Found: C, 63.92; H, 5.08; N, 7.27.

*2-(Benzo[d][1,3]dioxol-5-yl)-9-methoxy-2H-chromeno[2,3-d]pyrimidin-4(3H)-one (**3.2**).* Yellow solid, yield 31%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.91 (d, J = 1.2 Hz, 1H, –NH), 7.93 (s, 1H, H5), 7.21 (t, J = 7.2 Hz, 1H, H7), 7.20 (d, J = 7.2 Hz, 1H, H8), 7.13 (d, J = 8.0 Hz, 1H, H6), 6.88 (t, J = 1.6 Hz, 1H, H7′), 6.86 (s, 1H, H4′), 6.81 (dd, J_1 = 8.0, J_2 = 1.6 Hz, 1H, H6′), 6.13 (s, 1H, H2), 5.98 (s, 2H, H2′), 3.83 (s, 3H, –OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.06 (–$CONH_2$), 154.36 (C10a), 147.59 (C1′), 146.51 (C9), 142.73 (C9a), 136.45 (C3′), 133.60 (C5), 124.20 (C7), 121.24 (C6), 119.90 (C6′), 119.65 (C5a), 116.08 (C8), 114.77 (C4a), 108.25 (C4′), 106.98 (C7′), 101.29 (C2′), 70.84 (C2), 56.27 (–OCH_3). Anal. calcd for $C_{19}H_{14}N_2O_5$: C, 65.14; H, 4.03; N, 8.00. Found: C, 65.54; H, 4.14; N, 8.25.

*9-Methoxy-2-(pentan-3-yl)-2H-chromeno[2,3-d]pyrimidin-4(3H)-one (**3.3**).* Light-yellow solid, yield 25%, mp 260–262 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.49 (s, 1H, –NH), 7.81 (d, J = 1.2 Hz, 1H, H5), 7.21 (dd, J_1 = 1.6 Hz, J_2 = 7.6 Hz, 1H, H8), 7.16 (dd, J_1 = 1.6 Hz, J_2 = 7.6 Hz, 1H, H6), 7.11 (t, J = 7.6 Hz, 1H, H7), 5.27 (s, 1H, H2), 3.85 (s, 3H, –OCH_3), 1.42–1.33 (m, 2H), 1.08 (q, J_1 = 2 Hz, J_2 = 7.2 Hz, J_3 = 14 Hz, 2H), 0.89 (t, J =

7.6 Hz, 3H, –CH_3), 0.83 (t, J = 7.6 Hz, 3H, –CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.23 (–$CONH_2$), 157.45 (C10a), 146.55 (C9), 142.82 (C9a), 132.70 (C5), 124.32 (C7), 121.23 (C6), 119.72 (C5a), 116.00 (C8), 115.45 (C4a), 70.50 (C2), 56.30 (–OCH_3), 48.73 (C1′), 20.67 (C2′), 12.32 (C3′). Anal. calcd for $C_{17}H_{20}N_2O_3$: C, 67.98; H, 6.71; N, 9.33. Found: C, 67.74; H, 6.53; N, 9.54.

*2-(2-Bromo-5-hydroxy-4-methoxyphenyl)-7-methoxy-2H-chromeno[2,3-d]pyrimidin-4(3H)-one (**3.4**).* Yellow solid, yield 48%, mp 270 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.89 (s, 1H, –NH), 7.99 (s, 1H, H5), 7.26 (d, J = 2.8 Hz, 1H, H9), 7.11 (s, 1H, H6), 7.09 (d, J = 2.8 Hz, 1H, H8), 7.07 (s, 1H, H3′), 6.73 (s, 1H, H6′), 6.34 (s, 1H, H2), 3.75 (s, 6H, 2× –OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 159.44 (–$CONH_2$), 155.95 (C7), 154.90 (C10a), 148.62 (C4′), 147.80 (C9a), 146.67 (C5′), 133.54 (C5), 132.49 (C1′), 119.93 (C9), 119.57 (C5a), 116.83 (C6), 116.19 (C3′), 114.93 (C4a), 114.91 (C6′), 113.03 (C8), 109.66 (C2′), 70.90 (C2), 56.28 (–OCH_3), 55.93 (–OCH_3). Anal. calcd for $C_{2.1}H_{20}N_2O_6{\cdot}0.25H_2O$: C, 52.36; H, 3.55; N, 6.43. Found: C, 52.36; H, 3.61; N, 6.31.

*7-Methoxy-2-(2,4,6-trimethoxyphenyl)-2H-chromeno[2,3-d]pyrimidin-4(3H)-one (**3.5**).* Lime-green solid, yield 79%, mp 252–254 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.48 (d, J = 1.6 Hz, 1H, –NH), 7.86 (s, 1H, H5), 7.25 (d, J = 2.8 Hz, 1H, H6), 7.09–7.02 (m, 2H, H8 + H9), 6.52 (dd, J_1 = 0.4 Hz, J_2 = 1.6 Hz, 1H, H2), 6.21 (s, 2H, H3′ + H5′), 3.76 (s, 3H, –OCH_3), 3.75 (br s, 6H, 2× –OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.79 (3C, C2′ + C4′ + C6′), 159.61 (–$CONH_2$), 155.15 (C7), 154.72 (C10a), 147.61 (C9a), 130.56 (C5), 119.51 (C5a), 118.81 (C9), 116.78 (C4a), 116.40 (C8), 112.75 (C6), 109.24 (C1′), 91.29 (C3′ + C5′), 62.83 (C2), 56.06 (–OCH_3), 55.80 (–OCH_3), 55.68 (–OCH_3), 55.34 (–OCH_3). Anal. calcd for $C_{2.1}H_{20}N_2O_6$: C, 63.63; H, 5.09; N, 7.07. Found: C, 64.04; H, 5.13; N, 7.39.

*7-Bromo-2-(2,4,6-trimethoxyphenyl)-2H-chromeno[2,3-d]pyrimidin-4(3H)-one (**3.6**).* Light-yellow solid, yield 28%, mp 248–250 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.56 (s, 1H, –NH), 7.89 (s, 2H, H5, H6), 7.60 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H, H8), 7.11 (d, J = 8.8 Hz, 1H, H9), 6.53 (s, 1H, H2), 6.21 (s, 2H, H3′ + H5′), 3.75 (s, 3H, –OCH_3), 3.64 (br s, 6H, 2× –OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.96 (C2′ + C4′ + C6′), 159.32 (–$CONH_2$), 154.23 (C10a), 152.61 (C9a), 134.70 (C8), 131.50 (C6), 129.40 (C5), 121.31 (C5a), 117.84 (C9), 117.42 (C4a), 115.29 (C7), 108.84 (C1′), 91.32 (C3′ + C5′), 62.93 (C2), 55.82 and 55.41 (3× –OCH_3). Anal. calcd for $C_{20}H_{17}BrN_2O_5$: C, 53.95; H, 3.85; N, 6.29. Found: C, 53.49; H, 3.86; N, 6.36.

*9-Methoxy-2-phenyl-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.1**).* Off-white solid, yield 35%, mp 270 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.82 (s, 1H, –NH), 8.18 (d, J = 7.6 Hz, 2H, H2′ + H6′), 7.60 (dt, J_1 = 8.4, J_2 = 1.2 Hz, 1H, H4′), 7.54 (dt, J_1 = 8.0 Hz, J_2 = 1.6 Hz, 2H, H3′ + H5′), 7.06 (t, J = 8.0 Hz, 1H, H7), 6.96 (dd, J_1 = 8.0, J_2 = 1.2 Hz, 1H, H8), 6.84 (dd, J_1 = 7.6, J_2 = 1.6 Hz, 1H, H6), 3.83 (s, 3H, –OCH_3), 3.73 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.72 (C═O), 161.61 (C10a), 147.68 (C9), 139.30 (C9a), 131.97 (C4′), 128.73 (C2′ + C6′), 127.72 (C3′ + C5′), 124.39 (C7), 120.67 (C6), 120.31 (C5a, C2), 110.82 (C8), 96.83 (C4a), 55.72 (–OCH_3), 21.86 (C5). Anal. calcd for $C_{18}H_{14}N_2O_3$: C, 70.58; H, 4.61; N, 9.15. Found: C, 70.30; H, 4.50; N, 9.00.

*9-Methoxy-2-(p-tolyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.2**).* Light-yellow solid, yield 42%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.74 (s, 1H, –NH), 8.02 (dt, J = 8.0 Hz, 2H, H2′ + H6′), 7.33 (d, J = 8.0 Hz, 2H, H3′ + H5′), 7.05 (t, J = 8.0 Hz, 1H, H7), 6.94 (dd, J_1 = 8.0 Hz, J_2 = 0.8 Hz, 1H, H8), 6.83 (dd, J_1 = 7.6 Hz, J_2 = 1.2 Hz, 1H, H6), 3.83 (s, 3H, –OCH_3), 3.71 (s, 2H, H5), 2.38 (s, 3H, –CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.59 (C═O), 161.51 (C10a), 155.21 (C2), 147.65 (C9), 142.14 (C4′), 139.30 (C9a), 129.28 (C3′ + C5′), 127.62 (C2′ + C6′), 124.29 (C7), 120.63 (C6), 120.31 (C5a), 110.75 (C8), 94.41 (C4a), 55.67 (–OCH_3), 21.84 (C5), 20.98 (–CH_3). Anal. calcd for $C_{19}H_{16}N_2O_3$: C, 71.24; H, 5.03; N, 8.74. Found: C, 71.11; H, 5.07; N, 8.78.

