Research paper

Antiproliferative and pro-apoptotic activities of 2′- and 4′-aminochalcones against tumor canine cells

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ABSTRACT

In the present study, a series of 2′- and 4′-aminochalcones were synthesized and their antiproliferative activity against a canine malignant histiocytic cell line (DH82) was evaluated. Particularly amino-chalcones with a hydrophobic substituent on ring B proved to be potent antiproliferative agents. Among these compounds, aminochalcones **3**, **4** and **11** inhibited the growth of DH82 cells, with IC_{50} values of 34.4, 31.4 and 38.2 μM, respectively, and were three times more potent than etoposide (IC_{50} = 95.5 μM). The selected chalcones induced death through apoptosis rather than necrosis in DH82 and non-tumorigenic Madin-Darby canine kidney cells (MDCK). Further experiments suggested that the amino-chalcones interfere with the regulation of oncogenes/tumor suppressor genes. Aminochalcone **11** inhibited transcription of the *TOPOIIα* and TP53 genes and aminochalcone **4** down-regulated Sp1 protein expression in a concentration-dependent manner.

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1. Introduction

Cancer is an important disease in the canine population, but few wide-ranging epidemiological studies on the incidence of different types of cancer in pet dogs and its variations between breeds are available [1]. Among canine cancers, malignant histiocytosis (MH), also known as histiocytic sarcoma, is a fast-growing tumor that arises from myeloid cells, including dendritic cells and monocytes [2]. MH has a poor prognosis because of its high mortality, with survival ranging from 2 to 4 months, and the development of widespread metastasis to the spleen, lymph nodes, bone marrow, lungs and liver [3,4]. Veterinary therapeutic regimens for MH consist of combinations of DNA cross-linking alkylators (carboplatin and lomustine), doxorubicin and prednisone, which are often fatal to dogs. In cases of survival, tumor resistance to chemotherapy has been described [5,6]. Altogether, there is a perceived need to discover and develop substances with completely different molecular scaffolds from those of well-known *anti*-MH agents.

Chalcones are privileged scaffolds in Medicinal Chemistry that possess a simple typical skeleton, bearing two phenyls (rings A and B) spaced by a *trans*-enone bridge [7,8]. Interestingly, both synthetic and plant-derived chalcones exhibit potent activity against cancer cell growth and proliferation [9]. Mahapatra and co-authors [10] exhaustively revised the molecular targets of anticancer chal-cones, which include the inhibition of histone deacetylases, p53 degradation, and topoisomerase expression. Potent cytotoxic ef-fects have been reported for anticancer chalcones substituted by amino groups [11]. 2′-Aminochalcones with a methylenedioxy moiety exhibited high activity against pgP-expressing human nasopharyngeal epidermoid carcinoma (KB-VIN) [12]. Mai and co-authors [13] evaluated a range of chalcones with different electron-withdrawing and electron-donating substituents. The

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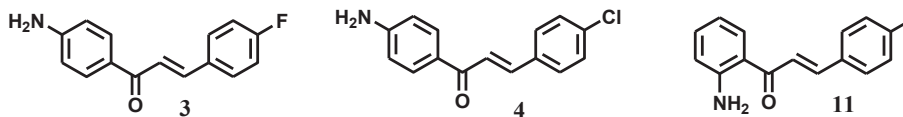[1] Both authors contributed equally to this work.

Fig. 1. Selected aminochalcones with potent antiproliferative activity.

authors observed potent and selective cytotoxic activity of 2′-aminochalcone bearing an unsubstituted B ring, as well as pro-apoptotic effects on 20 apoptotic markers. Few recent studies have evaluated the potential of compounds as cytotoxic agents against canine cancer cells. In a previous study from our group, we reported the potent activity of unsubstituted chalcone and 4′-methoxychalcone against a canine macrophage tumor cell line, which exhibited time-dependent and concentration-dependent cytotoxicity [14].

