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4′-Methoxy-2-styrylchromone a novel microtubule-stabilizing antimitotic agent

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ABSTRACT

4′-Methoxy-2-styrylchromone, a new synthetic chromone was identified as a selective proliferation inhibitor of human tumor (MCF-7 and NCI-H460) cell lines than to non-tumor cells (MRC-5). The antiproliferative activity of this chromone was also extensive to peripheral human lymphocytes. 4′-Methoxy-2-styrylchromone was found to block tumor cells in the G_2/M phase of the cell cycle. The G_2/M arrest of NCI-H460 cells was dose- and time-dependent, reaching a maximum after 12-h treatment while MCF-7 cells reached the maximum value of G_2/M accumulation only after a 24-h treatment. Chromone-treated cells evidenced a high frequency of cells in prometaphase, indicating progression beyond G_2 and arrest early in mitosis. This mitotic arrest was associated with abnormal mitotic spindles characterized by the formation of a monopolar structure, suggesting that the chromone interferes with microtubules. The results of an *in vitro* tubulin polymerization assay showed that this chromone stabilizes microtubules in a manner similar to paclitaxel.

1. Introduction

Antimitotic drugs are a major group of antitumoral agents that act either by preventing microtubule assembly, such as colchicine, nocodazole and the vinca alkaloids, or by preventing the depolymerization of tubulin such as taxanes and epothilone. The strategy of using tubulin as a target for cancer chemotherapy is based on the increased growth and division of cancer cells as well as on the death, by apoptosis, of cells blocked at mitosis [1]. These kinds of drugs induce growth arrest of proliferating cells but stationary phase cells are generally resistant. The importance of microtubules in mitosis and cell division, as well as the clinical success of microtubule-targeting drugs has made these highly dynamic structures to be considered as one of the most attractive targets for anticancer therapy [1,2].

Although all these agents can efficiently block cell cycle progression, only few have been used clinically. Despite the announced success many tubulin-targeting drugs discovered so far still have a long development phase before they can be

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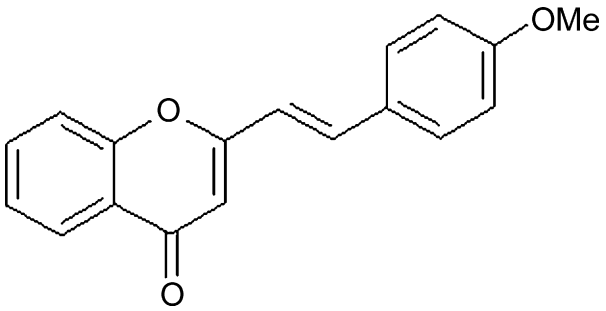

Fig. 1 – Chemical structure of 4′-methoxy-2-styrylchromone.

use in the clinic. The fact that microtubules are essential for a variety of cellular processes, the development of resistance to tubulin-targeting drugs and their toxicity has limited their efficacy across a range of cancers [3–9]. Therefore, much effort has been put into the discovery/development of new anti-mitotic drugs that more specifically target cells in mitosis. Thus, the development of more potent and selective anti-mitotic drugs is greatly needed, especially for the treatment of human cancers resistant to currently used drugs.

Recently, we have identified 4′-methoxy-2-styrylchromone (Fig. 1), a new synthetic chromone, as a potent growth inhibitor of human tumor cell lines that interferes with microtubules.

The aims of the present study were to investigate if 4′-methoxy-2-styrylchromone could be: (i) equally potent to non-tumor human cell lines; (ii) able to inhibit the proliferation of normal human peripheral lymphocytes; (iii) able to affect in a similar way confluent and non-confluent (exponential growing) cells. Another goal was to analyze how this chromone affects microtubules and microfilament network of cells and explore how it affects the polymerization of tubulin *in vitro*.

2. Material and methods

2.1. Sample

4′-Methoxy-2-styrylchromone was synthesized as previously described [10]. A stock solution of the compound was prepared in DMSO and kept at −20 °C and appropriate dilutions were freshly prepared just prior to every assay.

2.2. Cell cultures

Two human tumor cell lines, MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer), and one human non-tumor cell line, MRC-5 (diploid embryonic lung fibroblast) were used. They were grown as monolayer and routinely maintained in RPMI-1640 medium supplemented with 5% or 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL) (all from Gibco Invitrogen, Scotland, UK), at 37 °C in an humidified atmosphere containing 5% CO_2. NCI-H460 cell line was provided by the National Cancer Institute (NCI, Bethesda, USA), MCF-7 and MRC-5 cell lines were obtained from European Collection of Cell Cultures (ECACC, Salisbury, UK). Exponentially growing cells were obtained by plating 1.5×10^5 MCF-7 cells/mL and 0.75×10^5 NCI-H460 cells/mL followed by 24-h incubation. The effect of the vehicle solvent (DMSO) on the growth of cell lines was evaluated in all experiments by exposing untreated control cells to the maximum concentration of DMSO used in the different assays (0.5%).