*9-Methoxy-2-(2-methoxyphenyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.3**).* Light-yellow solid, yield 63%, mp 260–262 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.18 (s, 1H, –NH), 7.71 (dd, J_1 = 7.6, J_2 = 1.6 Hz, 1H, H6′), 7.54 (dt, J_1 = 7.6, J_2 = 1.2 Hz, 1H, H4′), 7.19 (d, J = 7.6 Hz, 1H, H3′), 7.09 (dt, J_1 = 7.6, J_2 = 0.8 Hz, 1H, H5′), 7.06 (t, J = 8.0 Hz, 1H, H7), 6.95 (dd, J_1 = 8.0 Hz, J_2 = 1.2 Hz, 1H, H8), 6.83 (dd, J_1 = 7.6 Hz, J_2 = 1.6 Hz, 1H, H6), 3.87 (s, 3H, –OCH_3), 3.82 (s, 3H, –OCH_3), 3.70 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.55 (C═O), 161.56 (C10a), 154.68 (C2), 147.70 (C9), 133.01 (C4′), 130.44 (C6′), 124.43 (C7), 120.97 (C5a), 120.72 (C6), 120.63 (C5′), 120.35 (C1′), 112.08 (C3′), 110.86 (C8), 96.82 (C4a), 55.92 (–OCH_3), 55.76 (–OCH_3), 21.87 (C5). Anal. calcd for $C_{19}H_{16}N_2O_4$: C, 67.85; H, 4.79; N, 8.33. Found: C, 68.22; H, 4.81; N, 8.42.

*9-Methoxy-2-(3-methoxyphenyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.4**).* Off-white solid, yield 34%, mp 296–298 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.79 (s, 1H, –NH), 7.71 (dd, J_1 = 7.6 Hz, J_2 = 1.6 Hz, 1H, H6′), 7.67 (t, J = 1.2 Hz, 1H, H2′), 7.44 (t, J = 8.0 Hz, 1H, H5′), 7.14 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 1H, H4′), 7.06 (t, J = 8.0 Hz, 1H, H7), 6.94 (d, J = 7.2 Hz, 1H, H8), 6.83 (d, J = 7.6 Hz, 1H, H6), 3.84 (s, 3H, –OCH_3), 3.83 (s, 3H, –OCH_3), 3.72 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.59 (C═O), 161.46 (C10a), 159.39 (C3′), 154.68 (C2), 147.66 (C9), 139.27 (C9a), 133.01 (C1′), 129.86 (C5′), 124.36 (C7), 120.65 (C6), 120.05 (C6′), 118.33 (C4′), 112.19 (C2′), 110.77 (C8), 96.62 (C4a), 55.67 (–OCH_3), 55.41 (–OCH_3), 21.87 (C5). Anal. calcd for $C_{19}H_{16}N_2O_4{\cdot}0.2H_2O$: C, 67.10; H, 4.80; N, 8.20. Found: C, 66.80; H, 4.60; N, 8.30.

*2-(2,5-Dimethoxyphenyl)-9-methoxy-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.5**).* Pinkish solid, yield 59%, mp 297–299 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.12 (s, 1H, –NH), 7.28 (d, J = 2.4 Hz, 1H, H6′), 7.14 (d, J = 2.4 Hz, 2H, H3′ + H4′), 7.06 (t, J = 8.0 Hz, 1H, H7), 6.94 (d, J = 7.2 Hz, 1H, H8), 6.83 (d, J = 7.6 Hz, 1H, H6), 3.84 (s, 3H, –OCH_3), 3.82 (s, 3H, –OCH_3), 3.77 (s, 3H, –OCH_3), 3.71 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.34 (C═O), 161.40 (C10a), 154.27 (C2), 152.91 (C5′), 151.35 (C2′), 147.62 (C9), 139.23 (C9a), 124.34 (C7), 121.18 (C1′), 120.64 (C6), 120.28 (C5a), 118.60 (C4′), 114.63 (C6′), 113.41 (C3′), 110.76 (C8), 96.88 (C4a), 56.24

(–OCH_3), 55.67 (–OCH_3), 55.65 (–OCH_3), 21.82 (C5). Anal. calcd for $C_{20}H_{18}N_2O_5$: C, 65.57; H, 4.95; N, 7.65. Found: C, 65.22; H, 5.03; N, 7.79.

*2-(4-Ethoxyphenyl)-9-methoxy-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.6**).* White solid, yield 37%, mp 295–297 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.62 (s, 1H, –NH), 8.09 (d, J = 8.8 Hz, 2H, H2′ + H6′), 7.04 (d, J = 8.8 Hz, 2H, H3′ + H5′), 7.04 (t, J = 8.0 Hz, 1H, H7), 6.93 (d, J = 7.6 Hz, 1H, H8), 6.82 (d, J = 7.2 Hz, 1H, H6), 4.11 (q, J = 7.6 Hz, 2H, CH_2), 3.83 (s, 3H, –OCH_3), 3.69 (s, 2H, H5), 1.34 (t, J = 7.2 Hz, –CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.37 (C═O), 161.59 (C10a), 161.44 (C4′), 154.92 (C2), 147.64 (C9), 139.58 (C9a), 129.28 (C2′ + C6′), 124.04 (C7), 123.64 (C5a), 120.53 (C6), 120.36 (C1′), 114.42 (C3′ + C5′), 111.11 (C8), 95.55 (C4a), 63.40 (CH_2), 55.79 (–OCH_3), 21.62 (C5), 14.16 (CH_3). Anal. calcd for $C_{20}H_{18}N_2O_4$: C, 68.56; H, 5.18; N, 8.00. Found: C, 68.52; H, 5.05; N, 8.21.

*2-(3-Hydroxyphenyl)-9-methoxy-3H-chromeno[2,3-d] pyrimidin-4(5H)-one (**4.7**).* White solid, yield 54%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.68 (s, 1H, –NH), 9.78 (s, 1H, OH), 7.56 (d, J = 6.4 Hz, 1H, H6′), 7.55 (s, 1H, H2′), 7.32 (t, J = 8.4 Hz, 1H, H5′), 7.06 (t, J = 8.0 Hz, 1H, H7), 6.94 (d, J = 8.4 Hz, 1H, H8), 6.92 (dd, J_1 = 9.6 Hz, J_2 = 2.0 Hz, 1H, H4′), 6.83 (dd, J_1 = 7.6 Hz, J_2 = 1.2 Hz, 1H, H6), 3.84 (s, 3H, –OCH_3), 3.72 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 157.59 (C3′), 147.67 (C9), 139.30 (C9a), 132.64 (C1′), 124.34 (C7), 120.66 (C6), 120.32 (C5a), 119.02 (C4′), 118.22 (C6′), 114.46 (C2′), 110.79 (C8), 96.67 (C4a), 55.72 (–OCH_3), 21.87 (C5), C2, C10a and C═O not seen. Anal. calcd for $C_{18}H_{14}N_2O_4$: C, 67.07; H, 4.38; N, 8.69. Found: C, 66.86; H, 4.66; N, 8.66.

*2-(2-Chlorophenyl)-9-methoxy-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.8**).* Off-white solid, yield 36%, mp 262–264 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.93 (s, 1H, –NH), 8.18 (d, J = 7.6 Hz, 2H, H2′ + H6′), 7.64–7.60 (m, 2H, H3′ + H6′), 7.56 (dt, J_1 = 8.0 Hz, J_2 = 1.6 Hz, 1H, H4′), 7.07 (t, J = 7.6 Hz, 1H, H7), 6.95 (d, J = 7.6 Hz, 1H, H8), 6.85 (d, J = 7.2 Hz, 1H, H6), 3.80 (s, 3H, –OCH_3), 3.73 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.82 (C═O), 161.28 (C10a), 155.11 (C2), 147.70 (C9), 139.20 (C9a), 132.63 (C2′), 132.07 (C4′), 131.29 (C1′), 130.85 (C6′), 129.79 (C3′), 127.34 (C5′), 124.56 (C7), 120.72 (C6), 120.27 (C5a), 110.94 (C8), 97.66 (C4a), 55.79 (–OCH_3), 21.81 (C5). Anal. calcd for $C_{18}H_{13}ClN_2O_3$: C, 63.44; H, 3.85; N, 8.22. Found: C, 63.70; H, 3.95; N, 8.54.

*2-(3-Chlorophenyl)-9-methoxy-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.9**).* White solid, yield 27%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.83 (s, 1H, –NH), 8.17 (t, J = 1.2 Hz, 1H, H2′), 8.09 (dd, J_1 = 8.0 Hz, J_2 = 1.2 Hz, 1H, H6′), 7.63 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 1H, H4′), 7.55 (t, J = 8.0 Hz, 1H, H5′), 7.05 (t, J = 7.6 Hz, 1H, H7), 6.94 (d, J = 7.6 Hz, 1H, H8), 6.84 (d, J = 7.2 Hz, 1H, H6), 3.85 (s, 3H, –OCH_3), 3.75 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.47 (C═O), 161.62 (C10a), 154.43 (C2), 147.61 (C9), 139.45 (C9a), 134.00 (C3′), 133.33 (C1′), 131.10 (C4′), 130.18 (C5′), 127.15 (C2′), 126.02 (C6′), 124.11 (C7), 120.48 (C6), 120.17 (C5a), 111.16 (C8), 96.88 (C4a), 55.76 (–OCH_3), 21.58 (C5). Anal. calcd for $C_{18}H_{13}ClN_2O_3$: C, 63.44; H, 3.85; N, 8.22. Found: C, 63.24; H, 3.78; N, 8.30.