In our continuing search for new veterinary anticancer agents from natural product-based synthetic compounds, we synthesized a series of chalcones substituted by amino groups at positions 2′ and 4′. The cytotoxicity of these compounds was evaluated against canine MH cells (DH82 cell line) to derive preliminary structure-activity relationships. The three most active aminochalcones, **3**, **4** and **11** (Fig. 1), were investigated regarding their selective toxicity and apoptosis induction in DH82 cells compared to a non-tumorigenic canine cell line (Madin-Darby canine kidney cells, MDCK). In addition, their effects on the expression of Sp1 protein and on the transcription of the *TOPOIIα*, Sp1 and TP53 genes were determined.

2. Results and discussion

2.1. Chemistry

The synthesis of aminochalcones was achieved by Claisen-Schmidt condensation of 2′- or 4′-aminoacetophenones with the corresponding aryl aldehydes substituted with electron-withdrawing and electron-donating groups, resulting in moderate to high yields of 60−92% (Scheme 1).

We found that benzaldehyde derivatives substituted with electron-donating groups provided higher yields of the respective chalcone products than those substituted with electron-withdrawing substituents. Electron-withdrawing groups or atoms pose some barrier and are responsible for lower yields. Variations in chalcone yields due to the electronic effect of substituents has been reported by Bahekar and co-authors [15].

The chalcones presented amino group substitutions on ring A at positions 2′ and 4′, respectively. The major substituents on ring B were halogens (F, Cl and Br), electron-donating groups (OCH$_3$ and CH$_3$), and an electron-withdrawing group (NO$_2$). For 4′-amino-chalcones, phenyl ring B was replaced with furan, thiophene and naphthalene rings (Table 1).

The structure of the aminochalcones was characterized by ^{1}H NMR and ^{13}C NMR spectral data, which were obtained from a Bruker® Avance III (14.095 T; 600 MHz) and Bruker® Avance III (9.397 T; 400 MHz) spectrometers. Compounds were dissolved in deuterated chloroform or DMSO, and their non-deuterated residues were used as internal standard for establishment of chemical shift values. The most characteristic signals observed in the ^{1}H NMR spectrum were those of the olefinic double bond hydrogens, which were present as pairs of doublets with coupling constants ranging from 15.0 to 15.6 Hz, indicating its *trans* configuration. In the ^{13}C NMR spectrum, the most downfield signals with chemical shifts ranging from 185.7 to 191.1 ppm were attributed to the carbonyl group of the *trans*-enone bridge. For all amino-chalcone structures, the NMR parameters (chemical shifts, integrations, multiplicities and coupling constants) corresponded to the structures proposed. In addition low-resolution MS (positive mode) spectral analysis of chalcones were performed on ESI-IT Bruker® equipment. First, peak attributed to protonated molecule [M+H]$^+$ were observed as base peak and selected to MS/MS experiments, which exhibited typical fragments of chalcone cleavage, including acylium-type cations, constituted by ring A-ketone or ring B-enone, as well as tropylium-type cation. Detailed spectral analyses were shown in the supplementary material.

2.2. Biological activity

2.2.1. Cytotoxicity and selectivity of aminochalcones in canine cells

The cytotoxicity of all aminochalcones (concentration of 15 μg/mL) was evaluated for 24 h by the methyl thiazolyl tetrazolium (MTT) assay using canine malignant histiocytic cells (DH82) (Table 1). In a previous study from our group, the chalcone with unsubstituted rings A and B (**1**) exhibited cytotoxic activity against DH82 cells [14]. In the present study, we used compound **1** as a framework to investigate the relationship between substitutions on chalcone rings A and B and the toxic effect on DH82 cells. At 15.0 μg/mL, compound **1** inhibited DH82 cell growth by 27%.
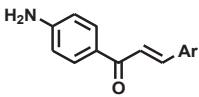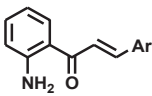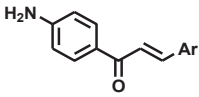
First, we compared the activities of **1** with 4′-aminochalcone (**2**), which suggested that the amino group improved cytotoxicity. This result indicates that the presence of an −NH$_2$ group on ring A is important for determining the anticancer potential. Amino group substitutions in the chalcone framework have been shown to play a central role in the anticancer activities of these compounds [15,16].