2.3. Cell growth assay

The effect of 4′-methoxy-2-styrylchromone on the growth of non-tumor cell line MRC-5 was evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) in the ''*In vitro* Anticancer Drug Discovery Screen'' that use the protein-binding dye sulforhodamine B (Sigma–Aldrich, Saint Louis, USA) to assess cell growth [11]. Briefly, exponentially growing cells in 96-well plates were exposed for 48 h to five serial concentrations of chromone (2.2, 6.8, 20, 60 and 180 μM). Following this incubation period adherent cells were fixed *in situ*, washed and stained with SRB. The bound stain was solubilized and absorbance was measured at 492 nm in a microplate reader (Bio-tek Instruments Inc., Powerwave XS, Winooski, USA). For each cell line a dose–response curve was obtained and the growth inhibition of 50% (GI_{50}), corresponding to the concentration of chromone that inhibited 50% of the net cell growth was calculated as described elsewhere [12].

2.4. Lymphocytes proliferation assay

The effect of 4′-methoxy-2-styrylchromone on the phytohemagglutinin-induced proliferation of human lymphocytes was evaluated using a modified version of the colorimetric MTT-assay [13], which was previously described by our group [14]. Human mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers by density centrifugation using Histopaque-1077 (Sigma–Aldrich) and adjusted to $(2–3) \times 10^6$ cells/mL in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine and 50 μg/mL of gentamicin. Mononuclear cells in 96-well plates were exposed for 4 days to seven serial concentrations of chromone (9.4, 18.8, 37.5, 75, 150, 300 and 600 μM). The concentration of chromone that inhibited 50% of lymphocyte proliferation (IC_{50}) was calculated.

2.5. Cell cycle analysis

Exponential MCF-7 and NCI-H460 cells growing in 25 cm^2 flasks were treated with different concentrations of 4′-methoxy-2-styrylchromone for 12, 24 and 48 h.

MRC-5 cells were treated with 100 μM 4′-methoxy-2-styrylchromone or 0.66 μM nocodazole for 12, 24 and 48 h. After treatment, attached cells were released by trypsinization and mixed with non-adherent cells. Cells were centrifuged, washed twice with PBS and fixed with 70% ice-cold ethanol. Fixed cells were resuspended in a DNA staining solution containing 50 μg/mL propidium iodide (Sigma–Aldrich), 0.5 mg/mL RNAse (Promega, Madison WI, USA) in 10 mM Tris and 5 mM $MgCl_2$. DNA cellular content was analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain

View, CA) with excitation at 488 nm. Data were acquired in a listmode data file, gated to 30,000 events in cell cycle, using the CellQuest Pro software, version 4.0.2 (Becton Dickinson) included in the system. Cell cycle was analyzed using the FlowJo 7.2.2 software (Tree Star Inc., Ashland, OR).

2.6. Analysis of 4′-methoxy-2-styrylchromone effect on confluent and non-confluent cells

Non-confluent (exponential growing) cells were obtained by plating 1.5×10^5 MCF-7 cells/mL and 0.75×10^5 NCI-H460 cells/mL followed by 24 h incubation. Confluent (non-proliferating) cells were obtained by plating the same density of cells followed by 72 h incubation. After this period the culture medium was replaced by RPMI-1640 supplemented with 0.5% heat-inactivated FBS. The cells were incubated for more 8 days. Confluent and non-confluent (exponential) MCF-7 and NCI-H460 cells, growing in 96-well microplates, were exposed to serial concentrations (2.2, 6.8, 20, 60 and 180 μM) of chromone for 48 h. Following this incubation period cell growth was assessed either by SRB or by [^{3}H] thymidine incorporation. By SRB assay cells were fixed *in situ*, washed and stained with this protein-binding dye as described, previously. The bound stain was solubilized and absorbance was measured at 492 nm in a microplate reader (Bio-tek Instruments, Inc., PowerWave X).

By [^{3}H] thymidine incorporation assay cells were treated with 1 μCi of [^{3}H] thymidine (Amersham, Illinois, EUA) and further incubated for more 4 h. Pulsed cells were then harvested on a glass filter 102 mm × 256 mm (Skatron, Norway) using a semiautomatic cell harvester (Skatron Instruments, Norway) and allowed to dry. Incorporation of [^{3}H] thymidine was determined in a scintillation counter LS 6500 (Beckman Instruments, CA, USA).

2.7. Determination of mitotic index

Exponential MCF-7 and NCI-H460 cells growing in coverglasses were exposed to 100 μM 4′-methoxy-2-styrylchromone or 0.66 μM nocodazole for 24 h or 12 h, respectively. After treatment, coverglasses were fixed with paraformaldehyde (4%) washed and mounted in slides using Vectashield containing DAPI (Vector Laboratories, UK). Slides were observed in a fluorescence microscope (Eclipse E400, Nikon, Japan). Images were processed with Adobe Photoshop CS (Adobe Microsystems, CA). For each sample a minimum of 500 cells from at least five different random areas of the slide were counted and mitotic cells were scored. All the different stages of mitosis (prophase, metaphase, anaphase, telophase) were considered.