*2-(3-Bromophenyl)-9-methoxy-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.10**).* Off-white solid, yield 22%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.86 (s, 1H, –NH), 8.31 (t, J = 1.2 Hz, 1H, H2′), 8.12 (d, J = 8.0 Hz, 1H, H6′), 7.78 (dd, J_1 = 8.0 Hz, J_2 = 0.8 Hz, 1H, H4′), 7.49 (t, J = 8.0 Hz, 1H, H5′), 7.06 (t, J = 8.0 Hz, 1H, H7), 6.94 (d, J = 7.2 Hz, 1H, H8), 6.83 (d, J = 7.6 Hz, 1H, H6), 3.83 (s, 3H, –OCH_3), 3.72 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.59 (C═O), 154.59 (C2), 147.70 (C9), 139.25 (C9a), 134.51 (C4′), 130.90 (C5′), 130.33 (C2′), 126.76 (C6′), 124.47 (C7), 121.98 (C3′), 120.48 (C6), 120.17 (C5a), 111.16 (C8), 96.88 (C4a), 55.76 (–OCH_3), 21.86 (C5), C10a and C1′ not seen. Anal. calcd for $C_{18}H_{13}BrN_2O_3{\cdot}0.2H_2O$: C, 55.51; H, 3.44; N, 7.20. Found: C, 55.41; H, 3.45; N, 7.32.

*2-(4-Bromophenyl)-9-methoxy-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.11**).* Off-white solid, yield 39%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.58 (s, 1H, –NH), 8.06 (d, 2H, J = 8.4 Hz, H2′ + H6′), 7.38 (d, 2H, J = 8.4 Hz, H3′ + H5′), 7.06 (t, J = 8.0 Hz, 1H, H7), 6.94 (d, J = 6.8 Hz, 1H, H8), 6.83 (d, J = 6.8 Hz, 1H, H6), 3.83 (s, 3H, –OCH_3), 3.72 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.59 (C═O), 161.48 (C10a), 154.72 (C2), 147.53 (C9), 139.40 (C9a), 131.25 (C3′ + C5′), 129.45 (C1′), 129.34 (C2′ + C6′), 123.96 (C7, C4′), 120.38 (C6), 120.12 (C5a), 111.05 (C8), 96.54 (C4a), 55.67 (–OCH_3), 21.52 (C5). Anal. calcd for $C_{18}H_{13}BrN_2O_3$: C, 56.12; H, 3.40; N, 7.27. Found: C, 56.52; H, 3.43; N, 7.27.

*2-(4-Fluorphenyl)-9-methoxy-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.12**).* White solid, yield 23%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.83 (s, 1H, –NH), 8.18 (dd, 2H, J_1 = 8.8 Hz, J_2 = 5.2 Hz, H3′ + H5′), 7.37 (dd, 2H, J_1 = 8.8 Hz, J_2 = 2.0 Hz, H2′ + H6′), 7.06 (t, J = 8.0 Hz, 1H, H7), 6.95 (d, J = 6.8 Hz, 1H, H8), 6.84 (d, J = 6.8 Hz, 1H, H6), 3.83 (s, 3H, –OCH_3), 3.72 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.40 (d, J = 248.9 Hz, C4′), 163.16 (C═O), 161.63 (C10a), 147.74 (C9), 139.33 (C9a), 130.50 (d, J = 9.2 Hz, C2′ + C6′), 128.24 (C1′), 124.49 (C7), 120.73 (C6), 120.34 (C5a), 115.87 (d, J = 21.9 Hz, C3′ + C5′), 110.89 (C8), 96.75 (C4a), 55.78 (–OCH_3), 21.88 (C5), C2 not seen. Anal. calcd for $C_{18}H_{13}FN_2O_3$: C, 66.66; H, 4.04; N, 8.64. Found: C, 66.98; H, 3.97; N, 8.91.

*2-(3,5-Di-tert-butyl-4-hydroxyphenyl)-9-methoxy-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.13**).* Lime-green solid, yield 17%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.76 (s, 1H, –NH), 7.83 (s, 2H, H2′ + H6′), 7.65 (s, 1H, OH), 7.07 (t, J = 8.0 Hz, 1H, H7), 6.95 (d, J = 6.8 Hz, 1H, H8), 6.83 (d, J = 6.8 Hz, 1H, H6), 3.83 (s, 3H, –OCH_3), 3.70 (s, 2H, H5), 1.43 (s, 9H, C3′ or C5′, *t*-Bu), 1.35 (s, 9H, C3′ or C5′, *t*-Bu). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.71 (C═O), 161.55 (C10a), 157.63 (C4′), 156.02 (C2), 147.62 (C9), 139.33 (C9a), 138.68 (C3′ + C5′), 124.79 (C2′ + C6′), 124.24 (C7), 122.60 (C1′), 120.64 (C6), 120.42 (C5a), 110.64 (C8), 95.45 (C4a), 55.59 (–OCH_3), 34.73 (–$C(CH_3)_3$), 30.20 (–CH_3), 30.13 (–CH_3), 21.85 (C5). Anal. calcd for $C_{2.6}H_{18}N_2O_4$: C, 71.87; H, 6.96; N, 6.45. Found: C, 71.96; H, 6.86; N, 6.75.

*9-Methoxy-2-styryl-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.14**).* Cream solid, yield 19%, mp 275 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.55 (s, 1H, –NH), 7.91 (d, J = 16.0 Hz, 1H, H2′), 7.64 (dd, J_1 = 8.4 Hz, J_2 = 1.6 Hz, 2H, H2′ + H6′), 7.48–7.42 (m, 3H, H3′ + H4′ + H5′), 7.05 (t, J = 8.0 Hz, 1H, H7), 6.94 (dd, J_1 = 8.8 Hz, J_2 = 0.8 Hz, 1H, H8), 6.82 (d, J = 16.0 Hz, 1H), 6.82 (dd, J_1 = 7.6 Hz, J_2 = 1.2 Hz 1H, H6), 3.83 (s, 3H, –OCH_3), 3.69 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.91 (C═O), 161.54 (C10a), 154.13 (C2), 147.68 (C9), 139.33 (C9a), 139.60, 134.62 (C1′), 130.14 (C3′ + C5′), 129.12 (C4′), 127.84 (C2′ + C6′), 124.32 (C7), 120.65 (C6), 120.34 (C5a), 119.41, 110.78 (C8), 96.42 (C4a), 55.72 (–OCH_3), 21.96 (C5). Anal. calcd for $C_{20}H_{16}N_2O_3$: C, 72.28; H, 4.85; N, 8.43. Found: C, 72.15; H, 5.16; N, 8.75.

*2-(Furan-2-yl)-9-methoxy-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.15**).* Brown solid, yield 33%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.35 (s, 1H, –NH), 7.99 (d, 1H, J = 1.2 Hz, H4′), 7.56 (d, 1H, J = 3.6 Hz, H2′), 7.05 (t, J = 8.0 Hz, 1H, H7), 6.95 (dd, J_1 = 8.4 Hz, J_2 = 1.2 Hz, 1H, H8), 6.83 (d, J = 7.6 Hz, 1H, H6), 6.74 (dd, J_1 = 3.6 Hz, J_2 = 1.6 Hz, 1H, H3′), 3.83 (s, 3H, –OCH_3), 3.69 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.15 (C═O), 161.46 (C10a), 159.87 (C2), 147.65 (C9), 146.90 (C4′), 145.74 (C1′), 139.26 (C9a), 124.33 (C7), 120.63 (C6), 120.30 (C5a), 115.20 (C2′), 112.76 (C3′), 110.81 (C8), 96.54 (C4a), 55.72 (–OCH_3), 21.89 (C5). Anal. calcd for $C_{16}H_{12}N_2O_4{\cdot}0.2H_2O$: C, 64.02; H, 4.13; N, 9.33. Found: C, 64.33; H, 4.40; N, 9.55.

*7-Methoxy-2-(2-methoxyphenyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.16**).* Beige solid, yield 81%, mp 268–270 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.15 (s, 1H, –NH), 7.69 (d, 1H, J_1 = 8.0 Hz, J_2 = 2.0 Hz, H6′), 7.53 (dt, 1H, J_1 = 7.2 Hz, J_2 = 2.0 Hz, H4′), 7.19 (d, 1H, J = 8.4 Hz, H3′), 7.07 (dt, 1H, J_1 = 7.6 Hz, J_2 = 0.8 Hz, H5′), 7.02 (d, J = 9.2 Hz, 1H, H9), 6.84 (d, J = 2.8 Hz, 1H, H6), 6.79 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, 1H, H8), 3.86 (s, 3H, –OCH_3), 3.72 (s, 2H, H5), 3.68 (s, 3H, OMe). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.61 (C═O), 161.81 (C10a), 157.27 (C2′), 155.99 (C2), 154.70 (C7), 133.01 (C4′), 130.39 (C6′), 121.01 (C1′), 120.66 (C5), 120.47 (C5a), 117.50 (C9), 113.83 (C6 + C8), 112.11 (C3′), 96.33 (C4a), 55.92 (–OCH_3), 55.52 (–OCH_3), 22.20 (C5). Anal. calcd for $C_{19}H_{16}N_2O_4$: C, 67.85; H, 4.79; N, 8.33. Found: C, 67.79; H, 4.86; N, 8.54.