Second, comparisons were made to determine whether the presence of electron-withdrawing/donating substituents on ring B (−F, −Cl, −NO$_2$, −CH$_3$ and −OCH$_3$), in addition to an amino group at position 4′ (ring A), leads to differences in activity. The presence of halogens and a methyl group at the *para*-position of ring B, which are electron-withdrawing atoms and electron-donating groups, respectively, improved cytotoxicity. On the other hand, the chalcone with the methoxyl group substituent (**6**), a strong electron-donating substituent, exhibited reduced cytotoxicity compared to **2**. We therefore concluded that the electronic effect of *para* substituents on ring B is not essential for activity. However, halogenated chalcones **3** and **4**, as well as methyl chalcone **5**, are more
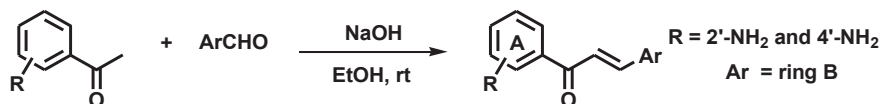
Scheme 1. Synthesis of 2′- and 4′-aminochalcones.

Table 1
In vitro effect of chalcones on DH82 cells.

Chalcone		Ar	Activity[a]
1	unsubstituted ring A	unsubstituted ring B	27.7 ± 7.3
2	H₂N—⟨ring⟩—C(=O)—CH=CH—Ar	phenyl	59.2 ± 4.2
3		4-fluorophenyl	88.0 ± 0.7
4		4-chlorophenyl	91.0 ± 1.3
5		4-methylphenyl	74.7 ± 1.7
6		4-methoxyphenyl	46.6 ± 1.0
7		4-nitrophenyl	52.4 ± 6.7
8	⟨ring⟩—C(=O)—CH=CH—Ar, NH₂	4-fluorophenyl	76.6 ± 0.1
9		4-chlorophenyl	83.2 ± 5.7
10		4-bromophenyl	87.0 ± 7.5
11		4-methylphenyl	88.9 ± 1.5
12	H₂N—⟨ring⟩—C(=O)—CH=CH—Ar	2-furyl	18.8 ± 4.0
13		2-thiophenyl	43.6 ± 3.7
14		1-naphthyl	40.3 ± 0.6
15		2-naphthyl	40.4 ± 3.7

[a] Results are expressed as the mean percentage of DH82 cell death ± SEM at 15.0 μg/mL after 24 h.

lipophilic and active than **2**, indicating a clear influence of hydrophobic substituents on ring B to increase bioactivity [17,18].

Third, two comparisons were made to establish whether the effect of hydrophobic substituents on ring B maintained bioactivity in the 2′-aminochalcone framework. Halogenated 2′-aminochalcones **8** and **9**, including *para*-bromochalcone **10**, exhibited cytotoxic activity similar to that of their halogenated 4′-aminochalcone analogues **3** and **4**. The comparison of cytotoxicity between methyl chalcones **5** and **11** suggests a slight increase in cytotoxicity for **11**. Taken together, the two comparisons suggested that the change of the amino group from position 4′ to 2′ in chalcones with a hydrophobic ring B does not reduce their antiproliferative activity.

Fourth, to evaluate hypothetical ring bioisosterism [17], comparisons were made to determine the effect of replacing benzene ring B in 4′-aminochalcones with heteroarylic rings with six π-electrons (2-furyl and 2-thiophenyl) and homoarylic rings with 10 π-electrons (1-naphthyl and 2-naphthyl). All ring analogues (**12**–**15**) were unable to inhibit DH82 cell growth when compared to **2**, indicating that π-electron density of ring B does not influence biological activity.

Aminochalcones **3**, **4** and **11** were selected for the subsequent cytotoxicity assays. Table 2 lists the IC$_{50}$ values of these aminochalcones against DH82 and MDCK cells, a non-tumorigenic cell line. Aminochalcones **3** (IC$_{50}$ = 34.3 μM), **4** (IC$_{50}$ = 31.4 μM) and **11** (IC$_{50}$ = 38.2 μM) were approximately three times more potent against DH82 cells than etoposide (IC$_{50}$ = 95.5 μM) [14], which was selected as the reference antitumor drug. Despite similar activity against DH82 cells, the selected aminochalcones exhibited different

Table 2
IC$_{50}$ values (μM) of aminochalcones in DH82 and MDCK cells and the corresponding selectivity ratios.

