Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 14 (2006) 153–163
Bioorganic & Medicinal Chemistry

# Antiproliferative properties of piperidinylchalcones

Xiaoling Liu and Mei-Lin Go*

*Department of Pharmacy, Medicinal Chemistry Program, Office of Life Sciences, National University of Singapore, Singapore*

Received 1 July 2005; revised 30 July 2005; accepted 1 August 2005
Available online 26 September 2005

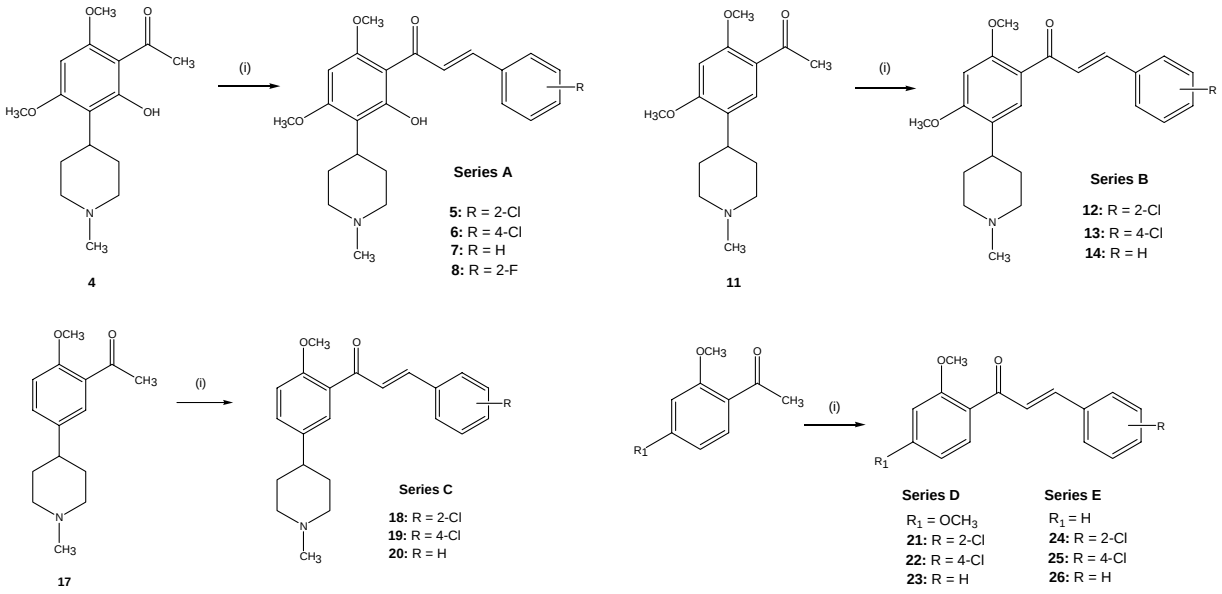
**Abstract**—Methoxylated chalcones bearing *N*-methylpiperidinyl substituents on ring A inhibited the growth of human tumour cell lines (MCF, HCT 116, and Jurkat) at $IC_{50}$ values of <5 μM. Investigations on a representative member (12) showed that antiproliferative activity was linked to the disruption of the cell cycle at G1 and G2/M phases. The effect was concentration dependent and was evident at the approximate $IC_{50}$ of 12. Down regulation of cell cycle regulatory components (CDK4, cyclin B, E2F, and phosphorylated Rb) were observed under similar conditions. Methoxylated chalcones without the piperidinyl substituent were found to exert equally potent and selective antiproliferative activity against HCT 116 tumour cells but did not interfere with cell cycle progression at their $IC_{50}$ concentrations. The presence of the piperidinyl substituent in the chalcone template is proposed to lend specificity to the mechanism of antiproliferative activity, in addition to promoting a more desirable physicochemical profile.


## 1. Introduction

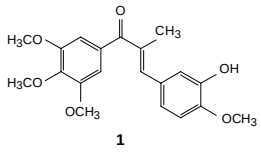
Chalcones are a class of privileged structures that have a wide range of biological properties.[1,2] An area of particular interest is their potential as anticancer agents,[3–6] for which several modes of action have been proposed. These include inhibition of angiogenesis,[7] interfering with p53–MDM2 interaction,[8,9] induction of mitochondrial uncoupling and membrane collapse,[10] and disruption of the cell cycle.[11–16] The antimitotic activity of chalcones has been attributed to the reactive enone moiety in the molecule which interacts with a critical thiol residue at the colchicine binding site on tubulin, leading to the inhibition of tubulin polymerization and disruption of mitosis.[14,15] The association of methoxylated chalcones with antimitotic activity is also noted in the literature, arising, in part, from the observation that known inhibitors of tubulin polymerization, such as combretastatin A and colchicine, are rich in methoxy groups.[11,14,15] The methoxylated chalcone, E-1-(3′,4′,5′-trimethoxy phenyl)-2-methyl-3-(3-hydroxyl-4-methoxyphenyl)prop-2-en-1-one (**1**), is probably the most potent cytotoxic chalcone reported to date.[16] It has an $IC_{50}$ of 0.21 nM against K562 human leukaemia cells and is a potent inhibitor of microtubule assembly ($IC_{50}$ 0.5 μM). Notwithstanding the beneficial contribution of methoxy groups to activity, their presence may serve to reduce aqueous solubility and drug-like character. One approach to moderate the hydrophobic influence of the methoxy groups would be to introduce hydrophilic substituents into the chalcone template. Basic amino functions, which are protonated at physiological pH, may be useful in this regard. In this investigation, we have synthesized a series of methoxylated chalcones bearing a basic *N*-methylpiperidinyl substituent on ring A (Scheme 1, Series A–C), with the aim of understanding how the presence of an ionizable basic function would influence the antiproliferative properties of these compounds. From a physicochemical standpoint, the *N*-methylpiperidine ring ($pK_a \sim 10$), which is protonated at physiological pH, enhances the aqueous solubility of a generally hydrophobic chalcone template. The basic character of the piperidine ring may also have the added advantage of promoting hydrogen bonding and other interactions at the putative target site. Interestingly, others have reported enhanced selectivity and potency in the biological properties of chalcones with basic amino functions.[17,18] In one report, 2′-aminochalcones demonstrated a significantly increased anti-tumour activity compared with chalcones that lack this function.[17] Another investigation showed how the introduction of cationic aliphatic amino groups in the chalcone scaffold enhanced selectivity and potency of the resulting compounds against Gram-positive and -negative pathogens.[18] Thus, it is of interest to elucidate further the contribution of the basic amino function to the biological activity of chalcones.

*Keywords*: Piperidinylchalcones; Antiproliferative activity; Cell cycle; Expression of cell cycle regulatory proteins.
* Corresponding author. Tel.: +65 687 426 54; fax: +65 677 915 5; e-mail: phagoml@nus.edu.sg