*7-Methoxy-2-(3-methoxyphenyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.17**).* Light-yellow solid, yield 65%, mp 246–248 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.79 (s, 1H, –NH), 0.14 (dd, J_1 = 8.0 Hz, J_2 = 2.4 Hz, 1H, H8), 7.70 (d, J = 7.6 Hz, 1H, H6′), 7.67 (s, 1H, H2′), 7.43 (t, J = 8.0 Hz, 1H, H5′), 7.07 (d, J = 8.0 Hz, 1H, H9), 6.87 (d, J = 3.2 Hz, 1H, H6), 6.83 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, 1H, H4′), 3.84 (s, 3H, –OCH_3), 3.74 (s, 2H, H5), 3.72 (s, 3H, –OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.61 (C═O), 161.66 (C10a), 159.37 (C3′), 155.90 (C7), 154.98 (C2), 143.78 (C10), 132.93 (C1′), 129.84 (C5′), 120.35 (C5a), 119.99 (C6′), 118.28 (C8), 117.44 (C9), 113.73 (C6, C4′), 112.77 (C2′), 96.39 (C4a), 55.43 (–OCH_3), 55.38 (–OCH_3), 22.18 (C5). Anal. calcd for $C_{19}H_{16}N_2O_4$: C, 67.85; H, 4.79; N, 8.33. Found: C, 67.56; H, 4.76; N, 8.56.

*7-Methoxy-2-(2,5-dimethoxyphenyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.18**).* Off-white solid, yield 74%, mp 205–207 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.15 (s, 1H, –NH), 7.26 (dd, J_1 = 2.4 Hz, J_2 = 0.8 Hz, 1H, H4′), 7.14 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 1H, H8), 7.12 (d, J = 2.4 Hz, 1H, H3′), 7.12 (s, 1H, H6′), 7.07 (t, J = 8.0 Hz, 1H, H9), 6.87 (d, J = 3.2 Hz, 1H, H6), 3.82 (s, 3H, –OCH_3), 3.75 (s, 3H, –OCH_3), 3.73 (s, 3H, –OCH_3), 3.67 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.48 (C═O), 161.70 (C10a), 155.97 (C7), 154.30 (C2), 152.98 (C2′), 151.45 (C5′), 143.79 (C9a), 121.17 (C1′), 120.42 (C5a), 118.66 (C3′), 117.48 (C9), 114.67 (C4′), 113.81 (C8), 113.80 (C6), 113.52 (C6′), 96.43 (C4a), 56.33 (–OCH_3), 55.71 (–OCH_3), 55.50 (–OCH_3), 22.19 (C5). Anal. calcd for $C_{20}H_{18}N_2O_5$: C, 65.57; H, 4.95; N, 7.85. Found: C, 65.79; H, 4.87; N, 7.76.

*7-Methoxy-2-(3-chlorophenyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.19**).* Off-white solid, yield 39%, mp 278 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.15 (s, 1H, –NH), 8.16 (t, J = 1.6 Hz, 1H, H2′), 8.07 (d, J = 7.6 Hz, 1H, H6′), 7.65 (dd, J_1 = 8.0 Hz, J_2 = 1.2 Hz, 1H, H4′), 7.56 (t, J = 8.0 Hz, 1H, H5′), 7.06 (d, J = 8.8 Hz, 1H, H9), 6.86 (d, J = 2.4 Hz, 1H, H6), 6.81 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H, H8), 3.76 (s, 2H, H5), 3.73 (s, 3H, –OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.74 (C10a), 155.95 (C7), 143.73 (C9a), 133.53 (C3′), 131.57 (C4′), 130.64 (C5′), 127.42 (C2′), 126.32 (C6′), 120.30 (C5a), 117.45 (C9), 113.80 (C6), 113.77 (C8), 96.78 (C4a), 55.46 (–OCH_3), 22.16 (C5), C2, C1′ and C═O not seen. Anal. calcd for $C_{18}H_{13}ClN_2O_3$: C, 63.44; H, 3.85; N, 8.22. Found: C, 63.48; H, 3.88; N, 8.33.

*2-Phenyl-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.20**).* Off-white solid, yield 39%, mp 298 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.82 (s, 1H, –NH), 8.11 (dd, J_1 = 8.0, J_2 = 1.2 Hz, 2H, H2′ + H6′), 7.59 (dt, J_1 = 7.2 Hz, J_2 = 1.2 Hz, 1H, H4′), 7.52 (dt, J_1 = 1.2 Hz, J_2 = 6.8 Hz, 2H, H3′ + H5′), 7.30 (dd, J_1 = 8.0 Hz, J_2 = 1.6 Hz, 1H, H6), 7.26 (dt, J_1 = 7.6 Hz, J_2 = 1.2 Hz, 1H, H8), 7.14–7.11 (m, 2H, H7 + H9), 3.73 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.63 (C═O), 161.62 (C10a), 155.43 (C2), 149.98 (C9a), 131.68 (C4′), 131.66 (C1′), 129.62 (C6), 128.72 (C3′ + C5′), 128.06 (C8), 127.68 (C2′ + C6′), 124.59 (C7), 119.57 (C5), 116.58 (C9), 96.97 (C4a), 21.75 (C5). Anal. calcd for $C_{17}H_{12}N_2O_2$: C, 73.90; H, 4.38; N, 10.14. Found: C, 74.01; H, 4.48; N, 10.09.

*2-(3,5-Di-tert-butyl-4-hydroxyphenyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.21**).* Off-white solid, yield 21%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.77 (s, 1H, –NH), 7.85 (s, 2H, H2′ + H6′), 7.65 (s, J = 7.6 Hz, 1H, OH), 7.28 (d, J = 7.6 Hz, 1H, H6), 7.25 (t, J = 7.2 Hz, 1H, H8), 7.14 (d, J = 8.0 Hz, 1H, H9), 7.12 (t, J = 7.6 Hz, 1H, H7), 3.73 (s, 2H, H5), 1.42 (s, 18H, 2× *t*-Bu). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.77 (C═O), 161.65 (C10a), 157.67 (C4′), 156.16 (C2), 150.09 (C9a), 138.86 (C3′ + C5′), 129.59 (C6), 124.81 (C2′ + C6′), 124.48 (C7), 122.49 (C1′), 119.70 (C5a), 116.59 (C9), 95.62 (C4a), 34.74 (C3′-*t*-Bu or C5′-*t*-Bu), 30.13 (C3′-*t*-Bu or C5′-*t*-Bu), 21.74 (C5). Anal. calcd for $C_{2.5}H_{28}N_2O_3$: C, 74.23; H, 6.98; N, 6.93. Found: C, 73.95; H, 6.84; N, 7.19.

*9-Methyl-2-phenyl-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.22**).* Off-white solid, yield 56%, mp > 300 °C. ^{1}H NMR (400

MHz, DMSO-d_6): δ 12.47 (s, 1H, –NH), 8.13 (dd, J_1 = 8.0 Hz, J_2 = 1.2 Hz, 2H, H2′ + H6′), 7.53 (dt, J_1 = 8.0 Hz, J_2 = 2.8 Hz, 1H, H4′), 7.53 (dt, J_1 = 7.6 Hz, J_2 = 1.6 Hz, 2H, H3′ + H5′), 7.10 (d, J = 7.6 Hz, 2H, H6 + H8), 7.01 (t, J = 8.0 Hz, 1H, H7), 3.75 (s, 2H, H5), 2.32 (s, 3H, –CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.19 (C═O), 162.27 (C10a), 161.58 (C2), 155.29 (C1′), 148.14 (C9a), 131.31 (C4′), 128.86 (C8), 128.19 (C3′ + C5′), 127.32 (C2′ + C6′), 126.55 (C6), 125.01 (C9), 123.56 (C7), 118.99 (C5a), 96.59 (C4a), 21.60 (C5), 15.04 (–CH_3). Anal. calcd for $C_{18}H_{14}N_2O_2$: C, 74.47; H, 4.86; N, 9.65. Found: C, 74.35; H, 4.98; N, 9.65.

*7-Methyl-2-phenyl-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.23**).* Off-white solid, yield 62%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.79 (s, 1H, –NH), 8.10 (d, J = 7.2 Hz, 2H, H2′ + H6′), 7.82 (t, J = 7.6 Hz, 1H, H5′), 7.59 (dt, J_1 = 7.2, J_2 = 1.2 Hz, 2H, H3′ + H5′), 7.09 (d, J = 1.6 Hz, 1H, H6), 7.04 (dd, J_1 = 7.6, J_2 = 1.6 Hz, 1H, H8), 7.01 (d, J = 7.6 Hz, 1H, H9), 3.68 (s, 2H, H5), 2.25 (s, 3H, –CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.64 (C═O), 161.65 (C10a), 155.24 (C2), 147.84 (C9a), 133.61 (C1′), 133.61 (C5a), 131.90 (C4′), 129.72 (C6), 128.69 (C3′ + C5′), 128.48 (C8), 127.64 (C2′ + C6′), 119.09 (C7), 116.31 (C9), 96.81 (C4a), 21.74 (C5), 20.21 (–CH_3). Anal. calcd for $C_{18}H_{14}N_2O_2$: C, 74.47; H, 4.86; N, 9.65. Found: C, 74.36; H, 5.00; N, 9.60.