2.8. Immunofluorescence staining of tubulin and actin

Exponential MCF-7 and NCI-H460 cells growing in coverglasses were exposed to 100 μM 4′-methoxy-2-styrylchromone for 24 h or 12 h, respectively. For tubulin detection, cells were fixed with 3.7% paraformaldehyde in PHEM buffer (25 mM Hepes, 50 mM Pipes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) for 10 min at room temperature, and permeabilized with PHEM containing 0.5% Triton X-100. Immunodetection of microtubules was performed using a mouse monoclonal antibody against α-tubulin, clone B-5-12 (Sigma–Aldrich), diluted 1:5000, and a secondary goat anti-mouse Alexa Fluor® 488 (Molecular Probes, Leiden, Holland) diluted 1:2000. Coverglasses were mounted in slides using Vectashield containing DAPI, and observed in a fluorescence microscope (Imager Z1, Carl Zeiss, Germany). Data stacks were deconvolved, using the Huygens Essential version 3.0.2p1 (Scientific Volume Imaging B.V., The Netherlands) and Images were processed with Adobe Photoshop CS (Adobe Microsystems). For actin detection cells were fixed with 3.7% paraformaldeyde in PBS for 10 min at room temperature, and permeabilized with PBS containing 0.1% Triton X-100. Immunodetection of actin filaments was performed using a fluorescent probe Alexa Fluor 488® Phalloidin (Molecular Probes). Coverglasses were mounted in slides using Vectashield containing DAPI, and observed in a fluorescence microscope (Eclipse E400, Nikon). Images were processed with Adobe Photoshop CS (Adobe Microsystems).

2.9. In vitro tubulin polymerization assay

The effect of 4′-methoxy-2-syrylchromone on tubulin polymerization was evaluated using the Tubulin Polymerization Assay Kit (Cytoskeleton Inc., Denver, USA) according to the supplier's recommended protocol. Briefly, in a 96-well plate purified bovine brain tubulin was incubated in the presence of chromone (100, 300 and 500 μM), nocodazole (10 μM), paclitaxel (10 μM) or DMSO (0.5%) at 37 °C. Absorbance values were taken every minute during 1 h at 340 nm in a microplate reader (Bio-tek Instruments, Inc., PowerWave X).

3. Results

3.1. Effect of 4′-methoxy-2-styrylchromone on the growth of the human non-tumor MRC-5 cell line

Exponentially growing MRC-5 cells were incubated with a range of concentrations (2.2–180 μM) of chromone for 48 h and the cellular growth assessed through cellular protein content. The effect of this chromone was also evaluated on the human tumor cell lines MCF-7 and NCI-H460 for a non-tumor cell line comparison. 4′-Methoxy-2-styrylchromone is seen to inhibit the growth of the tumor MCF-7 and NCI-H460 cells in a dose-dependent manner ($GI_{50} = 4.4 \pm 0.8$ and 5.2 ± 0.3 μM), respectively, while growth of the non-tumor MRC-5 cell line (GI_{50} value of 26.9 ± 3.2 μM) was six times less potent.

3.2. Effect of 4′-methoxy-2-styrylchromone on the proliferation of human lymphocytes

Since 4′-methoxy-2-styrylchromone showed less efficiency in inhibiting the growth of the non-tumor MRC-5 cells, we further evaluated if this effect was extensive to normal human proliferating lymphocytes. For that, peripheral human lymphocytes were stimulated with phytohemagglutinin and exposed to a serial range of chromone concentrations (9.4–600 μM) for 96 h. 4′-Methoxy-2-styrylchromone is also seen to be a weak inhibitor of human lymphocytes proliferation

(IC_{50} = 74.7 ± 4.8 μM), showing once more its lower efficacy in inhibiting the proliferation of normal cells.

3.3. *Effect of 4′-methoxy-2-styrylchromone on the cell cycle*

The discrepancy in sensitivity between tumor and non-tumor human cell lines to this chromone, as well as the lower efficacy in inhibiting the proliferation of human lymphocytes prompted us to carry out further experiments to analyze the mechanistic basis of this difference. In the first series of experiments we analyzed the effect of chromone on the cell cycle progression of the two tumor (MCF-7 and NCI-H460) and the non-tumor (MRC-5) cell lines. For this we performed FACS analysis on MCF-7 and NCI-H460 exponentially growing cells treated with serial concentrations of chromone (10, 20 40 and 100 μM) during 12, 24 and 48 h. MRC-5 cells were equally analyzed after 12-, 24- or 48-h treatments with 100 μM 4′-methoxy-2-styrylchromone. The effect of the antimitotic agent nocodazole (0.66 μM) was also evaluated for comparison in MRC-5 cells. The results show that chromone treatment affected differently the cell cycle progression of the three cell lines (Fig. 2). While 4′-methoxy-2-styrylchromone caused an