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.08.006



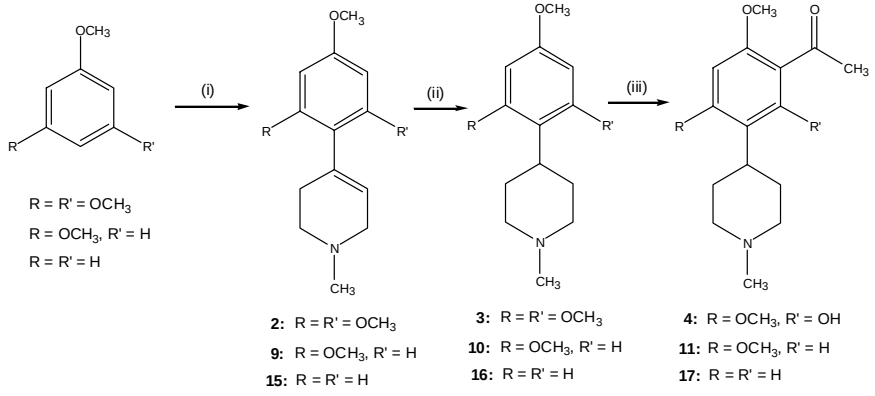

**Scheme 1.** Reagents and conditions: (i) Aldehyde, 3% w/v NaOH, methanol, 12 h, rt.



## 2. Results

### 2.1. Synthesis of chalcones

Five series of chalcones (A–E) were synthesized by a base-catalyzed Claisen–Schmidt reaction between an appropriately substituted benzaldehyde and a methoxylated acetophenone (Scheme 1). In the case of the Series A–C piperidinyl chalcones, the methoxylated 3-(*N*-methylpiperidinyl)acetophenones (**4**, **11**, and **17**) were synthesized according to procedures shown in Scheme 2. 1,3,5-Trimethoxybenzene was reacted with 1-methyl-4-piperidinone in the presence of hydrogen chloride gas in glacial acetic acid to give the unsaturated product **2** in moderate yield (65%).[19] Catalytic reduction of the double bond in **2** afforded **3**, which was treated with acetic anhydride and boron triflouride for the introduction of the acetyl function. In the course of reaction, it was found that one of the methoxy groups was lost through demethylation to give compound **4**. This compound was reacted with commercially available benzaldehydes to give the Series A chalcones **5**–**8** (Scheme 1). In a similar manner, 1,3-dimethoxybenzene and methoxybenzene were reacted with *N*-methyl-4-piperidinone to give compounds **9** and **15**, respectively. Catalytic hydrogenation gave compounds **10** and **16** in good yield and subsequent acetylation reaction was achieved without the loss of a methoxy group to give acetophenones **11** and **17**, respectively. Claisen–Schmidt condensation of compounds **11** and **17** with benzaldehydes gave the desired Series B (**12**–**14**) and Series C (**18**–**20**) chalcones in acceptable yields. Chalcones that lack the *N*-methylpiperidine ring (Series D and E) were also synthesized by base-catalyzed




**Scheme 2.** Reagents and conditions: (i) *N*-methylpiperidinone, HCl, acetic acid, 95–100 °C, 3 h; (ii) 10% Pd/C, 40 psi, rt; (iii) $BF_3$, acetic anhydride, DCM.

**Table 1.** Antiproliferative $IC_{50}$ values and selectivity indices of Series A–E chalcones

| | Antiproliferative activity $IC_{50}$ μM[a] | | | | | | Selectivity index | |
|---|---|---|---|---|---|---|---|---|
| | MCF7 | HCT 116 | Jurkat | Average[b] | CCL 186 | AG 1523 | $C_{50\ CCL}/IC_{50\ average}$ | $IC_{50\ AG\ 1523}/IC_{50\ average}$ |
| *Series A* | | | | | | | | |
| **5** | 3.1 | 3.1 | 1.6 | 2.6 | 3.1 | 6.4 | 1.2 | 2.5 |
| **6** | 6.7 | 2.6 | 2.4 | 3.9 | 6.1 | 9.8 | 1.6 | 2.5 |
| **7** | 5.7 | 4.2 | 2.2 | 4.0 | 7.3 | 9.3 | 1.8 | 2.3 |
| **8** | 3.4 | 3.0 | 1.7 | 2.7 | 4.6 | 5.2 | 1.7 | 1.9 |
| *Series B* | | | | | | | | |
| **12** | 2.7 | 3.4 | 2.5 | 2.9 | 7.6 | 7.1 | 2.6 | 2.4 |
| **13** | 2.5 | 2.9 | 1.8 | 2.4 | 3.6 | 6.9 | 1.5 | 2.9 |
| **14** | 3.4 | 3.9 | 2.9 | 3.4 | 7.8 | 13.2 | 2.3 | 3.9 |
| *Series C* | | | | | | | | |
| **18** | 2.9 | 2.3 | 2.0 | 2.4 | 6.0 | 11.3 | 2.5 | 4.7 |
| **19** | 3.2 | 3.3 | 1.4 | 2.6 | 5.2 | 9.4 | 2.0 | 3.6 |
| **20** | 4.2 | 3.6 | 2.9 | 3.6 | 9.8 | 14.0 | 2.7 | 3.9 |
| *Series D* | | | | | | | | |
| **21** | | 5.2 | | | 12.8 | | 2.4 | |
| **22** | | 6.2 | | | 11.3 | | 1.8 | |
| **23** | | 4.4 | | | 13.7 | | 3.1 | |
| *Series E* | | | | | | | | |
| **24** | | 5.2 | | | 11.7 | | 2.3 | |
| **25** | | 2.7 | | | 4.3 | | 1.6 | |
| **26** | | 5.5 | | | 18.0 | | 3.3 | |
| **27**[c] | | 25.0 | | | 30.0 | | 1.2 | |

[a] Determined by MTT assay, 72 h incubation with test compound. Mean of at least three separate determinations. Flavorpiridol was used as a control: $IC_{50}$ 0.02 μM (HCT116); 0.17 μM (CCL 186).
[b] Average of $IC_{50}$ values from MCF-7, HCT 116 and Jurkat cell lines.
[c] 2′,3′,4′-Trimethoxy-2,4-dimethoxychalcone.[20]

condensation of 2,4-dimethoxyacetophenone or 2-methoxyacetophenone with the relevant benzaldehydes (Scheme 1).

## 2.2. Inhibition of cell proliferation

The $IC_{50}$ values of the target chalcones were determined against three human cancer cell lines (HCT 116, MCF-7 and Jurkat) and two human diploid fibroblast cell lines (fetal lung CCL 186, foreskin AG 1523) by the microculture tetrazolium assay, which is based on the ability of metabolically active cells to reduce the yellow tetrazolium salt to a coloured formazan product.[20] The results are given in Table 1, together with the average $IC_{50}$ values against the three cancer cell lines and the selectivity indices of these compounds when evaluated against the normal human cells.

As seen from Table 1, the *N*-methylpiperidinyl chalcones (Series A–C) had moderately good antiproliferative activity, with average $IC_{50}$ values less than 5 μM. An interesting observation is the limited variation in $IC_{50}$ values across Series A–C, despite the different substitution patterns on their rings A and B. Thus, equally potent members were found in all three series, as can be seen from the $IC_{50\ average}$ values of **5** (Series A, 2.6 μM), **13** (Series B, 2.4 μM) and **18** (Series 2.4 μM). Clearly, the number of methoxy groups or the inclusion of an additional hydroxyl group on ring A (for the same substitution pattern on ring B) made little difference to activity. However, this conclusion should be tempered with the observation that substitution on ring B had essentially been limited to halogens (chlorine, fluorine) in this study. A different finding may emerge when other groups are introduced to ring B.

The importance of the *N*-methylpiperidine substituent on ring A was explored by comparing the antiproliferative activities of Series B and C compounds (with *N*-methylpiperidine) with those in Series D and E (without *N*-methylpiperidine). Based on $IC_{50}$ values for HCT116, it is seen that with one exception (compounds **19** and **25**), the presence of the *N*-methylpiperidine substituent served to enhance activity only to a modest degree. As in the case of the Series A–C chalcones, Series D and E compounds do not show marked differences in their $IC_{50}$ values. In this respect, the poor activity of compound **27** (2′,3′,4′-trimethoxy-2,4-dimethoxychalcone, $IC_{50}$ 25 μM)[21] is notable, as it suggests an important role for the substitution pattern on ring B.

The selective activity of the target chalcones was also evaluated and expressed as selectivity indices against the relevant normal cell line (Table 1). The Series A–C compounds were 1.9–4.7 times more selective against cancer cell lines than AG 1523, but slightly less selective (1.2–2.7) when compared against CCL 186. The absence of the *N*-methylpiperidine ring in Series D and E

chalcones did not alter selectivity to a great extent ($SI_{CCL\ 186}$ = 1.6–3.3).

Of the five series evaluated, the compounds in Series B and C were seen to combine the advantageous features of potency and selectivity, as exemplified by compounds **12** and **18**.

### 2.3. Effect on cell cycle analysis

The antiproliferative activity of the chalcones may stem from interference with different stages of cell cycle. Methoxylated chalcones are known to block cells in the G2/M phase of the cell cycle, which is consistent with their ability to disrupt the mitotic spindle by interaction with the protein tubulin.[11,16] It would be of interest to see if the current methoxylated chalcones behave in a similar manner. This aspect was investigated by fluorescence-activated cell sorter (FACS) analysis using flow cytometry. The time-related changes in the distribution of cells at each phase of the cell cycle were determined by monitoring the DNA content of the cells. Three compounds were investigated for their effects on the cell cycle: compound **12** (a representative *N*-methylpiperidinyl chalcone with acceptable potency and selectivity), compound **22** (a representative chalcone without the *N*-methylpiperidinyl substituent) and compound **27** (a highly methoxylated chalcone with low potency and selectivity).