*2-(2,5-Dimethoxyphenyl)-7-methyl-3H-chromeno[2,3-d] pyrimidin-4(5H)-one (**4.24**).* White solid, yield 73%, mp 207–209 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.06 (s, 1H, –NH), 7.17 (dd, J_1 = 2.4 Hz, J_2 = 0.8 Hz, 1H, H6′), 7.03 (d, J = 3.2 Hz, 1H, H3′), 7.03 (dd, J_1 = 3.2 Hz, J_2 = 0.8 Hz, 1H, H4′), 7.00 (dd, J_1 = 8.8 Hz, J_2 = 1.2 Hz, 1H, H8), 6.94 (dd, J_1 = 8.4 Hz, J_2 = 1.6 Hz, 1H, H6), 6.91 (d, J = 8.0 Hz, 1H, H9), 3.74 (s, 3H, –OCH_3), 3.67 (S, 3H, –OCH_3), 3.58 (s, 2H, H5), 2.17 (s, 3H, –CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.37 (C═O), 161.56 (C10a), 154.26 (C2), 152.91 (C2′), 147.80 (C9a), 133.61 (C5a), 129.72 (C8), 128.47 (C6), 121.14 (C1′), 119.09 (C7), 118.56 (C4′), 116.28 (C9), 114.62 (C6′), 113.42 (C3′), 96.92 (C4a), 96.92 (C4a), 56.26 (C5′–OCH_3), 55.64 (C2′–OCH_3), 21.73 (C5), 20.21 (–CH_3). Anal. calcd for $C_{20}H_{18}N_2O_4$: C, 68.56; H, 5.18; N, 8.00. Found: C, 68.78; H, 5.17; N, 8.07.

*7-Bromo-2-phenyl-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.25**).* Off-white solid, yield 22%, mp 290–292 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.84 (s, 1H, –NH), 8.10 (dd, J_1 = 7.6, J_2 = 1.2 Hz, 2H, H2′ + H6′), 7.59 (dt, J_1 = 7.2, J_2 = 1.2 Hz, 1H, H4′), 7.58 (d, J = 2.4 Hz, 1H, H6), 7.54 (dt, J_1 = 7.6, J_2 = 2.0 Hz, 2H, H3′ + H5′), 7.42 (dd, J_1 = 8.8, J_2 = 2.4 Hz, 1H, H8), 7.11 (d, J = 8.4 Hz, 1H, H9), 3.75 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.34 (C═O), 161.43 (C10a), 155.60 (C2), 149.32 (C9a), 132.02 (C6, C4′), 130.79 (C8), 128.72 (C3′ + C5′), 127.68 (C2′ + C6′), 122.42 (C5a), 118.78 (C9), 116.07 (C7), 96.71 (C4a), 21.62 (C5). Anal. calcd for $C_{17}H_{11}BrN_2O_2{\cdot}2H_2O$: C, 52.19; H, 3.86; N, 7.16. Found: C, 52.31; H, 4.12; N, 7.16.

*7-Bromo-2-(3,4-dimethoxyphenyl)-3H-chromeno[2,3-d] pyrimidin-4(5H)-one (**4.26**).* Off-white solid, yield 24%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.84 (s, 1H, –NH), 7.79 (dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz, 1H, H6′), 7.74 (d, J = 2.4 Hz, 1H, H5′), 7.52 (d, J = 2.4 Hz, 1H, H6), 7.40 (dd, J_1 = 8.4, J_2 = 2.4 Hz, 1H, H8), 7.10 (d, J = 8.4 Hz, 1H, H9), 7.08 (dd, J_1 = 8.8, J_2 = 1.2 Hz, 1H, H2′), 3.87 (s, 3H, –OCH_3), 3.85 (s, 3H, –OCH_3), 3.72 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.06 (C═O), 161.14 (C10a), 154.84 (C2), 152.04 (C4′), 149.22 (C9a), 148.61 (C3′), 131.55 (C6), 130.33 (C8), 123.59 (C1′), 122.25 (C5a), 121.16 (C6′), 118.32 (C9), 115.59 (C7), 111.63 (C2′), 111.03 (C5′), 95.32 (C4a), 55.66 (–OCH_3), 55.56 (–OCH_3), 21.31 (C5). Anal. calcd for $C_{19}H_{15}BrN_2O_4$: C, 54.96; H, 3.64; N, 6.75. Found: C, 54.86; H, 3.52; N, 7.06.

*2-(Benzo[d][1,3]dioxol-5-yl)-7-bromo-3H-chromeno[2,3-d] pyrimidin-4(5H)-one (**4.27**).* Off-white solid, yield 21%, mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.66 (s, 1H, –NH), 7.74 (dd, J_1 = 8.0 Hz, J_2 = 1.6 Hz, 1H, H2′), 7.63 (d, J = 1.6 Hz, 1H, H6′), 7.49 (dd, J_1 = 3.2 Hz, J_2 = 0.8 Hz, 1H, H6), 7.40 (dd, J_1 = 8.8 Hz, J_2 = 0.8 Hz, 1H, H8), 7.06 (d, J = 8.4 Hz, 1H, H9), 7.00 (d, J = 8.4 Hz, 1H, H3′), 6.13 (s, 2H, H7′), 3.72 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.11 (C═O), 161.22 (C10a), 154.97 (C2), 150.18 (C4′), 149.23 (C9a), 147.46 (C5′), 131.48 (C6), 130.33 (C8), 125.43 (C1′), 122.71 (C2′), 122.21 (C5a), 118.28 (C9), 115.62 (C7), 107.85 (C3′), 107.07 (C6′), 101.48 (C7′), 95.49 (C4a), 21.27 (C5). Anal. calcd for $C_{18}H_{11}BrN_2O_4$: C, 54.16; H, 2.78; N, 7.02. Found: C, 54.55; H, 2.73; N, 7.11.

*7-Chloro-2-(2-methoxyphenyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.28**).* Light-yellow solid, yield 65%, mp 274–276 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.25 (s, 1H, –NH), 7.67 (dd, J_1 = 7.6 Hz, J_2 = 1.6 Hz, 1H, H6′), 7.54 (dt, J_1 = 8.8 Hz, J_2 = 1.6 Hz, 1H, H4′), 7.40 (s, 1H, H6), 7.28 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H, H8), 7.18 (d, J = 8.4 Hz, 1H, H3′), 7.13 (d, J = 8.8 Hz, 1H, H9), 7.08 (t, J = 7.6 Hz, 1H, H5′), 3.86 (s, 3H, –OCH_3), 3.72 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.46 (C═O), 161.41 (C10a), 154.97 (C2), 148.88 (C9a), 133.11 (C4′), 130.40 (C6′), 129.20 (C6), 128.21 (C7), 127.98 (C8), 122.03 (C5a), 120.89 (C1′), 120.67 (C5′), 118.47 (C9), 112.13 (C3′), 96.69 (C4a), 55.95 (–OCH_3), 21.79 (C5). Anal. calcd for $C_{18}H_{13}ClN_2O_3$: C, 63.54; H, 3.85; N, 8.22. Found: C, 63.59; H, 3.96; N, 8.55.

*7-Chloro-2-(3-methoxyphenyl)-3H-chromeno[2,3-d]pyrimidin-4(5H)-one (**4.29**).* Light-yellow solid, yield 32%, mp 290 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.86 (s, 1H, –NH), 7.72 (d, J = 8.4 Hz, 1H, H6′), 7.68 (t, J = 2.0 Hz, 1H, H2′), 7.43 (t, J = 8.0 Hz, 1H, H5′), 7.39 (d, J = 8.4 Hz, 1H, H6), 7.28 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H, H8), 7.15 (d, J = 8.8 Hz, 1H, H9), 7.14 (dd, J_1 = 8.0 Hz, J_2 = 2.4 Hz, 1H, H4′), 3.85 (s, 3H, –OCH_3), 3.76 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.69 (C10a), 159.24 (C3′), 154.20 (C2), 148.70 (C9a), 132.82 (C1′), 129.45 (C5′), 128.72 (C6), 127.90 (C7), 127.53 (C8), 121.72 (C5a), 119.78 (C6′), 118.03 (C9), 117.97 (C4′), 112.28 (C2′), 96.33 (C4a), 55.43 (–OCH_3), 21.48 (C5), C4 not seen. Anal. calcd for $C_{18}H_{13}ClN_2O_3$: C, 63.54; H, 3.85; N, 8.22. Found: C, 63.95; H, 3.79; N, 8.58.

*7-Chloro-2-(2,5-dimethoxyphenyl)-3H-chromeno[2,3-d] pyrimidin-4(5H)-one (**4.30**).* Light-yellow solid, yield 62%, mp 260–262 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.12 (s, 1H, –NH), 7.41 (d, J = 2.8 Hz, 1H, H6), 7.29 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, 1H, H8), 7.25 (dd, J_1 = 2.4 Hz, J_2 = 1.2 Hz, 1H, H4′),

7.16 (s, 1H, H6′), 7.12 (d, J = 6.8 Hz, 1H, H3′), 7.12 (d, J = 8.8 Hz, 1H, H9), 3.82 (s, 3H, C2′–OCH_3), 3.75 (s, 3H, C5′–OCH_3), 3.72 (s, 2H, H5). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.25 (C10a), 154.52 (C2), 152.93 (C5′), 151.40 (C2′), 148.80 (C9a), 129.13 (C6), 128.13 (C7), 127.89 (C8), 121.95 (C5a), 121.05 (C1′), 118.68 (C3′), 118.40 (C6′), 114.63 (C4′), 113.47 (C9), 96.74 (C4a), 56.28 (–OCH_3), 55.67 (–OCH_3), 21.74 (C5), C4 not seen. Anal. calcd for $C_{19}H_{15}ClN_2O_4 \cdot 1.5H_2O$: C, 57.31; H, 4.52; N, 7.03. Found: C, 57.51; H, 4.13; N, 6.92.