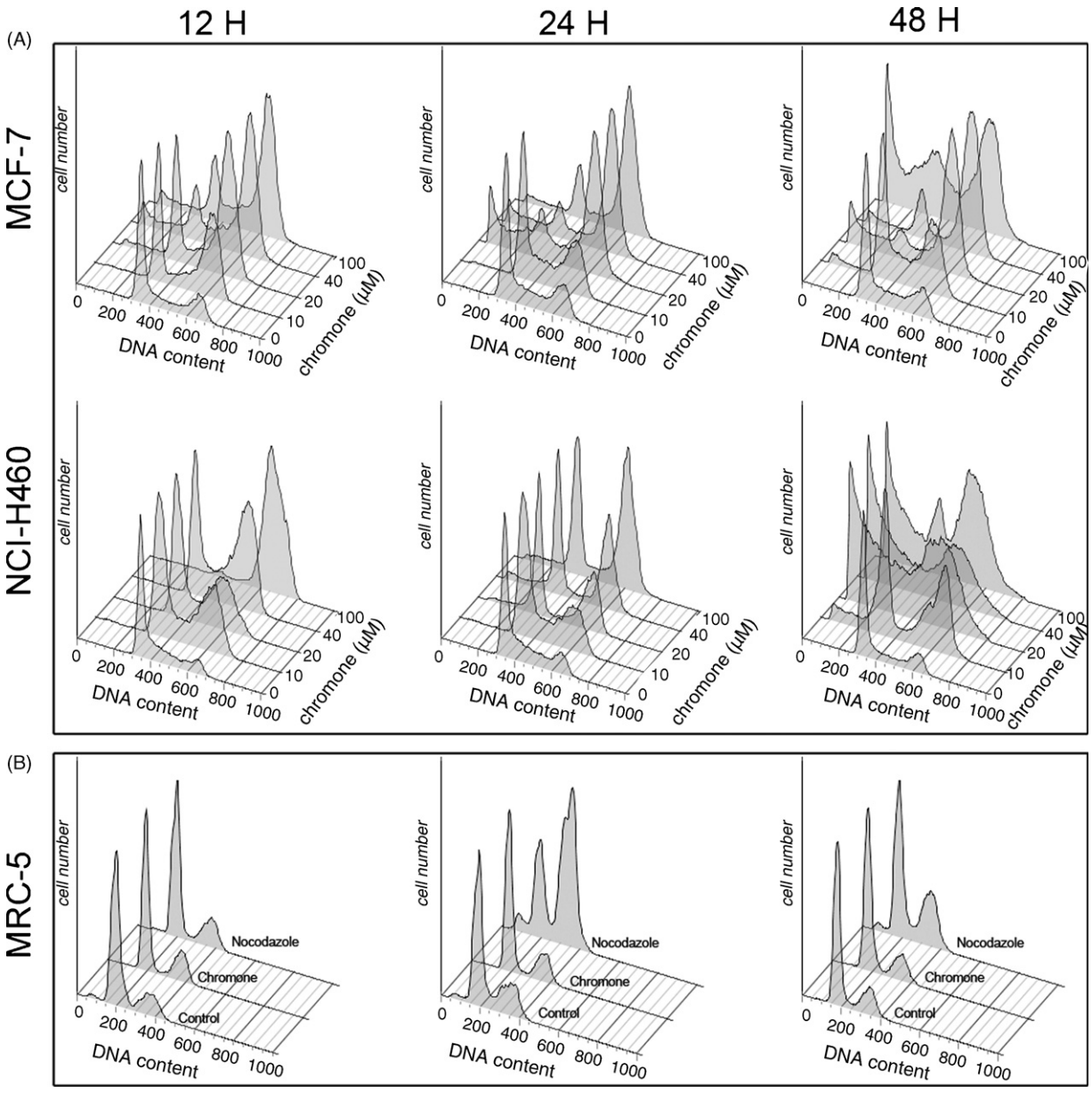

Fig. 2 – Effect of 4′-methoxy-2-styrylchromone on the cell cycle of MCF-7, NCI-H460 and MRC-5 cells. (A) Untreated MCF-7 and NCI-H460 cells (DMSO vehicle) and cells treated with various concentrations of 4′-methoxy-2-styrychromone for 12, 24 and 48 h. (B) Untreated MRC-5 cells and cells treated with 100 μM 4′-methoxy-2-styrylchromone or with 0.66 μM nocodazole for 12, 24 and 48 h. Cells were stained with propidium iodide and analyzed by flow cytometry for DNA content. The percentage of cells in each phase was determined using the program CellQuest Pro and FlowJo. Results are from a representative experiment, performed in duplicate, of two carried out independently.

Table 1 – Percentage of MCF-7 and NCI-H460 cells in G_2/M phase of the cell cycle

	% of cells in G_2/M phase				
	Control	4′-Methoxy-2-styrylchromone (μM)			
		10	20	40	100
MCF-7					
12 h	21.0	47.2	56.1	56.9	57.3
24 h	25.6	34.5	48.7	63.3	69.1
48 h	24.5	36.9	61.6	67.8	40.6
NCI-H460					
12 h	12.8	26.1	18.5	31.6	81.4
24 h	14.9	22.4	41.7	44.0	54.2
48 h	18.3	44.1	40.9	38.9	45.5

arrest at the G_2/M phase of the cell cycle in the tumor MCF-7 and NCI-H460 cells (Fig. 2A), the non-tumor MRC-5 cells seemed not to be affected by this chromone showing a cell cycle profile similar to untreated controls (Fig. 2B). Moreover, the arrest of tumor cell lines at the G_2/M phase was seen to be dose- and time-dependent. Indeed, accumulation of NCI-H460 cells reached a maximum value after 12-h treatment with 100 μM (81.4%) (Table 1) and after extended incubation periods the G_2/M peak reduces due to the increase of dead cells (sub G_1 peak in Fig. 2A), indicating apoptosis. In MCF-7 cells, the maximum value of G_2/M accumulation was reached later on, only after an exposure of 24 h with 100 μM (69.1%) (Table 1). An increase of dead cells was also observed with extended incubation periods (48 h) (Fig. 2A).