Aminochalcone	DH82 cells	MDCK cells	Selectivity ratio
3	34.4	70.1	2.0
4	31.4	57.2	1.8
11	38.2	23.3	0.6
Etoposide[a]	95.5	not tested	not calculated

[a] Reference antitumor drug [14].

effects on MDCK cells, with IC$_{50}$ values ranging from 23.3 to 70.1 μM. Therefore, the selectivity ratios (IC$_{50}$ MDCK/IC$_{50}$ DH82) indicated the following order of selectivity: **3** > **4** > **11**.

2.2.2. Aminochalcones induce cell death through apoptosis rather than necrosis in canine cells

Antineoplastic drugs induce tumor cell death through apoptosis or necrosis, with apoptosis being the preferred mechanism of cell death [19,20]. Apoptosis induction has been observed in different cancer cell lines treated with chalcones [21–23].

In order to understand the mode of cytotoxicity of the selected aminochalcones in DH82 and MDCK cells, annexin V/PI staining for flow cytometry was used to assess whether the cells were dying due to apoptosis or necrosis induction. Aminochalcones **3** and **11** induced 22.7% ± 5.8 and 25.9% ± 2.0 apoptotic events in DH82 cells, respectively, a 12- and 14-fold increase compared to the untreated negative control (1.8% ± 0.3 events). In MDCK cells, **3** induced 12.1% ± 1.7 apoptotic events and **11** induced 24.4% ± 2.7 apoptotic events. The highest induction of apoptosis in DH82 cells was observed for **4**, with 42.2% ± 6.5 apoptotic events or a 23-fold increase compared to the negative control. In MDCK cells, **4** also induced a high percentage of apoptotic events (23.1% ± 0.8). Finally, the selected aminochalcones induced lower levels of necrosis events in both cell lines compared to doxorubicin (positive control), which was used as the reference antineoplastic drug (Fig. 2).

2.2.3. Aminochalcone **11** inhibits mRNA expression of TOPOIIα and TP53 genes

Topoisomerase IIα is a key enzyme required for generating transient DNA double-stranded breaks, thus playing an important role in DNA replication, transcription, chromosome separation, and segregation [24]. The gene encoding topoisomerase IIα (*TOPOIIα*) is commonly altered at both gene copy number and gene expression level in cancer cells and is therefore a target for the development of
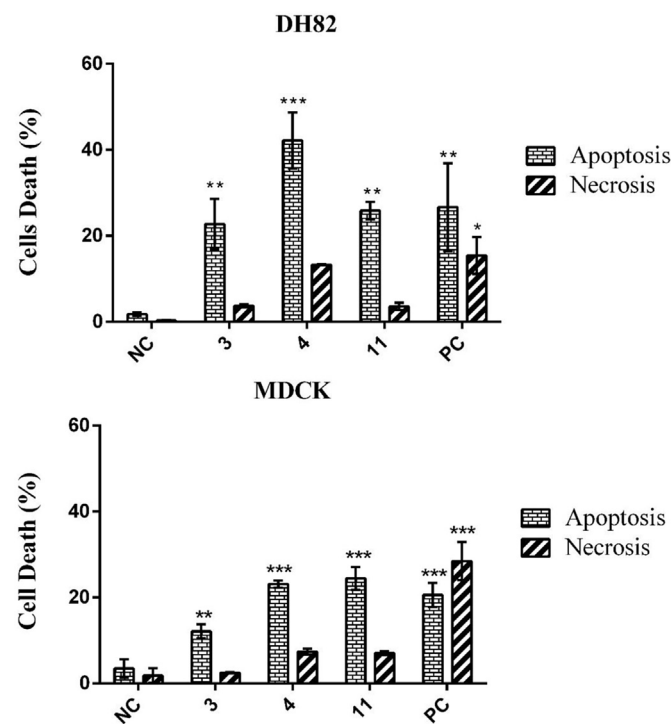