The cell cycle progression experiments were designed to examine synchronized cell populations at G1 or G2/M phase through one transit of the cell cycle. G1-synchronized cells were obtained by arresting the cells at the G1 phase by growing to confluence and then stimulating their re-entry into the cell cycle by sub-culturing at lower densities. Cells immediately released from a G1 block were exposed to different concentrations of the test compound and progression through the cell cycle was monitored for over 48 h. Twenty-four hours after release from the G1 block, untreated cells get aligned at the G2/M phase. These G2/M-synchronized cells were collected and similarly exposed to the test compounds.

Figure 1 shows the cell cycle phase distribution in HCT 116 cells initially synchronized at G1 over a period of 48 h. Within 24 h of serum treatment, the control cells showed significant progression into the G2/M phase (Fig. 1, panel 3). This was not observed for cells exposed to compounds **12** (10 μM), **22** (20 μM) and **27** (50 μM) where the proportion of cells in the G1 phase remained largely unchanged 24 h after the start of the experiment. Lower concentrations of compound **12** (5 μM, but not 0.5 μM) also arrested cells at G1. Notably, compound **12** arrested the cell cycle at a concentration close to its $IC_{50}$ (3.4 μM). In contrast, compounds **22** and **27** caused G1 arrest only at concentrations that were at least twice their $IC_{50}$ values (Table 2A).

When control cells were synchronized at G2/M, progression to the G1 phase was attained after 8 h (Fig. 2, panel 2). Compounds **12**, **22** and **27** arrested the cells in the G2/M phase and halted their progression to G1. This effect was observed at 10 and 20 μM of compound **12**, but not at lower concentrations (0.5, 5 μM) (Table 2B). One possibility is that the G2/M → G1 progression was less sensitive to **12**. As for compounds **22** and **27**, the arrest of cells at the G2/M phase was again observed at concentrations greater than their $IC_{50}$ values (Table 2B).

The effect of compound **12** on the cell cycle was also investigated on a normal cell line (CCL 186) to avoid the confounding effects of loss of checkpoint control in

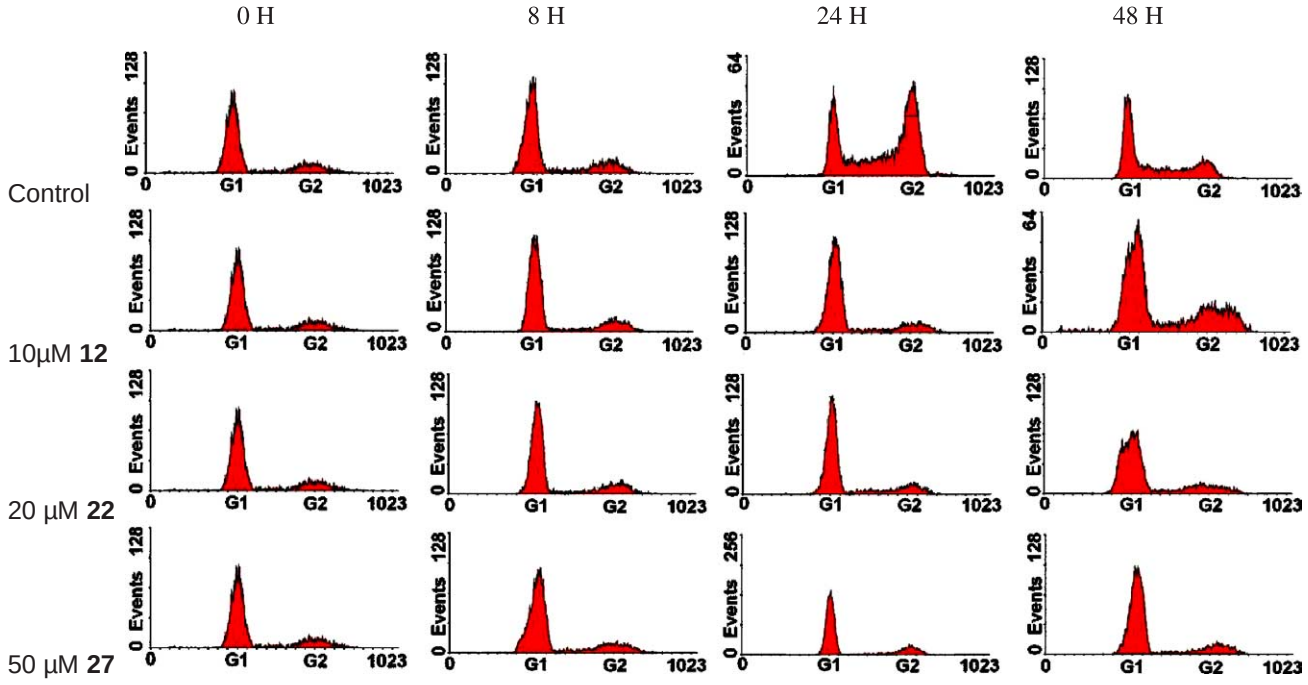



**Figure 1.** FACS diagram showing the effect of compounds **12**, **22** and **27** in G1-arrested HCT 116 cells.

**Table 2.** Effect of compounds **12**, **22** and **27** on distribution of HCT 116 and CCL 186 cells in various phases of the cell cycle