3.4. *Effect of 4′-methoxy-2-styrylchromone on the growth of confluent and non-confluent cells*

Accumulation of MCF-7 and NCI-H460 cells at the G_2/M phase of the cell cycle suggests that 4′-methoxy-2-styrylchromone might specifically affect mitosis, therefore, it was expected that exponential (non-confluent) growing cells were more sensitive to the chromone than non-proliferating (confluent) cells. To test this assumption, confluent and non-confluent cells were treated with several concentrations of the chromone (180, 60, 20, 6.6 and 2.2 μM) for 48 h, and their cellular growth was assessed either by protein content (SRB assay) or by DNA synthesis ([^{3}H] thymidine incorporation assay) (Fig. 3). When the growth of both cell lines was compared in terms of protein content (Fig. 3A) we observed that confluent cells were less sensitive to the effect of chromone treatment than non-confluent as demonstrated by their high cellular protein content similar to untreated control cells. However, a typical dose-dependent inhibition of cell growth was seen on non-confluent cells. This differential behavior was much more evident when cell growth was assessed by DNA synthesis as exemplified for the NCI-H460 cell line (Fig. 3B). The increase of chromone concentration leads to a dramatic fall in the synthesis of DNA of non-confluent cells, but confluent cells were not affected by chromone treatment as demonstrated by thymidine incorporation values similar to those of untreated control cells. On MCF-7 cells this different behavior was difficult to observe since the culture conditions chosen to reach confluence, that were efficient for

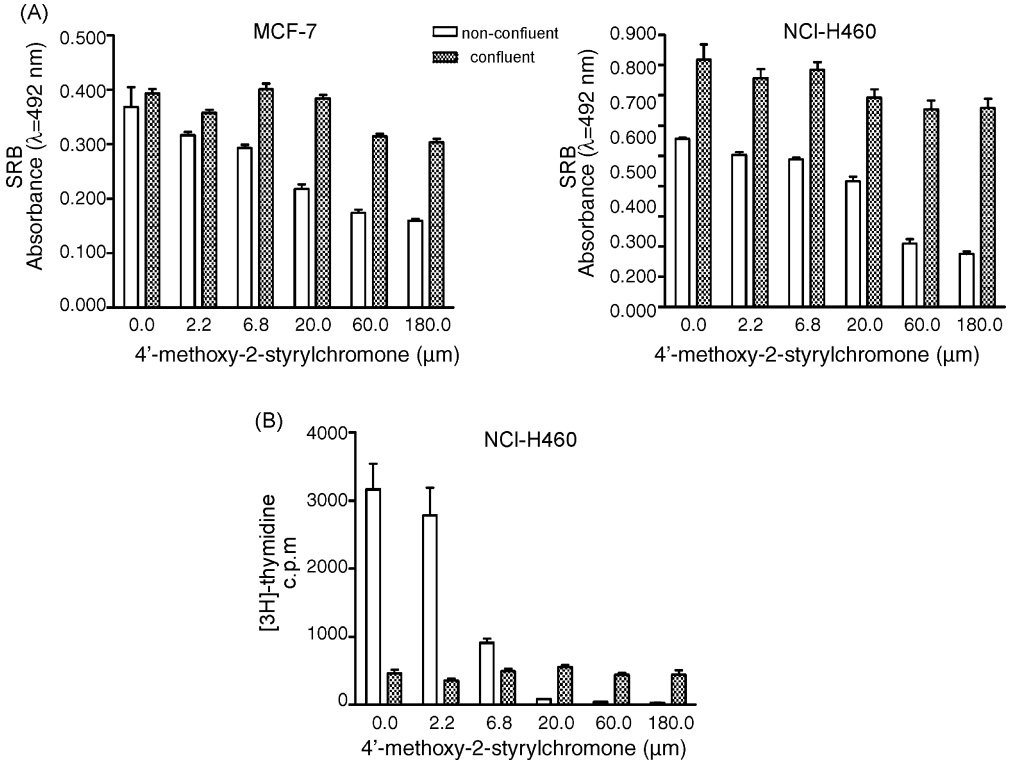

Fig. 3 – Effect of 4′-methoxy-2-styrylchromone on confluent and non-confluent MCF-7 and NCI-H460 cells, after 48-h treatment, determined by SRB assay (A) and [^{3}H] thymidine incorporation assay (B). Results represent the mean ± S.E.M. of a representative assay performed in triplicate of two carried out in independent experiments.