*Synthesis of 2-amino-6-bromo-4-(2-oxo-2-phenylethyl)-4H-chromene-3-carbonitrile (**5**).* The synthesis of compound **5** was performed according to the procedure described in the literature.[12] Aqueous NaOH (3 M, 4.0 mL) was added to a solution of 5-bromosalicylaldehyde **1** (1.2 equiv.) and acetophenone (10 mmol) in ethanol. The solution was stirred at room temperature for 3 h. Addition of concentrated HCl (37%) to pH 7, led to a suspension. After cooling at 4 °C for 2 h, the solid was filtered and washed with cold ethanol, water and diethyl ether, leading to a yellow solid identified as 3-(5-bromo-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (2.06 g, 6.8 mmol, 68%) by comparison of the ^{1}H NMR spectrum with that of an authentic sample.[12]

Malononitrile (1.81 g, 27.4 mmol, 2 equiv.) and triethylamine (7.61 mL, 4 equiv.) were added to a suspension of the crude product 3-(5-bromo-2-hydroxyphenyl)-1-phenylprop-2-en-1-one (4.13 g, 13.6 mmol) in ethanol (8.0 mL). The solution was stirred for 1 h. After standing in an ice bath for 2 h, the suspension was filtered, washed with cold ethanol and diethyl ether, and the white solid was identified as 2-amino-6-bromo-4-(2-oxo-2-phenylethyl)-4*H*-chromene-3-carbonitrile **5** (2.71 g, 7.36 mmol, 54%). Mp 192–194 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.88 (dd, J_1 = 8.4, J_2 = 1.2 Hz, 2H, H2′ + H6′), 7.60 (dt, J_1 = 8.8, J_2 = 1.2 Hz, 1H, H4′), 7.48 (t, J = 7.6 Hz, 2H, H3′ + H5′), 7.46 (dd, J_1 = 2.4, J_2 = 0.8 Hz, 1H, H5), 7.35 (dd, J_1 = 8.8, J_2 = 2.4 Hz, 1H, H7), 6.94 (d, J = 8.8 Hz, 1H, H8), 6.78 (s, 2H, –NH_2), 4.10 (t, J = 5.6 Hz, 1H), 3.42 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 197.91 (C═O), 161.45 (C2), 148.83 (C8a), 136.71 (C1′), 133.49 (C4′), 130.88 (C7), 130.67 (C5), 128.85 (C3′ + C5′), 128.12 (C2′ + C6′), 126.51 (C4a), 120.47 (–CN), 118.25 (C8), 116.03 (C6), 53.90 (C3), 46.90 (–CH_2), 30.87 (C4). Anal. calcd for $C_{18}H_{13}BrN_2O_2$: C, 58.56; H, 3.55; N, 7.59. Found: C, 58.48; H, 3.44; N, 7.48.

*Synthesis of ethyl N-(6-bromo-3-cyano-4-(2-oxo-2-phenylethyl)-4H-chromen-2-yl)formimidate (**6**).* 4*H*-Chromene **5** (0.82 g, 2.17 mmol) was added to a mixture of triethyl orthoformate (2.58 g, 17.4 mmol, 2.90 mL, 8 equiv.) and acetic anhydride (0.66 g, 6.51 mmol, 615 μL, 3 equiv.), and the mixture was refluxed for 35 minutes. After complete consumption of the starting material **5** (confirmed by TLC, AcOEt/*n*-hexane 1 : 1), 10 mL of *n*-hexane were added to the mixture. The brown solution led to a suspension after standing in an ice bath for 2 h. The light-pink solid was filtered, washed with cold *n*-hexane and dried. The product was identified as ethyl *N*-(6-bromo-3-cyano-4-(2-oxo-2-phenylethyl)-4*H*-chromen-2-yl)formimidate **6** (2.07 mmol, 0.88 g, 96%). Mp 164–166 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.91 (dd, J_1 = 8.8, J_2 = 1.6 Hz, 2H, H2′ + H6′), 7.61 (t, J = 7.2 Hz, 1H, H4′), 7.56 (d, J = 2.0 Hz, 1H, H5), 7.49 (t, J = 8.0 Hz, 2H, H3′ + H5′), 7.41 (dd, J_1 = 8.8, J_2 = 2.4 Hz, 1H, H7), 7.07 (d, J = 8.8 Hz, 1H, H8), 4.31 (q, J = 6.8 Hz, 2H), 4.30 (t, J = 5.6 Hz, 1H), 3.74 (dd, J_1 = 18.4, J_2 = 5.6 Hz, 1H), 3.53 (dd, J_1 = 18.4, J_2 = 5.2 Hz, 1H), 1.30 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 197.30 (C═O), 161.55 (C2), 158.66 (C2), 148.83 (C8a), 136.40 (C1′), 133.57 (C4′), 131.17 (C7), 130.63 (C5), 128.79 (C3′ + C5′), 128.06 (C2′ + C6′), 124.76 (C4a), 118.86 (C8), 117.84 (–CN), 116.85 (C6), 78.32 (C3), 64.02, 44.71, 31.53 (C4), 13.93. MS (ESI) m/z = 425 and 427 (MH^+). HRMS calcd for $C_{2.1}H_{18}N_2O_3{}^{79}Br$ (MH^+): 425.0495; found, 425.0492.

*Synthesis of N′-(6-bromo-3-cyano-4-(2-oxo-2-phenylethyl)-4H-chromen-2-yl)-N-phenylformimidamide (**7**).* A suspension of imidate **6** (0.15 g, 0.35 mmol), in a mixture of ethanol/water 1 : 1 (2 mL), was combined with 2 equivalents of aniline (0.07 g, 0.71 mmol, 65 μL) and 0.5 equivalents of acetic acid (0.01 g, 0.18 mmol, 12 μL). The mixture was stirred at 80 °C for 5 h. The dense suspension was then cooled to room temperature for 5 minutes, and in an ice bath for 2 h. The suspension was filtered, washed with cold ethanol and water, and a few drops of diethyl ether, and dried. The beige solid was identified as *N′*-(6-bromo-3-cyano-4-(2-oxo-2-phenylethyl)-4*H*-chromen-2-yl)-*N*-phenylformimidamide **7** (*x* g, *y* mmol, 85%). Mp 187–189 °C, MS (ESI) m/z = 472 and 474 (MH^+). HRMS calcd for $C_{2.5}H_{18}N_3O_2{}^{79}Br$ (MH^+): 472.0655; found, 472.0658. Two tautomeric forms were identified, by NMR, for compound **7**. Tautomer 1: ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.90 (d, J = 12.0 Hz, 1H, –NH), 8.95 (d, J = 11.2 Hz, 1H), 7.94 (d, J = 8.0 Hz, 2H, H2′ + H6′), 7.61 (t, J = 7.2 Hz, 1H, H4′), 7.48 (t, J = 7.6 Hz, 2H, H3′ + H5′), 7.40 (m, 2H, H5, H7), 7.32 (d, J = 8.8 Hz, 2H, H2″ + H6″), 7.30 (t, J = 8.0 Hz, 2H, H3″ + H5″), 7.24 (d, J = 8.4 Hz, 1H, H8), 7.08 (t, J = 7.6 Hz, 1H, H4″), 4.29 (t, J = 5.2 Hz, 1H, H4), 3.66 (dd, J_1 = 14.0, J_2 = 5.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 197.46 (C═O), 160.46 (C2), 151.27, 149.21 (C8a), 139.12 (C1″), 136.51 (C1′), 133.44 (C4′), 130.92 (C7), 130.61 (C5), 128.76 (C3″ + C5″), 128.73 (C3′ + C5′), 128.04 (C2′ + C6′), 125.36 (C4a), 123.96 (C4″), 119.42 (–CN), 118.90 (C8), 117.10 (C2″ + C6″), 116.11 (C6), 73.63 (C3), 45.30, 31.95 (C4). Tautomer 2: ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.85 (d, J = 5.2 Hz, 1H, –NH), 8.36 (d, J = 4.8 Hz, 1H), 7.94 (d, J = 8.0 Hz, 4H, H2′ + H6′, H2″ + H6″), 7.61 (t, J = 7.2 Hz, 1H, H4′), 7.54 (m, 2H, H5, H7), 7.48 (t, J = 7.6 Hz, 2H, H3′ + H5′), 7.32 (t, J = 8.0 Hz, 2H, H3″ + H5″), 7.13 (d, J = 8.8 Hz, 1H, H8), 7.08 (t, J = 7.6 Hz, 1H, H4″), 4.29 (t, J = 5.2 Hz, 1H), 3.52 (dd, J_1 = 17.2, J_2 = 4.4 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 197.46 (C═O), 160.63 (C2), 149.11 (C8a), 148.65, 138.96 (C1″), 136.51 (C1′), 133.44 (C4′), 130.85 (C7), 130.46 (C5), 129.52 (C3″ + C5″), 128.73 (C3′ + C5′), 128.04 (C2′ + C6′), 125.36 (C4a), 123.84 (C4″), 120.04 (C2″ + C6″), 119.40 (–CN), 118.64 (C8), 116.04 (C6), 74.23 (C3), 45.37, 31.65 (C4).