| | Concentration (μM) | % Cells in phase of cell cycle | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 8 h[a] | | | 24 h[a] | | | 48 h[a] | | |
| | | G1 | S | G2/M | G1 | S | G2/M | G1 | S | G2/M |
| *(A) HCT 116 cells synchronized at G1 phase* | | | | | | | | | | |
| **12**[b] | 0.5 | 62.0 (65.6) | 6.4 (6.0) | 27.8 (25.9) | 32.9 (34.1) | 14.8 (15.8) | 48.4 (46.8) | 40.0 (49.7) | 22.7 (11.5) | 31.6 (34.3) |
| | 5 | 61.4 (65.6) | 5.8 (6.0) | 30.7 (25.9) | 50.2 (34.1) | 13.0 (15.8) | 33.7 (46.8) | 49.1 (49.7) | 11.1 (11.5) | 36.4 (34.3) |
| | 10 | 74.3 (72.7) | 3.8 (5.2) | 21.7 (21.3) | 75.8 (28.8) | 4.7 (13.7) | 18.0 (54.5) | 57.2 (57.9) | 7.6 (14.5) | 33.1 (27.4) |
| | 20 | 69.2 (72.7) | 5.8 (5.2) | 18.6 (21.3) | 78.4 (28.8) | 3.6 (13.7) | 17.0 (54.5) | 41.4 (57.9) | 6.6 (14.5) | 11.8 (27.4) |
| **22**[b] | 10 | — | — | — | 54.3 (35.5) | 13.6 (27.9) | 31.8 (36.8) | 49.5 (54.3) | 18.5 (11.4) | 30.7 (33.7) |
| | 20 | 75.5 (72.7) | 3.2 (5.2) | 20.9 (21.3) | 75.4 (28.8) | 5.6 (13.7) | 18.4 (54.5) | 71.5 (57.9) | 5.6 (14.5) | 20.4 (27.4) |
| **27**[b] | 20 | — | — | — | 45.8 (35.5) | 19.5 (27.9) | 34.5 (36.8) | 51.1 (54.3) | 17.4 (11.4) | 31.2 (33.7) |
| | 50 | 74.9 (72.7) | 4.1 (5.2) | 19.9 (21.3) | 73.5 (28.8) | 3.0 (13.7) | 23.0 (54.5) | 76.7 (57.9) | 3.4 (14.5) | 19.6 (27.4) |
| | Concentration (μM) | % Cells in phase of cell cycle | | | | | | | | |
| | | 8 h[c] | | | 24 h[c] | | | 48 h[c] | | |
| | | G1 | S | G2/M | G1 | S | G2/M | G1 | S | G2/M |
| *(B) HCT 116 cells synchronized at the G2 phase* | | | | | | | | | | |
| **12**[b] | 0.5 | 39.4 (38.8) | 12.5 (14.3) | 44.0 (43.4) | 42.2 (49.7) | 14.8 (11.5) | 38.4 (34.3) | 54.6 (52.0) | 7.1 (6.5) | 33.4 (37.9) |
| | 5 | 38.7 (38.8) | 12.5 (14.3) | 45.1 (43.4) | 41.8 (49.7) | 13.4 (11.5) | 41.3 (34.3) | 46.9 (52.0) | 22.7 (6.5) | 22.9 (37.9) |
| | 10 | 24.0 (45.9) | 13.4 (18.2) | 62.4 (35.8) | 22.2 (57.9) | 8.4 (14.5) | 68.8 (27.4) | 28.6 (66.0) | 9.1 (7.6) | 60.4 (25.8) |
| | 20 | 24.2 (45.9) | 11.2 (18.2) | 64.4 (35.8) | 20.0 (57.9) | 7.7 (14.5) | 72.1 (27.4) | 21.7 (66.0) | 8.6 (7.6) | 68.0 (25.8) |
| **22**[b] | 10 | 36.6 (46.0) | 16.0 (15.4) | 47.4 (38.5) | 53.0 (54.3) | 17.1 (11.4) | 29.6 (33.7) | 54.8 (68.4) | 13.3 (3.9) | 31.7 (26.9) |
| | 20 | 13.8 (45.9) | 8.2 (18.2) | 77.2 (35.8) | 20.9 (57.9) | 12.3 (14.5) | 64.8 (27.4) | 27.7 (66.0) | 16.4 (7.6) | 42.7 (25.8) |
| **27**[b] | 20 | 46.5 (46.0) | 15.7 (15.4) | 37.6 (38.5) | 53.6 (54.3) | 13.5 (11.4) | 32.7 (33.7) | 60.0 (68.4) | 4.2 (3.9) | 34.6 (26.9) |
| | 50 | 32.7 (45.9) | 12.1 (18.2) | 54.8 (35.8) | 46.1 (57.9) | 4.0 (14.5) | 48.9 (27.4) | 48.3 (66.0) | 3.7 (7.6) | 46.2 (25.8) |
| | Concentration μM | % Cells in phase of cell cycle | | | | | | | | |
| | | 24 h[d] | | | 48 h[d] | | | | | |
| | | G1 | S | G2/M | G1 | S | G2/M | | | |
| *(C) CCL 186 cells* | | | | | | | | | | |
| Cells synchronized at G1 phase | | | | | | | | | | |
| **12**[e] | 0.5 | 56.9 (42.8) | 10.3 (16.0) | 32.7 (40.5) | 79.0 (72.7) | 3.4 (6.3) | 16.2 (19.9) | | | |
| | 5.0 | 69.3 (42.8) | 8.5 (16.0) | 21.2 (40.5) | 76.3 (72.7) | 3.0 (6.3) | 17.2 (19.9) | | | |
| Cells synchronized at G2 phase | | | | | | | | | | |
| **12**[e] | 0.5 | 67.7 (72.7) | 7.1 (6.3) | 24.2 (19.9) | 75.4 (75.1) | 6.9 (5.7) | 17.6 (19.0) | | | |
| | 5.0 | 38.4 (72.7) | 14.6 (6.3) | 43.7 (19.9) | 36.1 (75.1) | 14.1 (5.7) | 46.9 (19.0) | | | |

[a] Time (h) after cells were released from the G1 block. Values in parentheses represent control values in concurrent experiments.
[b] $IC_{50\ HCT\ 116}$: **12** 3.4 μM; **22**: 6.2 μM; **27**: 25.0 μM.
[c] Time (h) after cells were synchronized at G2/M. Untreated cells progressed to G1 within 8–12 h. Values in parentheses represent control values in concurrent experiments.
[d] Time (h) after cells were released from G1 block or time (h) after cells were synchronized at G2/M. Values in parentheses represent control values in concurrent experiments.
[e] $IC_{50\ CCL\ 186}$: 7.6 μM.

tumour-derived cell lines. It was noted that compound **12** at 2 μM also interfered with cell cycle progression in both G1-arrested and G2/M-synchronized cell populations.

## 2.4. Effect of compound 12 on the expression of cell cycle regulatory proteins

Since compound **12** affected cell cycle progression at concentrations that were close to its $IC_{50}$, it was of interest to see if its effect on the cell cycle was mediated through interference with the expression of key cell cycle regulatory proteins. HCT116 cells synchronized at the G1 phase were incubated with compound **12** at 2 μM for 24 h and the expression of selected regulatory proteins was examined by Western blot analysis. The proteins investigated were cyclins D and B, CDK4, E2F and the retinoblastoma protein Rb. Cyclin D and CDK4 are important for initiating the phosphorylation of Rb (retinoblastoma protein). In its unphosphorylated state, Rb is bound to and inactivates the E2F family of transcription factors, thereby repressing DNA synthesis. Once Rb is phosphorylated, E2F dissociates from Rb and activates the genes necessary for S phase entry and progression. Progression from G2 to M phase is mainly regulated by cyclin B and its CDK partner (CDK 1).

The results of the Western blot analysis are shown in Figure 3. Serum-starved cells did not undergo the cell cycle and showed light bands of CDK4, cyclin D, cyclin