Fig. 2. Apoptosis induction by the selected aminochalcones in DH82 and MDCK cells. Statistically significant: *$p < 0.05$ **$p < 0.01$ and ***$p < 0.001$ vs. control. NC: negative control; PC: positive control.

anticancer drugs. The regulation of *TOPOIIα* expression involves transcription factors p53 and Sp1, which have binding sites in the promoter of the *TOPOIIα* gene. While p53 is a tumor suppressor protein that is frequently inactivated in a variety of human and canine cancers and is a negative regulator of *TOPOIIα* expression [25], Sp1 is often overexpressed in different types of cancer and acts mainly as a transcriptional activator of *TOPOIIα* expression [23]. The mechanism of action of important antineoplastic drugs such as doxorubicin and etoposide involves stabilization of the topoisomerase IIα-DNA cleavage complex, with a consequent increase in double stranded breaks that ultimately results in cell death [26]. *In vitro* topoisomerase II activity assays with aminochalcones **3**, **4** and **11** did not result in inhibition of the enzyme (data not shown). On the other hand, we found that **1** inhibits *TOPOIIα* mRNA transcription. This inhibition is probably related to the modulation of p53 and Sp1 by **1**, which reduces Sp1 protein expression and increases p53 expression [14,22].

Considering the important roles of p53, Sp1 and topoisomerase IIα in cell cycle progression and apoptosis [21,22,27] and the higher cytotoxic and pro-apoptotic effects of the aminochalcones compared to **1** in DH82 cells (Table 1), we used quantitative RT-PCR to investigate mRNA expression of the corresponding TP53, Sp1 and *TOPOIIα* genes in DH82 and MDCK cells treated with aminochalcones **3**, **4** and **11** for 24 h (Fig. 3).
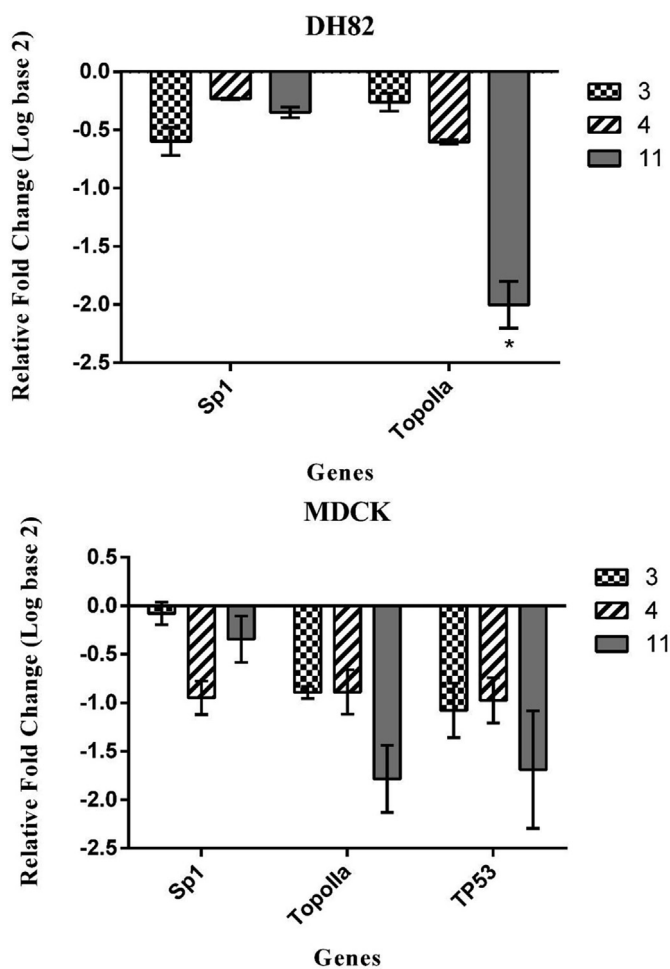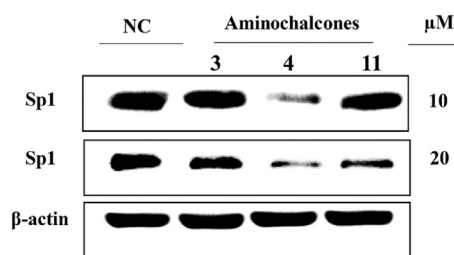

Fig. 4. Effect of the selected aminochalcones on the expression of Sp1 protein in DH82 cells.