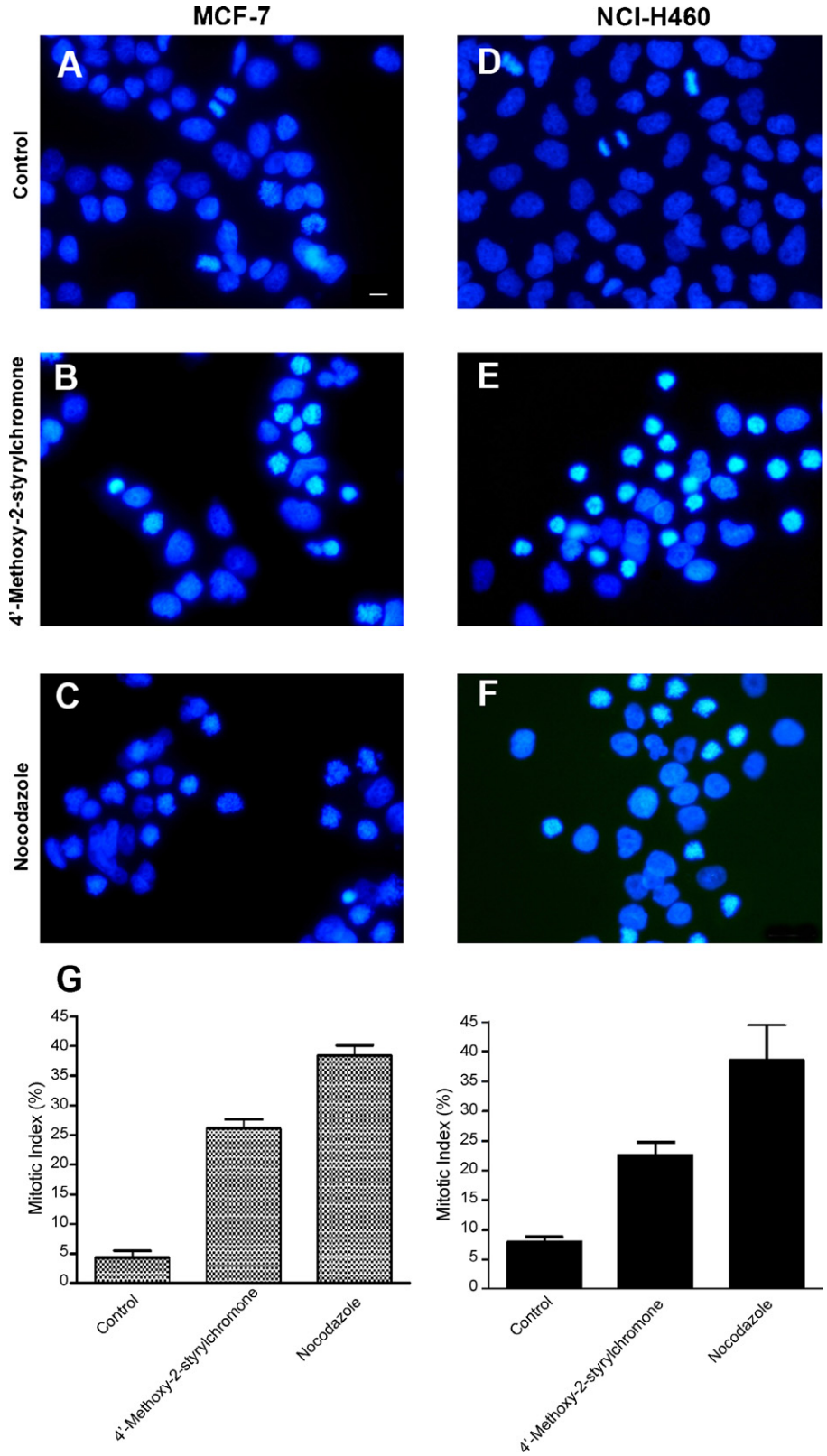

Fig. 4 – Effect of 4′-methoxy-2-styrylchromone on mitotic progression of MCF-7 and NCI-H460 cells. Fluorescent microscopic appearance of nuclei DAPI stained MCF-7 and NCI-H460 cells. Untreated control cells (A and D), cells treated with 100 μM 4′-methoxy-2-styrylchromone (B and E) and treated with 0.66 μM of nocodazole (positive control) (C and F) and for 24 and 12 h, respectively. The figures represent the increase on mitotic figures in the presence of chromone. (G) Quantification of mitotic cells. Scale bars are 10 μM. Results show means ± S.E.M. of three to six independent experiments.