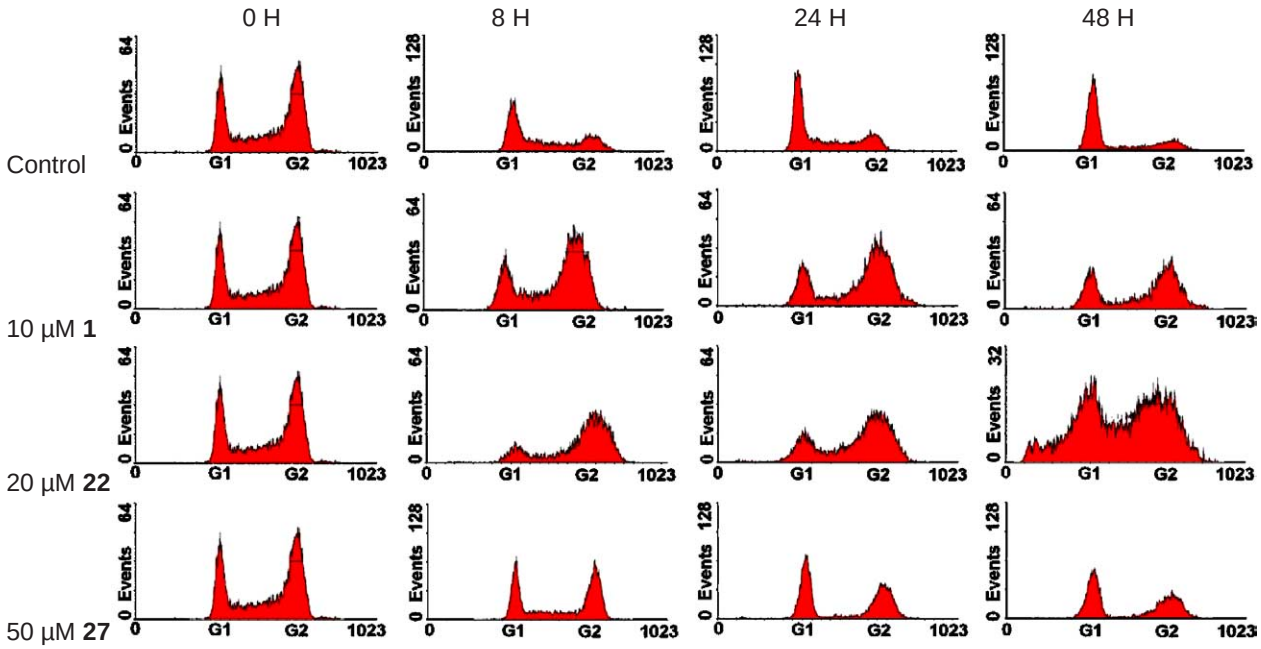



**Figure 2.** FACS diagram showing the effect of compounds **12**, **22** and **27** in G2-synchronized HCT 116 cells.

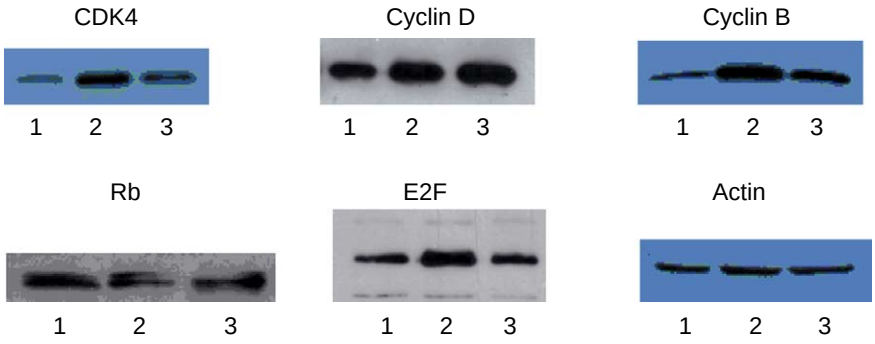



**Figure 3.** Expression of cell cycle regulatory proteins in HCT 116 cells synchronized at G1 phase. Lane 1, serum-starved cells; lane 2, 24 h after addition of 10% serum to serum starved cells; lane 3, 24 h after addition of compound 12 (2 μM) and 10% serum to serum-starved cells. Protein content of cellular extracts was standardized for uniformity of content.

B, E2F and Rb (hypophosphorylated). Upon addition of serum, the intensity of these bands increased and both states (phosphorylated and unphosphorylated) of Rb were detectable. Incubation of the cells with compound **12** caused the CDK4, cyclin B and E2F bands to decrease noticeably in intensity. The band corresponding to hyperphosphorylated Rb band diminished but the hypophosphorylated Rb band remained detectable. On the other hand, there was no discernible change in the level of cyclin D. The decreases in CDK4 and E2F indicated an interference with the early G1 phase while the decrease in cyclin B suggested that the G2/M phase was also affected. The unchanged levels of cyclin D which is also involved in the G1 phase transition could not be readily explained.

## 3. Discussion

The piperidinylchalcones of Series A–C showed promising antiproliferative activity against three cancer cell lines, with $IC_{50\ average}$ values less than 5 μM. Omission of the piperidinyl group to give Series D and E compounds resulted in only a slight decrease in potency, with little change in selective activity. Despite the limited variations in activity, the Series B and C compounds can be identified as useful leads for future structural modifications because several members combine the advantageous features of potency and selectivity.

While these results point to a limited contribution by the piperidine ring to antiproliferative activity, their effects on cell cycle progression suggested otherwise. Notably, compound **12**, a representative piperidinyl chalcone, caused a buildup of cells in the G1 phase, blocking normal progression through G2/M, at 5 μM—a concentration that was close to its $IC_{50}$ value (3.4 μM). On the other hand, compounds **22** and **27**, which lack the basic functionality, had no effect on cell cycle progression at their $IC_{50}$ concentrations. The presence of the basic piperidine ring in **12** may have contributed to this small but significant difference.

At 5 μM, compound **12** slowed the progression of the G1-synchronized HCT 116 cells to the G2/M stage but had less effect on the transition of the G2-synchronized cells to G1. Since higher concentrations of **12** blocked the cell cycles of synchronized G1 and G2/M cells, it may be that the G1 → G2/M transition was more sensitive to intervention by this compound. However, Western blot analyses pointed to multiple effects of **12** on the cell cycle, involving both the early G1 phase (decrease in CDK4 and E2F) and the later G2/M phase (decrease in cyclin B).

The ability of methoxylated chalcones to arrest the cell cycle at the G2/M phase had been reported by other investigators.[11,16] In one report, methoxylated chalcones arrested G2/M progression when investigated at five times their $IC_{50}$ values on K562 human leukaemia cells.[11] These compounds were subsequently shown to disrupt microtubule assembly. Licochalcone A, a naturally occurring chalcone found in liquorice root, had pronounced effects on cell cycle progression, arresting a prostate cancer cell line (PC-3) in G2/M.[12] The induction of G2/M arrest was associated with changes in the levels of cell cycle regulatory proteins (a reduction of cyclin D1, CDK4 and CDK6) and the transcription factor E2F. These observations were made at 25 μM licochalcone, a concentration that was approximately twice its $IC_{50}$ against PC-3 cells.

Like licochalcone, the antiproliferative activity by compound **12** has been shown in this investigation to be linked to disruption of the cell cycle and changes in the expression of cell cycle regulatory proteins. However, unlike licochalcone, these effects were observed at a concentration close to its $IC_{50}$ value and after a relatively short incubation period (24 h). In addition, unusual for a methoxylated chalcone, compound **12** interfered with both G1 and G2/M phase transitions, with possibly a greater effect on the former.

## 4. Conclusion

This investigation has shown that presence of basic piperidine ring on the methoxylated chalcone template did not significantly affect antiproliferative potency but may influence the mechanism of antiproliferative activity. This can be seen from the antiproliferative activity of the piperidinylchalcone **12** which inhibited the cell cycle at both G1 and G2/M phases at a concentration that was close to its $IC_{50}$. The downregulation of cell cycle regulatory components (CDK4, cyclin B, E2F and phosphorylated Rb) lends further support to this conclusion. In contrast, chalcones **22** and **27** that lack the basic functionality disrupted the cell cycle at higher concentrations ($\geqslant 2 \times IC_{50}$). It is possible that these chalcones do not specifically disrupt cell cycle progression and interfere with cell replication by other means. Thus, besides moderating the physicochemical characteristics of solubility and lipophilicity, the basic piperidine function may have an important role in influencing the mechanism of antiproliferative activity of methoxylated chalcones. Further investigations will focus on the inclusion of other basic moieties besides *N*-methylpiperidine in the chalcone template and investigate their effects on antiproliferative activity. Possible candidates are other nitrogen-containing heterocycles (*N*-methylpiperazine, *N*-methylpyrrolidine) and aliphatic amines designed as open chain analogues of *N*-methylpiperidine.

## 5. Experimental

### 5.1. Chemistry

Melting points were determined in open glass capillary tubes and are uncorrected. $^1$H NMR spectra were recorded on a Bruker (DPX 300 MHz) spectrometer and are reported in $\delta$ (ppm), relative to TMS as an internal standard. Mass spectra were collected on a VG Micromass 7035 E mass spectrometer by chemical ionization. Analytical thin-layer chromatography (TLC) was carried out on precoated plates (silica gel F 254) and column chromatography was performed with silica gel 60 (70–230 mesh). Combustion analyses (C,H) were carried out by the Department of Chemistry, National University of Singapore.

**5.1.1. 1-Methyl-4-(2,4,6-trimethoxyphenyl)-1,2,3,6-tetrahydropyridine (2).** *N*-Methylpiperidone (0.15 mol) was added with stirring to a solution of 1,3,5-trimethoxybenzene (0.15 mol) in glacial acetic acid (200 ml) with temperature maintained below 25 °C. At the end of addition, HCl gas was bubbled through the reaction mixture for 1 h. The reaction mixture was then stirred for 24 h at 25 °C, and at 95–100 °C for 3 h. The solvent was removed under reduced pressure and the residue was diluted with water. The aqueous solution was extracted with ether, the ethereal layer was separated off and the aqueous layer was rendered alkaline with 1 M NaOH. A precipitate was obtained, filtered off, washed with water and dried. Recrystallization from ether/hexane (2/8) gave **2**, mp 121–123 °C (mp 118–121 °C[19]). $^1$H NMR ($CDCl_3$): 6.15 (2H, s), 5.53–5.52 (1H, m), 3.83 (3H, s), 3.77 (6H, s), 3.11–3.08 (2H, m), 2.68–2.64 (2H, t), 2.41 (3H, s), 2.39–2.37 (2H, m).

**5.1.2. *N*-Methyl-4-(2,4-dimethoxyphenyl)-1,2,3,6-tetrahydropyridine (9).** *N*-Methylpiperidone (0.15 mol) was similarly reacted with 1,3-dimethoxybenzene (0.15 mol) in glacial acetic acid (200 ml) under conditions described for **2**. On workup, **9** was obtained as a colourless oil (65%) and purified by column chromatography with 0.1% triethylamine in ethyl acetate as mobile phase. $^1$H NMR ($CDCl_3$): 7.10–7.07 (1H, m), 7.45–7.43 (2H, m), 5.74–5.72 (1H, m), 3.80 (3H, s), 3.78 (3H, s), 3.10–3.08 (2H, m), 2.65–2.62 (2H, m), 2.57–2.56 (2H, m), 2.40 (3H, s).