2'-Aminochalcone **11** exerted an inhibitory effect on the expression of *TOPOIIα* in DH82 (fold change = −2.0 ± 0.2) and MDCK cells (fold change = −1.79 ± 0.3), while a lower inhibitory effect was observed for 4'-aminochalcones **3** and **4** (fold change < 1.0). The TP53 gene is deleted in DH82 [28], but the results obtained for the MDCK cell line also indicated an inhibitory effect of **11** on TP53 expression (fold change = −1.69 ± 0.6), while lower effects were observed for the 4'-aminochalcones (fold change < 1.0). The selected aminochalcones exerted no significant effect on the modulation of Sp1 gene expression in either cell line. These results indicate that the 2'-aminochalcone **11** alters *TOPOIIα* and TP53 mRNA expression, while weak effects on the expression of these genes were found for the 4'-aminochalcones **3** and **4**, suggesting that the position of the amino group on ring A influences the mode of action of these compounds.

2.2.4. Aminochalcone 4 down-regulates Sp1 protein expression

The transcriptional inhibition of the *TOPOIIα* gene in the absence of p53 in the DH82 cell line treated with the 2'-aminochalcone **11** suggests that other transcriptional factors are important for the regulation of *TOPOIIα*. Sp1 is a transcriptional activator of *TOPOIIα*, but the quantitative RT-PCR experiments did not indicate inhibition of this gene at the transcriptional level. We used Western blot analysis to determine whether aminochalcones **3**, **4** and **11** exerted an inhibitory effect on the expression of the corresponding Sp1 protein, which could contribute to the inhibition of *TOPOIIα*. After treatment with 10 and 20 μM of the selected aminochalcones for 24 h, Sp1 was down-regulated by aminochalcone **4** in a concentration-dependent manner, while **3** and **11** exhibited a significant effect only at 20 μM. Thus, compound **11** reduced the levels of *TOPOIIα* mRNA and of Sp1 protein in DH82 cells (Fig. 4).

3. Conclusion

A series of chalcones containing amino groups on ring A and electron-withdrawing and electron-donating substituents on ring B were synthesized as a continuation of our ongoing veterinary anticancer research project. Among the most active compounds, aminochalcones with hydrophobic substituents on ring B (**3**, **4** and **11**) were more potent than etoposide. The death of DH82 cells caused by the selected aminochalcones was mainly due to apoptosis rather than necrosis when compared to doxorubicin. In addition, preliminary assays on the molecular targets of aminochalcones **3**, **4** and **11** were carried out. Aminochalcone **11** inhibited transcription of the *TOPOIIα* and TP53 genes and aminochalcone **4** down-regulated Sp1 protein expression. Taken together, our results corroborate the anticancer potential of chalcones as a privileged framework, which possess completely different molecular targets from classical chemotherapeutic agents.

Fig. 3. Effect of the selected aminochalcones on the expression of *TOPOIIα*, Sp1 and TP53 genes in DH82 and MDCK cells. Data are reported as the mean ± SD of three independent experiments. (*) Statistically significant at *p* < 0.01 between treatments.

4. Experimental

4.1. Chemistry

Fifteen chalcone derivatives (**1**–**15**) were synthesized by Claisen-Schmidt aldol condensation using protocols reported in previous studies from our group [29–32].

4.2. Biological activity

4.2.1. Cell lines and culture

The canine cell lines DH82 (ATCC CRL-10389) and MDCK (ATCC CCL-32) were obtained from the Rio de Janeiro Cell Bank (BCRJ, Federal University of Rio de Janeiro, Brazil). The cells were cultured in DMEM (HyClone™) supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL penicillin-streptomycin (Sigma-Aldrich®) in a humidified incubator at 37 °C under 5% CO_2.