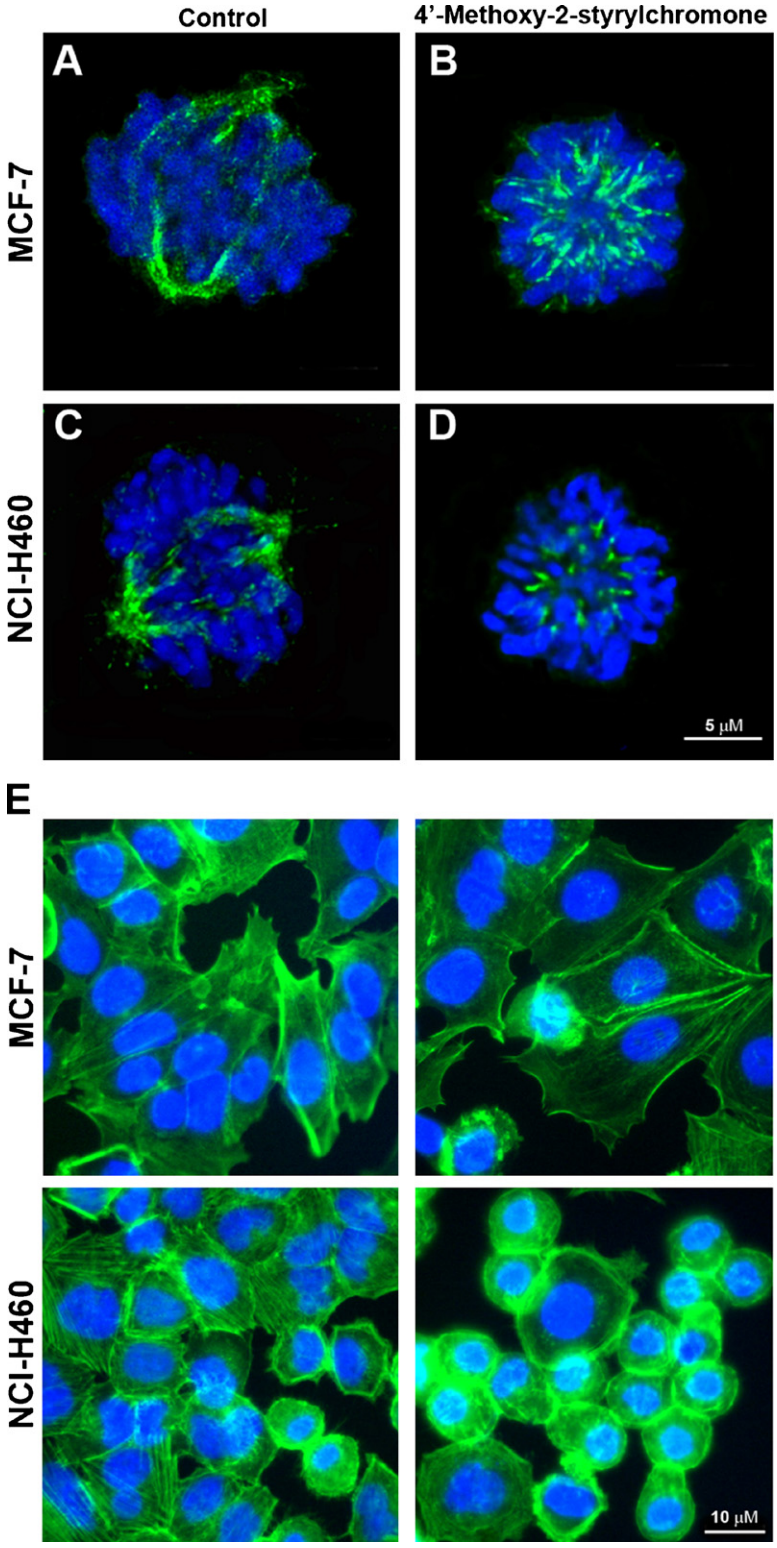

Fig. 5 – Effect of 4′-methoxy-2-styrylchromone on microtubules and microfilament network of MCF-7 and NCI-H460 cells. Immunofluorescence of untreated control cells and cells treated with 100 μM of 4′-methoxy-2-styrylchromone for 24 and 12 h, respectively. For immunodetection of microtubules cells was stained with an anti-α-tubulin antibody to visualize microtubules (green) and DAPI to counterstained DNA (blue). Immunodetection of actin filaments was performed using a fluorescent probe Alexa Fluor 488® Phalloidin. DNA was stained with DAPI.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

NCI-H460 cells, proved to be inadequate for MCF-7 cells which continued to synthesized DNA (data not shown).

3.5. *Effect of 4′-methoxy-2-styrylchromone on mitotic index*

Subsequently, we used fluorescent microscopy to ascertain the cell cycle stage and cellular morphology after chromone treatment. Cells were treated with 100 μM of chromone and then fixed and stained with DAPI to reveal the organization of the chromatin. This high concentration of chromone was chosen because it showed the highest values of G_2/M accumulation.

MCF-7- and NCI-H460-treated cultures showed an abundance of mitotic cells with particularly high frequency of cells in a prometaphase-like state (Fig. 4B and E), where clearly individualized and condensed can be easily distinguished but are rarely aligned at the equatorial plate, as observed in untreated control cells (Fig. 4A and D). This effect was similar to that caused by nocodazole treatment (Fig. 4C and F). The elevated number of prometaphase mitotic figures and the absence of later mitotic stages was the main feature observed after 100 μM 4′-methoxy-2-styrylchromone treatment. Quantification of mitotic cells showed that after chromone treatment cultures displayed mitotic index values of 26.1% and 22.6% for MCF-7 and NCI-H460, respectively, in contrast to 4.4% and 8.0% of untreated control cells (Fig. 4G). For nocodazole the number of cells in mitosis was 38.4% for MCF-7 and 38.6% for NCI-H460.

3.6. *Effect of 4′-methoxy-2-styrylchromone on microtubule and microfilament morphology*

When studying the effects of 4′-methoxy-2-styrylchromone on cytoskeleton components (actin filaments and microtubules) during interphase, we observed that the microtubule cytoskeleton appeared rich and intact in both MCF-7 and NCI-H460 cells similarly to untreated control cells (data not shown). However, mitotic cells exhibited severe alterations including monopolar microtubule arrays (Fig. 5B and D) and rarely normal mitotic spindles were found (Fig. 5A and C). No significant changes on actin filaments were detected after treatment of MCF-7 and NCI-H460 with 100 μM 4′-methoxy-2-styrylchromone (Fig. 5E) suggesting that 4′-methoxy-2-styrylchromone affects specifically microtubules during mitosis.

3.7. *Effect of 4′-methoxy-2-styrylchromone on tubulin polymerization in vitro*

Since 4′-methoxy-2-styrylchromone induced severe alterations on the mitotic microtubule network we tested further its ability to interfere with microtubule assembly monitoring turbidity changes over time. Microtubule assembly was induced by increasing the temperature to 37 °C and was detected by the increase of absorbance at 240 nm. We compared the behavior of tubulin when exposed to the chromone or to depolymerizing (nocodazole) and stabilizing (paclitaxel) agents. As expected, paclitaxel (10 μM) strongly promoted microtubule polymerization and nocodazole (10 μM) strongly inhibited microtubule polymerization (Fig. 6). Significantly, 4′-methoxy-2-styrylchromone enhanced slightly tubulin polymerization when compared with control (DMSO) in a dose-dependent manner. The maximal plateau of tubulin polymerization was achieved with 500 μM, the highest 4′-methoxy-2-styrylchromone concentration tested. When the curves of paclitaxel and 4′-methoxy-2-styrylchromone were compared, it became evident that the initial rate of tubulin polymerization induced by chromone is lower than paclitaxel, but the higher absorbance values observed after 20 min could indicate a greater tubulin stabilizing capacity of this compound.