**5.1.3. *N*-Methyl-4-(4-methoxyphenyl)-1,2,3,6-tetrahydropyridine (15).** As described for **2**, *N*-methylpiperidone (0.15 mol) was added with stirring to a solution of methoxybenzene (0.15 mol) in glacial acetic acid (200 ml). Crude **15** was recrystallized from hexane to give the pure product in 72% yield (mp 102–104 °C). $^1$H NMR

($CDCl_3$) 7.33 (2H, d, $J$ = 9.0 Hz), 6.87 (2H, d, $J$ = 9.0 Hz), 5.97–5.94 (1H, m), 3.81 (3H, s), 3.24–3.22 (2H, m), 2.81–2.77 (2H, m), 2.64 (2H, m), 2.50 (3H, s).

**5.1.4. *N*-Methyl-4-(2,4,6-trimethoxyphenyl)piperidine (3).** Compound **2** (5 g) was hydrogenated in acetic acid–water (10:1, 150 ml) with 10% Pd/C (0.5 g) as catalyst at normal pressure (30–40 psi) and room temperature for 24 h. The reaction mixture was filtered over Celite and the solvent was evaporated under reduced pressure to give a residue that was diluted with water and rendered alkaline with 10 M NaOH solution. The precipitate thus obtained was filtered off, washed with water, dried and used without further purification for the next step. Yield: 96%. Mp 109–111 °C. $^1$H NMR ($CDCl_3$) 6.15 (2H, s), 3.81 (3H, s), 3.79 (6H, s), 3.14–3.05 (1H, m), 2.96–2.92 (2H, m), 2.48–2.34 (2H, m), 2.31 (3H, s), 2.05–1.97 (2H, m), 1.50–1.48 (2H, m).

**5.1.5. *N*-Methyl-4-(2,4-dimethoxyphenyl)piperidine (10).** Compound **9** (10 g) was hydrogenated in acetic acid–water (10:1, 200 ml) with 10% Pd/C (1 g) as catalyst under similar conditions as described for **2**. The aqueous solution was rendered alkaline with 10 M NaOH solution and extracted with ether. The solvent ether was removed and the residue was dissolved in 1 M HCl solution. The product was purified by reverse-phase column chromatography using a $C_{18}$ stationary phase (125 Å, 55–105 μM) with water–methanol as mobile phase. Elution was made progressively, starting with pure water and moving to 5% methanol/water, 10% methanol/water and finally 100% methanol. Compound **10** was obtained as a colourless liquid (92% yield). Accurate mass $[M+H]^+$ 236.1679 (236.1651). $^1$H NMR ($CDCl_3$) 7.13–7.10 (1H, m), 6.50–6.46 (2H, m), 3.82 (3H, s), 3.81 (3H, s), 2.98–2.95 (2H, m), 2.86–2.83 (1H, mb), 2.33 (3H, s), 2.12–2.04 (2H, m), 1.80–1.76 (4H, m).

**5.1.6. *N*-Methyl-4-(4-Methoxyphenyl)piperidine (16).** Compound **15** (5 g) was hydrogenated in acetic acid–water (10:1, 150 ml) using 10% Pd/C (0.5 g) as catalyst as described for **2** and worked up in a similar way. Compound **16** was obtained as a colourless liquid and used for the next step of reaction without further purification. Yield: 93%. $^1$H NMR ($CDCl_3$): 7.17 (2H, d, $J$ = 8.6 Hz), 6.87 (2H, d, $J$ = 8.6 Hz), 3.80 (3H, s), 2.99–2.96 (2H, m), 2.46–2.42 (1H, m), 2.33 (3H, s), 2.09–2.01 (2H, m), 1.83–1.74 (4H, m).

**5.1.7. 4-(3-Acetyl-4,6-dimethoxy-2-hydroxy)phenyl-1-methyl-piperidine (4).** Boron trifluoride dimethyl etherate (6 ml, 0.065 mol) was added dropwise to a stirred solution of **3** (1.8 g, 0.007 mol) in $CH_2Cl_2$ (50 ml), which had been cooled in an ice bath. Acetic anhydride (5 ml) was then added dropwise to the solution and stirring was continued for 24 h at room temperature. The reaction mixture was diluted with water, rendered alkaline with $Na_2CO_3$ and extracted with $CH_2Cl_2$. Removal of solvent in vacuo gave a solid residue that was recrystallized with methanol–water (2:1) to give **4** (83% yield, mp 208–210 °C). $^1$H NMR ($CDCl_3$): 14.17 (1H, OH), 5.94 (1H, s), 3.89 (3H, s), 3.87 (3H, s), 3.18–3.07 (1H, m), 2.94–2.90 (2H, m), 2.61 (3H, s), 2.48–2.34 (2H, m), 2.29 (3H, s), 2.05–1.96 (2H, m), 1.48–1.44 (2H, m).

**5.1.8. 4-(5-Acetyl-2,4-dimethoxy)phenyl-1-methyl-piperidine (11).** Compound **10** (3 g) was similarly reacted with boron trifluoride dimethyl etherate (11.7 ml, 0.13 mol) and acetic anhydride (9.6 ml) in $CH_2Cl_2$ (100 ml) as described for **3**. Compound **11** was obtained as a solid residue and recrystallized with methanol–water (2:1) to give the desired product in 85% yield. Mp 181–182 °C. $^1$H NMR ($CDCl_3$): 7.77 (1H, br s), 6.45 (1H, br s), 3.92 (3H, s), 3.89 (3H, s), 2.97–2.93 (2H, m), 2.82–2.72 (1H, m), 2.56 (3H, s), 2.30 (3H, s), 2.08–2.00 (2H, m), 1.87–1.76 (4H, m).

**5.1.9. 4-(3-Acetyl-4-methoxy)phenyl-1-methyl-piperidine (17).** Compound **16** (1.8 g) was similarly reacted with boron trifluoride dimethyl etherate (6 ml, 0.065 mol) and acetic anhydride (5 ml) in $CH_2Cl_2$ (50 ml) as described for **3**. Compound **17** was obtained as a solid residue and recrystallized with methanol–water (2:1) to give the desired product in 81% yield. Mp 63–65 °C. $^1$H NMR ($CDCl_3$): 7.62 (1H, d, $J$ = 2.4 Hz), 7.36 (1H, dd, $J_1$ = 2.3 Hz, $J_2$ = 8.4 Hz), 6.94 (1H, d, $J$ = 8.4 Hz), 3.91 (3H, s), 3.00–2.96 (2H, m), 2.62 (3H, s), 2.56–2.44 (1H, m), 2.33 (3H, s), 2.10–2.01 (2H, m), 1.85–1.75 (4H, m).

### 5.2. General procedure for the preparation of Series A–E chalcones

A solution of the aldehyde (1.2 mmol) in methanol (5 ml) was added dropwise to a stirred solution of the acetophenone (1 mmol) dissolved in 3% (w/v) NaOH in methanol (20 ml). The solution was stirred at room temperature (28 °C) for 12 h, after which the solvent was removed under reduced pressure. The resulting residue was dissolved in 1 M HCl and extracted with ether. The aqueous layer was rendered alkaline with saturated aqueous $Na_2CO_3$ solution to give a precipitate that was filtered, washed with water and dried. Recrystallization from methanol–water gave the desired product.

**5.2.1. 3-(2-Chlorophenyl)-1-[2-hydroxy-4,6-dimethoxy-3-(*N*-methylpiperidin-4-yl)phenyl]-prop-2-en-1-one (5).** Yield: 54%. Mp 194–195 °C. Accurate mass: $[M+H]^+$ 416.1626 (416.1629). $^1$H NMR ($CDCl_3$): 14.14 (1H, s), 8.15 (1H, d, $J$ = 15.6 Hz), 7.86 (1H, d, $J$ = 15.6 Hz), 7.73–7.64 (1H, m), 7.48–7.44 (1H, m), 7.35–7.31 (2H, m), 6.01 (1H, s), 3.96 (3H, s), 3.92 (3H, s), 3.19–3.14 (1H, m), 2.99–2.90 (2H, m), 2.47–2.44 (2H, m), 2.33 (3H, s), 2.10–2.02 (2H, m), 1.59 (2H, m).