4.2.2. Cytotoxic activity assay

Cytotoxicity of the compounds against DH82 and MDCK cells was evaluated by the MTT assay [14]. Cells were seeded in 96-well plates (2×10^4 cells/well) containing medium and the compounds at 15 μg/mL and were cultured for 24 h at 37 °C in a 5% CO_2 atmosphere. For the most potent compounds (**3**, **4** and **11**), this assay was performed using different concentrations ranging from 0 to 60 μM for DH82 and from 0 to 160 μM for MDCK and the IC_{50} values were determined for both cell lines. Etoposide was used as the reference antineoplastic drug.

4.2.3. Pro-apoptotic activity assay

Cells were seeded in 6-well plates (2×10^5 cells/well), cultured for 24 h, and treated with 40 μM of the compounds in medium containing 10% FBS for an additional 24 h. Adherent and floating cells were collected together. Apoptotic cells were determined by flow cytometry in a FACSCanto II Flow Cytometer (BD) after double staining (annexin V/propidium iodide) using the FITC Annexin V Apoptosis Detection kit I (BD Pharmigen™) according to manufacturer instructions. Doxorubicin (2.0 μM) was used as positive control.

4.2.4. Inhibition assay of TOPOIIα, Sp1 and gene expression

DH82 and MDCK cells cultured in 25^6 cm^2 flasks (1×10^6 cells) were treated for 24 h in serum-free medium containing 40 μM of aminochalcones **3**, **4** and **11** or 0.5% DMSO as negative control. Total RNA was isolated using the Illustra RNAspin Mini RNA Isolation kit (GE Healthcare). RNA concentration and purity were measured with a NanoPhotometer™ P360 (Implen). After DNase I (Sigma-Aldrich) treatment, 1 μg of total RNA was used for reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer instructions. The TaqMan® Gene Expression Assays (Applied Biosystems) were used for gene expression analysis. The reactions were carried out separately for each gene in a final volume of 20 μL containing 1.0 μL 20X TaqMan® Gene Expression Assay of each gene, 10 μL 2X TaqMan® Gene Expression Master Mix, 1 μL cDNA, and 8 μL RNase-free water. All reactions were run in triplicate and reagent contamination was verified by replacing sterile water for cDNA. The reactions were incubated in a Mx3005P real-time thermocycler (Stratagene, La Jolla, CA, USA) using the following cycling parameters: 1 denaturation cycle at 95 °C for 10 min, followed by 40 cycles of one denaturation step at 95 °C for 15 s and one single step for annealing and extension at 60 °C for 1 min. Threshold cycle (CT) results were subsequently normalized to RPL32 (reference gene). Data are reported as fold change using the comparative CT method, $2^{-\Delta\Delta CT}$ of the average and standard deviation of three independent experiments in which the test (aminochalcone-treated cells) was calibrated against the control cell level (0.5% DMSO-treated cells).

4.2.5. Inhibition assay of Sp1 protein expression

After treatment with the selected aminochalcones at 10 and 20 μM or with the negative control (0.5% DMSO) for 24 h, the cells were washed with PBS and lysed by sonication ($3 \times$ for 5 s each) in RIPA buffer (Sigma-Aldrich®). The cell lysate was centrifuged at 13,000 *g* for 20 min at 48 °C and total protein concentration in the supernatant was determined using Pierce BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL). An aliquot of total proteins (30 μg) were subjected to electrophoresis on 12% SDS-PAGE gel for 90 min (100 V, 360 mA) and then transferred to nitrocellulose membrane (Pall Corporation, Pensacola, FL). The membranes were blocked with TBST buffer (25 mM Tris, 3 mM KCl, 0.14 M NaCl, 0.05% Tween 20) containing 5% nonfat milk at room temperature for 1 h. The membranes were then incubated with the primary Sp1 and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBST-5% nonfat milk buffer (1:1000) overnight at 48 °C. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and washed with TBST. Proteins were visualized by chemiluminescence detection using the ECL Western Blotting Detection Reagent (Amersham Biosciences, Piscataway, NJ) in an LAS4000 luminescence analyzer (Fujifilm Medical Systems, Stanford, CT).

4.2.6. Statistical analysis

All data are reported as the mean ± standard deviation of a minimum of three independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Tukey test using the GraphPad software (GraphPad, San Diego, CA). The results were considered statistically significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2017.06.049.

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