*Synthesis of 10-bromo-2,4-diphenyl-4,6a-dihydro-7-oxa-3,4,6-triazabenzo[de]anthracene (**8**).* Amidine **7** (0.10 g, 0.21 mmol) was added to 2 mL of ethanol and the suspension was heated to 80 °C. The beige suspension slowly turned yellow after 30 minutes.

After 4 h, 1 equivalent of DBU (0.03 g, 0.21 mmol, 26 μL) was added, while stirring at the same temperature, and the suspension turned orange after 30 minutes. After 2 h and 30 minutes, the dark-orange suspension was allowed to cool to room temperature for 5 minutes, and then in an ice bath for 3 h. The suspension was filtered, and the solid was washed with cold ethanol and dried overnight. The light-yellow solid was identified as compound **8** (0.04 g, 0.09 mmol, 42%). Mp 290 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.52 (d, J = 2.4 Hz, 1H, H11), 8.31 (s, 1H, H1), 8.01 (dd, J_1 = 8.0, J_2 = 2.8 Hz, 2H, H2′ + H6′), 7.59 (dd, J_1 = 8.4, J_2 = 2.4 Hz, 1H, H9), 7.57–7.56 (m, 5H, H3′ + H5′, H2″ + H6″, H4″), 7.46 (t, J = 7.6 Hz, 1H, H4″), 7.34 (d, J = 2.0 Hz, 1H, H5), 7.13 (d, J = 8.4 Hz, 1H, H8), 6.79 (d, J = 2.0 Hz, 1H, H6a). ^{13}C NMR (100 MHz, DMSO-d_6): δ 155.40 (C2), 153.59 (C7a), 146.41 (C3a), 145.87 (C5), 138.31 (C1″), 138.19 (C11b), 137.80 (C1′), 134.66 (C9), 129.51 (C4′), 129.11 (C4″), 128.55 (C3′ + C5′), 127.70 or 127.74 (C3′ + C5′), 127.70 or 127.74 (C11), 127.34 (C2″ + C6″), 126.66 (C2′ + C6′), 122.35 (C11a), 120.32 (C8), 114.27 (C10), 109.58 (C1), 103.61 (C3b), 82.79 (C6a). Anal. calcd for $C_{2.5}H_{16}BrN_3O$: C, 66.09; H, 3.55; N, 9.25. Found: C, 66.25; H, 3.70; N, 9.40. MS (ESI) m/z = 454 and 456 (MH^+). HRMS calcd for $C_{2.5}H_{17}N_3O^{79}Br$ (MH^+): 454.0550; found, 454.0552.

4.2 Biology

4.2.1 Cell lines and culture conditions. Three human breast cancer cell lines Hs578t (triple-negative), MDA-MB-231 (triple-negative), and MCF-7 (luminal-A), and a non-neoplastic breast cell line MCF-10A, were obtained from American Type Culture Collection (ATCC). The Hs578t, MDA-MB-231 and MCF-7 breast cancer cell lines were cultured in Dulbecco's modified Eagle medium, 4.5 g L^{-1} glucose (DMEM, Gibco), supplemented with 10% heating activated fetal bovine serum (FBS, Gibco) and 1% antibiotic solution (penicillin–streptomycin, Gibco). The non-neoplastic breast cell line MCF-10A was cultured in Dulbecco's modified Eagle medium with nutrient mixture F-12 (DMEM/F-12, Gibco). This medium was supplemented with 5% heat-inactivated fetal bovine serum (FBS, Gibco), 1% penicillin–streptomycin (Gibco), 1% steroid hormone (hydrocortisone, Sigma-Aldrich), 0.1% peptide hormone (insulin, Sigma-Aldrich) and 0.01% protein complex (cholera toxin, Sigma). Cells were grown in a humidified incubator at 37 °C and 5% CO_2. Dimethyl sulfoxide (DMSO, Sigma-Aldrich) was used as control for all assays (vehicle). For all experiments, sub-confluent cells were used. Cells were first rinsed with phosphate-buffer saline (PBS 1×) and then detached from the flasks using trypsin (TrypLE™ Express, Gibco) at 37 °C. The culture medium was added to the flasks to inactivate trypsin and cells collected and centrifuged during 5 minutes at 900 rpm, at 4 °C. Afterward, cells were resuspended in fresh medium and 10 μL of cell suspension collected into an Eppendorf, in which 20 μL of trypan blue (Trypan Blue Solution, 0.4%, Gibco) was added to count the cells in a Neubauer chamber, for subsequent density calculation.

4.2.2 Cell viability assay. For the initial cell viability screening studies and IC_{50} values determination of the compounds in Hs578t, MDA-MB-231, MCF-7 and MCF-10A cell lines, the cells were plated in 96-well plates, with a density of 3000 cells per well (100 μL). Cells were allowed to adhere for 18 to 20 h (overnight) and where then exposed to the compounds at different concentrations, for a total of 72 h. Controls were performed using DMSO (0.3%). Then, sulforhodamine B (SRB, TOX-6 Sigma-Aldrich) was used to evaluate the effect of the studied compounds on cell viability. Spectrophotometric measurement was performed after SRB treatment and the absorbance was read at 490 nm, using 690 nm as background absorbance (Tecan Infinite M200). The software GraphPad Prism 6 was used to calculate the compounds' IC_{50} values, from at least three independent experiments, each in triplicate, applying a sigmoidal dose–response (variable slope) non-linear regression, after logarithmic transformation.

Selectivity index (SI): the SI value was calculated using the IC_{50} values of the compounds for the cell lines, using the following mathematical formula: SI = (IC_{50} MCF-10A cell line − IC_{50} cancer cell line)/IC_{50} cancer cell line; for SI values >1, cytotoxicity for cancer cell line is higher than for non-neoplastic cell line.

4.2.3 Proliferation assay. Hs578t cells were seeded in 96-well plates at a density of 8000 cells/100 μL per well and grown overnight at 37 °C in a 5% CO_2 humidified atmosphere. Adherent cells were treated with compounds **4.25** and **8** at IC_{50} and $\frac{1}{2}IC_{50}$ concentrations and controls were treated with 0.5% DMSO (vehicle), for 24 and 48 h. After the incubation period, cells were labeled with 5 μL per well of BrdU labeling solution (final concentration of 20 μM) and reincubated for 6 h, enabling BrdU to replace thymidine during DNA synthesis. Then, cells were fixed, and DNA was denatured by incubation with 200 μL of FixDenat solution for 30 minutes at room temperature. After removal of this solution, 100 μL of anti-bromodeoxyuridine-peroxidase (anti-BrdU-POD) diluted in antibody solution (1 : 100) were added and incubated for 90 minutes at room temperature, allowing the antibody conjugate to bind to the recently incorporated BrdU in the cell DNA. Then, the wells were rinsed three times with 200 μL of PBS 1×, followed by addition of 100 μL of substrate solution (tetramethyl-benzidine). This solution allows the detection of the immune complexes, acting until color development (5 minutes) at room temperature. The colorimetric reaction was stopped by adding 25 μL per well of 1 M H_2SO_4. The reaction product was quantified by measuring the absorbance at 450 nm in a microplate reader (Tecan Infinite M200). A blank control was used in each experimental time point, containing only 100 μL of culture medium, 10 μL of BrdU and 100 μL of anti-BrdU-POD. At least three independent experiments were performed, in triplicate, and the results were analyzed using the GraphPad Prism 6 software.

4.2.4 Annexin/PI cell death assay. Hs578t cells (8.0×10^4–2.0×10^5 cells per well) were seeded in 6-well plates and

incubated overnight at 37 °C in 5% CO_2 humidified atmosphere. Cells were treated with IC_{50} concentration of compounds **4.25** and **8** or 0.5% DMSO (control) for 24 and 48 h. Triplicates were performed in each experiment. Both adherent and floating cells were collected and centrifuged at 1000 rpm, 5 minutes at 4 °C. After supernatant removal, 300 μL of binding buffer were added. 8 μL of fluorescein isothiocyanate (FITC) annexin V (BD Pharmingen) and 30 μL of PI (50 μg mL^{-1}, P1304.13P, Invitrogen), were added to this solution. Samples were incubated for 15 minutes at room temperature, in the dark. PI signal was measured using a FACS LSRII flow cytometer (BD Biosciences®) with a 488 nm excitation laser. The annexin V signal was collected through a 488 nm blocking filter, a 550 nm ion-pass dichroic with a 525 nm band pass. Signals were captured and FACS Diva was used as the acquisition software. The percentage of cells in each phase was analyzed using the FlowJo™ 10 (Tree Star®) software. At least three independent experiments were performed.