4. Discussion

In the present work, we investigated if 4′-methoxy-2-styrylchromone, a new synthetic chromone identified as a potent inhibitor of the growth of a variety of human tumor cell lines,

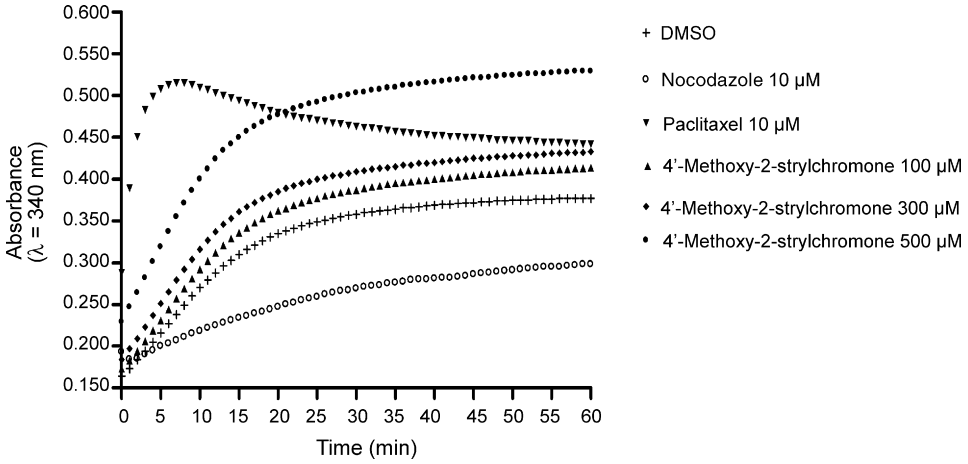

Fig. 6 – Effect of 4′-methoxy-2-styrylchromone on tubulin polymerization *in vitro*. Purified bovine brain tubulin was incubated in the presence of 4′-methoxy-2-styrylchromone, nocodazole, paclitaxel or DMSO (vehicle) at 37 °C, and absorbance were recorded each minute for 1 h. Results are from a representative experiment, performed in duplicate, of three carried out independently.

was equally potent against the non-tumor human fibroblast cell line MRC-5 and if the effect was extensive to normal human proliferating lymphocytes. We showed that 4′-methoxy-2-styrylchromone was less efficient in inhibiting normal cells which confirmed the higher sensitivity of tumor cell lines to chromone effect. Although the methods used to study the antiproliferative activity on cell lines and blood lymphocytes were different it seemed that lymphocytes are at least three-fold less sensitive than the cell lines studied.

The demonstration that 4′-methoxy-2-styrylchromone is not so efficient in inhibiting confluent than non-confluent cells led us to conclude that this chromone seems to be acting preferentially on proliferating cells. Studies carried out to clarify the mechanism underlying 4′-methoxy-2-styrylchromone potent tumor cell growth inhibitory effect showed a specific blockage of MCF-7 and NCI-H460 cells at the G_2/M phase of the cell cycle and a consequent increase in mitotic figures. The increase of cells in G_2/M phase is clearly seen after treatment with the lowest concentration tested, two-fold GI_{50}, indicating that the antiproliferative effect detected in the SRB assay could be correlated with this mitotic blockage. These effects resemble those caused by antimitotic agents such as taxol or nocodazole [15–17]. Interestingly, the G_2/M arrest was not observed on the non-tumor cell line MRC-5, resembling the high selectivity of this chromone to tumor cells, which provides interesting perspectives for this compound to be developed as a new anticancer agent.

Agents that target microtubules disrupt mitotic spindle dynamics, preventing metaphase/anaphase transition, leading to mitotic arrest and subsequently apoptosis [18,19]. Mitotic spindles formed in the presence of the chromone were abnormal showing a monopolar configuration. This disruption of the mitotic apparatus induced by the chromone treatment led to mitotic arrest, as demonstrated by the increase of cells in G_2/M, as well as by the increase of mitotic indices. The appearance of a subdiploid peak of cells after FACS analysis suggests that presumably as a consequence of the prolonged mitotic blockage induced by this chromone many cells enter apoptosis.

In order to clarify how 4′-methoxy-2-styrylchromone interfered with tubulin, studies were carried out to evaluate if this chromone acts on the polymerization dynamics of microtubules, either acting as microtubule-destabilizing agent, or stimulating microtubule polymerization. The data showed that high concentrations of 4′-methoxy-2-styrylchromone considerably increase the turbidity, suggesting that this chromone could affect the *in vitro* assembly of tubulin into microtubules in a manner similar to that reported for paclitaxel, acting as a microtubule-stabilizing agent [17,20]. However, electron microscopy observation of the product induced by chromone treatment is needed to assure that this increase in turbidity is due to the formation of microtubule structure, and not merely microtubule aggregation.

Many antimitotic agents that cause mitotic arrest via mechanisms involving tubulin disruption, particularly stabilization of microtubules have been reported [15,20–24].

The identification of 4′-methoxy-2-styrylchromone as a potential microtubule-stabilizing agent provides the first evidence of this biological activity of chromones and its ability to preferentially suppress the growth of tumor cells over normal cells. This behavior raises interesting perspectives for this family of compounds adding to the small number of microtubule-stabilizing agents available, a new structurally distinct chemical group that could be explored for the development of new anticancer strategies and antitumoral agents.

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