**5.2.2. 3-(4-Chlorophenyl)-1-[2-hydroxy-4,6-dimethoxy-3-(*N*-methylpiperidin-4-yl)phenyl]-prop-2-en-1-one (6).** 49% yield. Mp 189–190 °C. Accurate mass: $[M+H]^+$ 416.1634 (416.1629). $^1$H NMR ($CDCl_3$): 14.18 (1H, s), 7.85 (1H, d, $J$ = 15.8 Hz), 7.73 (1H, d, $J$ = 15.8 Hz), 7.56–7.53 (2H, d, $J$ = 8.6 Hz), 7.41–7.38 (2H, d, $J$ = 8.4 Hz), 6.01 (1H, s), 3.97 (3H, s), 3.92 (3H, s), 3.23–3.18 (1H, m), 2.98–2.95 (2H, m), 2.48–2.41 (2H, m), 2.33 (3H, s), 2.10–2.02 (2H, m), 1.59 (2H, m).

**5.2.3. 1-[2-Hydroxy-4,6-dimethoxy-3-(*N*-methylpiperidin-4-yl)phenyl]-3-phenyl-prop-2-en-1-one (7).** Yield: 56%. Mp 199–200 °C. Accurate mass: $[M+H]^+$ 382.2017

(382.2018). $^1$H NMR ($CDCl_3$): 14.20 (1H, s), 7.86 (1H, d, $J$ = 15.6 Hz), 7.76 (1H, d, $J$ = 15.6 Hz), 7.62–7.58 (2H, m), 7.42–7.38 (3H, m), 5.99 (1H, s), 3.95 (3H, s), 3.89 (3H, s), 3.20–3.10 (1H, m), 2.96–2.92 (2H, m), 2.50–2.42 (2H, m), 2.30 (3H, s), 2.10–1.96 (2H, m), 1.53 (2H, m).

**5.2.4. 3-(2-Fluorophenyl)-1-[2-hydroxy-4,6-dimethoxy-3-(*N*-methylpiperidin-4-yl)phenyl]-prop-2-en-1-one (8).** Yield: 62%. Mp 192–194 °C. Accurate mass: $[M+H]^+$ 400.1930 (400.1924) $^1$H NMR ($CDCl_3$,) 14.18 (1H, s), 7.99 (1H, d, $J$ = 15.8 Hz), 7.85 (1H, d, $J$ = 15.8 Hz), 7.63–7.58 (1H, m), 7.38–7.11 (3H, m), 6.01 (1H, s), 3.97 (3H, s), 3.92 (3H, s), 3.19–3.14 (1H, m), 2.95 (2H, m), 2.48–2.43 (2H, m), 2.33 (3H, s), 2.09–2.02 (2H, m), 1.59 (2H, m).

**5.2.5. 3-(2-Chlorophenyl)-1-[2,4-dimethoxy-5-(*N*-methylpiperidin-4-yl)phenyl]-prop-2-en-1-one (12).** Yield: 57%. Mp 99–100 °C. Accurate mass: $[M+H]^+$ 400.1687 (400.1679). $^1$H NMR ($CDCl_3$): 8.06 (1H, d, $J$ = 15.8 Hz), 7.51 (1H, d, $J$ = 15.8 Hz), 7.74–7.69 and 7.46–7.29 (5H, m), 6.48 (1H, br), 3.95 (3H, s), 3.94 (3H, s), 3.01–2.97 (2H, m), 2.83 (1H, m), 2.34 (3H, s), 2.08–2.04 (2H, m), 1.86–1.79 (4H, m).

**5.2.6. 3-(4-Chlorophenyl)-1-[2,4-dimethoxy-5-(*N*-methylpiperidin-4-yl)phenyl]-prop-2-en-1-one (13).** Yield: 52%. Mp 161–163 °C. Accurate mass: $[M+H]^+$ 400.1676 (400.1679). $^1$H NMR ($CDCl_3$): 7.69–7.37 (7H, m), 6.48 (1H, br), 3.96 (3H, s), 3.94 (3H, s), 3.00–2.97 (2H, m), 2.83–2.82 (1H, m), 2.34 (3H, s), 2.08 (2H, m), 1.86–1.79 (4H, m).

**5.2.7. 1-[2,4-Dimethoxy-5-(*N*-methylpiperidin-4-yl)phenyl]-3-phenylprop-2-en-1-one (14).** Yield: 60%. Mp 132–134 °C. Accurate mass: $[M+H]^+$ 366.2072 (366.2069). $^1$H NMR ($CDCl_3$) 7.70 (1H, d, $J$ = 15.8 Hz), 7.54 (1H, d, $J$ = 15.8 Hz), 7.68, 7.62–7.59 (3H, m), 7.41–7.39 (3H, m), 6.48 (1H, br), 3.95 (3H, s), 3.93 (3H, s), 3.00–2.96 (2H, m), 2.82–2.81 (1H, m), 2.32 (3H, s), 2.08–2.03 (2H, m), 1.85–1.78 (4H, m).

**5.2.8. 3-(2-Chlorophenyl)-1-[2-methoxy-5-(*N*-methylpiperidin-4-yl)phenyl]prop-2-en-1-one (18).** Yield: 84%. Mp 90–91 °C. Accurate mass: $[M+H]^+$ 370.1577 (370.1574). $^1$H NMR ($CDCl_3$): 8.03 (1H, d, $J$ = 16.1 Hz), 7.73–7.70 (1H, m), 7.49 (1H, d, $J$ = 16.2 Hz), 7.52–7.30 (5H, m), 6.99 (1H, d, $J$ = 8.7 Hz), 3.91 (3H, s), 3.22 (2H, m), 2.54–2.44 (1H, m), 2.33 (3H, s, *N*-methyl), 2.40 (2H, m), 2.08–1.92 (4H, m).

**5.2.9. 3-(4-Chlorophenyl)-1-[2-methoxy-5-(*N*-methylpiperidin-4-yl)phenyl]prop-2-en-1-one (19).** Yield: 42%. Mp 115–117 °C Accurate mass: $[M+H]^+$ 370.1582 (370.1574). $^1$H NMR ($CDCl_3$): 7.64–7.38 (8H, m), 7.01 (1H, d, $J$ = 8.3 Hz), 3.92 (3H, s), 3.57–3.53 (2H, m), 2.76 (3H, s, *N*-methyl), 2.76–1.93 (7H, m).

**5.2.10. 1-[2-Methoxy-5-(*N*-methylpiperidin-4-yl)phenyl]-3-phenyl-prop-2-en-1-one (20).** Yield: 48%. Accurate mass: $[M+H]^+$ 336.1970 (336.1964). $^1$H NMR ($CDCl_3$): 7.69–7.40 (9H, m), 7.02–6.99 (1H, d, $J$ = 8.3 Hz), 3.92 (3H, s), 3.59–3.55 (2H, m), 2.78 (3H, s, *N*-methyl), 2.78–2.71 (1H, m), 2.47–2.02 (6H, m).

**5.2.11. 3-(2-Chlorophenyl)-1-(2,4-dimethoxyphenyl)prop-2-en-1-one (21).** Yield: 36.9%. Mp 119–120 °C. C (calcd 67.44, found 67.38) H (calcd 4.99, found 5.30). $^1$H NMR ($CDCl_3$): 8.04 (1H, d, $J$ = 15.8 Hz), 7.78 (1H, d, $J$ = 8.7 Hz), 7.71–7.68 (1H, m), 7.49 (1H, d, $J$ = 15.8 Hz), 7.46–7.40 (1H, m), 7.30–7.26 (2H, m), 6.58 (1H, d, $J$ = 8.7 Hz), 6.50 (1H, s), 3.90 (3H, s), 3.87 (3H, s).