4.2.5 Protein extraction and Western blot. Hs578t cells were grown overnight (18–20 h) in 6-well plates in a humidified incubator at 37 °C and 5% CO_2. Then, cells were treated with the respective compounds **4.13**, **4.29**, **4.25** and **8** at their IC_{50} value concentration or 0.3% DMSO (controls) for 24 h and 48 h. After the treatment period, cells were washed with PBS 1× and treated with lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 10 mM NaF, 1% NP-40, 1% Triton-X100 and 1/7 protease inhibitor cocktail (Roche Applied Sciences)). Cells were collected by scraping and incubated for 15 minutes on ice and then centrifuged at 13 000 rpm for 15 minutes at 4 °C. The supernatant was collected for protein determination using the Bradford method.

Thirty μg of total protein of each sample were separated on a 12% polyacrylamide gel (100 V for 90 minutes) and transferred to a nitrocellulose membrane (100 V for 30 minutes). Membranes were blocked with 5% milk in Tris-buffered saline (TBS) 1× for 1 h before overnight incubation with primary antibodies at 4 °C (Table S2†). After washing for 5 minutes (twice) and a further 15 minutes (once) with TBS 1× 0.1% Tween 20, blots were incubated for 1 h with the respective secondary antibodies, at room temperature (Table S2†). Washing with TBS/0.1% Tween 20 solution was performed as before and immunoreactive bands were detected with chemiluminescent WesternBright™ Sirius (Advansta, USA) on Sapphire Biomolecular Imager (Azure Biosystems).

4.2.6 Cell cycle analysis. Hs578t cells were seeded in 6-well plates 8.0×10^4–2.0×10^5 cells/2 μL per well and incubated overnight at 37 °C in 5% CO_2 humidified atmosphere. Then, cells were treated with compounds **4.25** and **8**, at the respective IC_{50} concentration or 0.5% DMSO (controls) for 24 and 48 h. The culture medium and PBS 1× (500 mL), used to wash the wells, were collected to the respective tubes and 300 μL Accutase® (Grisp) solution was added to the wells for 10–15 minutes at room temperature. After centrifugation at 1000 rpm for 5 minutes, ethanol solution (70% v/v) was added to fix the cells for 30 minutes at 4 °C. PBS 1× was used twice to rinse the ethanol followed by centrifugation (1200 rpm, 6 minutes, 4 °C) and removal of the supernatant. Fixed cells were labeled with a solution containing PBS 10× (100 μL mL^{-1}), PI (50 μg mL^{-1}, Invitrogen), RNase A (20 mg mL^{-1}, Invitrogen), Triton-X100 (1 μL mL^{-1}) in deionized water. Samples were incubated for 1 h in the dark at 50 °C. PI signal was measured using a FACS LSRII flow cytometer (BD Biosciences®) with a 488 nm excitation laser, captured and FACS Diva was used as the acquisition software. The percentage of cells in each phase was analyzed using the FlowJo™ 10 (Tree Star®) software. At least three independent experiments were performed.

4.2.7 *Caenorhabditis elegans* (*C. elegans* strains), maintenance, and toxicity assay. *C. elegans* wild-type strain N2 (Bristol strain) was cultured in nematode growth medium (NGM) plates seeded with *Escherichia coli* OP_{50} strain, at 20 °C. The *in vivo* toxicity of the compounds was determined using the food clearance assay and performed as previously described.[50,51] Adult animals were bleached with 20% alkaline hypochlorite to obtain a population of eggs. In 96-well plates, eggs were plated in the presence of inactivated OP_{50} (inactivation performed by 3 cycles of freezing in liquid nitrogen followed by water bath at 37 °C; optical density (OD 595 nm) adjusted for 0.7) and with different concentrations of each compound (from 50 μM to 0.0001 μM). Plates were maintained at 20 °C with agitation and the consumption of food (OD 595 nm) was measured daily for 7 days. DMSO was used as drug vehicle, at 1% (final concentration) and each concentration of the compounds was tested in quintuple with 25–30 animals per well. Animals treated with 1% and 5% DMSO were used as non-toxic and toxic concentration controls, respectively. GraphPad Prism 7 was used for non-linear regression analysis of $\log IC_{50}$ and HillSlope parameters in sigmoid curves of the following conditions: DMSO 1% and compound concentrations tested.

4.2.8 Chick chorioallantoic membrane (CAM) assay. This assay was performed to test the *ex vivo* efficacy of compounds **4.25** and **8**. For this purpose, eggs were incubated at 37 °C (day 0). On the 3rd day of embryo development, a small window was open in the eggshell to detach the CAM membrane and the eggs were incubated at 37 °C. On the 9th day of embryo development, 2.0×10^6 Hs578t breast cancer cells were mixed with 10 μL of Matrigel and grafted on top of the CAM, and again incubated at 37 °C. On the 13th day of embryo development, the shell windows were opened again, and the formed tumors photographed *in ovo* and 20 μL of the IC_{50} solutions of each compound were added over the tumor and the window closed again. On the 17th day of development, the eggs were opened, and the tumors were photographed *in ovo*. Then, embryos were sacrificed at −80 °C for 20 minutes and the CAM was removed from the egg and photographed *ex ovo*. The images were obtained using a stereomicroscope (Olympus S2 × 16) and a digital camera (Olympus DP71) and the tumor perimeters were determined using ImageJ.

4.2.9 Toxicity evaluation in mice

Mouse maintenance. Wild-type male mice (n = 5/group, C57BL6/J background) with ages between 2 and 4 months of age were used to evaluate the toxicity of the chromene **8**, the most promising chromene based on the CAM assay. Vehicle- and drug-treated animals were alternately assigned and housed in groups of 5 animals in filter-topped polysulfone cages 267 × 207 × 140 mm (370 cm^2 floor area) (Tecniplast, Buguggiate, Italy), with corncob bedding (Scobis Due, Mucedola SRL, Settimo Milanese, Italy) in a conventional animal facility. All animals were maintained under standard laboratory conditions: 12 h artificial light/dark cycle (lights on from 8 a.m. to 8 p.m.), ambient temperature of 21 ± 1 °C and relative humidity of 50–60%. Mice were given a standard diet (4RF25 during gestation and postnatal days and 4FR21 after weaning) (Mucedola SRL, Settimo Milanese, Italy) and water *ad libitum*. All procedures were conducted in accordance with European regulations (European Union Directive 86/609/EEC). Animal facilities and the people directly involved in animal experiments (S.G.) were certified by the Portuguese regulatory entity – Direção-Geral da Alimentação e Veterinária (DGAV). All the protocols performed were approved by the joint Animal Ethics Committee of the Life and Health Sciences Research Institute, University of Minho. Health monitoring was performed according to FELASA guidelines,[52] confirming the specified pathogen free (SPF) health status of sentinel animals maintained in the same animal room. Humane endpoints for experiment were defined: 20% reduction of body weight, inability to reach food or water, presence of wounds in the body, severe dehydration.

Drug administration and animal welfare assessment. Animals were treated with one of the two dosages (10 and 50 mg kg^{-1}) of chromene **8** daily, through intraperitoneal (i.p.) injection, for 7 days. Control littermates were given a vehicle injection (90% saline, 5% dimethyl sulfoxide (DMSO) and 5% Tween-80) with the same frequency. Specific amounts of food (300 g) and water (250 mL) were added in each cage in the beginning of the experiment and the remaining quantity measured at the end, to monitor water and food intake throughout the experiment.

Mice body weight and welfare were assessed every day, starting before treatment initiation, while colonic temperature of the animals was measured before the first injection and at the end of the treatment. Also, the animals were recorded before the first injection and 20 minutes after, to clarify if there was any impact in animals' behavior. To evaluate mouse welfare, we used an already established protocol for phenotypic assessment based on the SHIRPA protocol. This protocol mimics the diagnostic process of general psychiatric evaluation in humans.[4,6] Briefly, each animal was placed in a transparent viewing jar (15 cm diameter) for 3 minutes to assess vertical activity (number of rears), body position, eyelid opening, fur quality, grooming, respiratory rate, tremors, and piloerection. Then, they were transferred to a square arena (55 × 33 × 18 cm) labelled with 15 squares, for 1 minute, to evaluate horizontal activity (number of squares travelled), reaction to transfer (time the animal takes to start walking after being tossed to the arena) and Straub tail reaction.[53] In the end, eye reflexes (in response to the approximation of a pen to the eye), clasping (extension/contraction of the hindlimbs when picked by the tail) and hydration status (skin picking) were also assessed. Throughout all these tests, spontaneous activity (activity status) was evaluated and the number of bolus fecalis counted.

4.2.10 Statistical analysis. All graphs and statistical analysis were performed with the GraphPad Prism 6 software. Statistical significance was assessed by the t-test and results are presented as normalized means ± SEM. The results of viability studies and CAM assays are expressed as means ± SD and were analysed using one-way ANOVA with GraphPad Prism 6 software and SPSS 22.0 (IBM Corp., Armonk, New York). Differences between groups were considered significant for $*p < 0.05$, $**p < 0.01$, $***p < 0.005$ and $****p < 0.001$. All assays were performed in triplicate.

Author contributions

For the Chemistry section, FPL: design, methodology, data collection and analysis, original draft – writing and editing; FP: conceptualization, supervision, data analysis, writing and reviewing. For the Biology section: LC, MCerqueira, AS, MCosta: design, methodology, data collection and analysis, original draft writing, reviewing and editing; OP, SOP, SGranja: data collection and analysis; SGuerreiro, MDC, PM: methodology, investigation, data analysis, and writing and reviewing of the *in vivo* toxicity experiments; ALF, FB, MCosta: conceptualization, supervision, writing and reviewing. All authors have read and agreed to the published version of the manuscript. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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