**5.2.12. 3-(4-Chlorophenyl)-1-(2,4-dimethoxyphenyl)prop-2-en-1-one (22).** Yield: 63.7%. Mp 118–120 °C. C (calcd 67.44, found 67.41) H (calcd 4.99, found 5.34). $^1$H NMR ($CD_3OD$): 7.76 (1H, d, $J$ = 8.7 Hz), 7.68 (1H, d, $J$ = 15.0 Hz), 7.65 (1H, d, $J$ = 15.4 Hz), 7.58 (2H, d, $J$ = 8.6 Hz), 7.42 (2H, d, $J$ = 8.6 Hz), 6.67–6.64 (2H, m), 3.95 (3H, s), 3.91 (3H, s).

**5.2.13. 1-(2,4-Dimethoxyphenyl)prop-2-en-1-one (23).** Yield: 43.2%. Mp 75–76 °C. C (calcd 76.10, found 76.29) H (calcd 6.01, found 5.95). $^1$H NMR ($CDCl_3$): 7.77 (1H, d, $J$ = 8.7 Hz), 7.68 (1H, d, $J$ = 15.8 Hz), 7.62–7.58 (2H, m), 7.51 (1H, d, $J$ = 15.8 Hz), 7.41–7.38 (3 H, m), 6.59–6.55 (1H, dd, $J$ = 8.7 Hz, 2.3 Hz), 6.51 (1H, d, $J$ = 2.3 Hz), 3.91 (3H, s), 3.88 (3H, s).

**5.2.14. 3-(2-Chlorophenyl)-1-(2-methoxyphenyl)prop-2-en-1-one (24).** Yield: 27.8%. Mp 65–67 °C. C (calcd 70.46, found 70.33) H (calcd 4.80, found 4.48). $^1$H NMR ($CD_3OD$): 8.01 (1H, d, $J$ = 15.8 Hz), 7.72–7.69 (1H, m), 7.63 (1H, d, $J$ = 7.5 Hz), 7.48 (1H, t, $J$ = 8.7 Hz), 7.44–7.41 (1H, m), 7.35 (1H, d, $J$ = 15.8 Hz), 7.34–7.29 (2H, m), 7.08–6.99 (2H, m), 3.91 (3H, s).

**5.2.15. 3-(4-Chlorophenyl)-1-(2-methoxyphenyl)prop-2-en-1-one (25).** Yield: 41.7%. C (calcd 70.46, found 70.56) H (calcd 4.80, found 4.90). $^1$H NMR ($CDCl_3$): 7.64–7.60 (1H, m), 7.53 (1H, d, $J$ = 7.9 Hz), 7.52 (2H, d, $J$ = 8.3 Hz), 7.48 (1H, d, $J$ = 15.8 Hz), 7.37 (2H, d, $J$ = 8.3 Hz), 7.35 (1H, d, $J$ = 15.8 Hz), 7.06 (1H, dd, $J$ = 7.5 Hz, 1.1 Hz), 7.01 (1H, d, $J$ = 7.9 Hz), 3.90 (3H, s).

**5.2.16. 1-(2-Methoxyphenyl)prop-2-en-1-one (26).** Yield: 72.8%. C (calcd 80.65, found 80.47) H (calcd 5.92, found 5.68). $^1$H NMR ($CD_3OD$): 7.63 (1H, d, $J$ = 15.8 Hz), 7.65–7.56 (3H, m), 7.51–7.45 (1H, m), 7.38 (1H, d, $J$ = 15.8 Hz), 7.40–7.35 (3H, m), 7.06 (1H, d, $J$ = 7.5 Hz), 7.01 (1H, d, $J$ = 8.3 Hz), 3.91 (3H, s).

### 5.3. Materials for cell culture

HCT 116 (transformed human colon cancer cell line) was a gift from Dr. Balram Chowbay, National Cancer Centre, Singapore. MCF-7 (human breast cancer cell line), Jurkat (human leukaemic cell line), CCL 186 (normal human diploid embryonic lung fibroblast) and AG 01523C (normal human foreskin diploid fibroblast) were purchased from American Type Culture Collection (Rockville, MD, USA). The following cell cycle regulatory proteins were obtained from BD Biosciences, Pharmingen, Ont., Canada: cyclin D1, cyclin B, CDK 4, retinoblastoma pRb (a.a. 332–344) E2F-1 and β-actin. pRb was also purchased from Santa Cruz Bio-

technology, Inc., CA, USA. Luminol was purchased from Supersignal West Pico, Pierce, IL, US. RNase A and propidium iodide were from Sigma Chemical Co, USA.

### 5.4. MTT assay for determination of antiproliferative activity

The growth inhibitory activity of Series A–C chalcones was determined on the following cell lines: HCT 116 (human colon cancer cell line), MCF-7 (human breast cancer cell line), Jurkat (human leukaemic cell line), CCL 186 (normal human diploid embryonic lung fibroblast) and AG 01523C (normal human foreskin diploid fibroblast) using a modified version of the microculture tetrazolium assay.[20] Series D and E compounds were only tested on HCT116 and CCL 186 cells. HCT116 cells were cultured in McCoy's 5A modified medium with 1.5 mM L-glutamine (ATCC) supplemented with 10% FBS and 1% antibiotics, and cells of passages 19–22 were used. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% antibiotics, and cells of passages 3–8 were used and CCL 186 in minimum essential medium α medium (MEM) with 2 mM L-glutamine (Gibco), supplemented with 1% 100× non-essential amino acids, 1.5 g/L sodium bicarbonate, 10% FBS and 1% antibiotics, and cells of passages 14–18 were used. AG 01523C cells were cultured in minimum essential medium (MEM) with non-essential amino acids, supplemented with 10% FBS and 1% antibiotics but without L-glutamine. Cells of passages 10–14 were used.

Once the cells reached 90% confluency, a cell suspension was prepared by mechanical disaggregation of the floating cells (Jurkat) or by trypsinization of monolayer cultures (other cell lines). Cell counts were performed, and the suspensions were diluted accordingly to give $1 \times 10^5$ cells/ml with the appropriate media. Aliquots (100 μl) of the cell suspension were added to each well in a 96-well microtitre plate. The cells were incubated for 24 h (37 °C, 5% $CO_2$) for incubation. Stock solutions of the test compound were prepared in DMSO and serial dilutions were made with media to over a 100-fold concentration range. Not more than 1% DMSO (final concentration) was present in each well. The test sample was incubated with the cells for 72 h. Wells without cells and those with cells in culture media/DMSO were examined in parallel. 0.1% SDS and dextran were included as positive and negative controls, respectively. At the end of the incubation period, the medium was decanted and replaced with 100 μl MTT solution (0.5 mg/ml in 1× phosphate-buffered saline solution (PBS)). The cells were incubated for another 3 h after which the medium was removed from each well by pipetting and the cells were carefully washed with PBS (100 μl). DMSO (150 μl) was added to each well to lyse the cells and to dissolve the purple formazan crystals. The absorbance of the formazan product was measured within 30 min at 590 nm on a microtitre plate reader. The absorbance values obtained at each concentration (triplicates for each run and three independent runs were carried out using cells of different passage numbers) were averaged, adjusted by subtraction of blank values (wells without cells) and expressed as a percentage of the average absorbance obtained from control wells (in the absence of test compound). $IC_{50}$ values were determined from logarithmic plots of the % absorbance versus concentration generated using Prism GraphPad (San Diego, CA, USA).

### 5.5. Cell cycle analysis

Human colon carcinoma cells (HCT 116) and normal human lung fibroblast cells (CCL 186) were grown to confluence and maintained in this state for a minimum of 5 days. The cells were then trypsinized and subcultured at 8000 cells/$cm^2$ in growth medium containing 10% fetal calf serum and the test compound was added either immediately (G1 release) or 24 h later (G2 synchronized). The cells were incubated in the presence of the test compound for up to 48 h. At indicated time points after compound addition, the cells were harvested, trypsinized and fixed in 70% ice-cold ethanol for a minimum of 24 h. After centrifugation, the supernatant was discarded and the pellet was treated with RNase A (200 μg/ml) for 30 min at room temperature, followed by cell staining using propidium iodide at a final concentration of 20 μg/ml. The stained cells were then analyzed for cell cycle distribution in the Go/G1, S and G2/M phases on a FACScan flow cytometer (Beckman Coulter CA, USA) equipped with an argon-ion laser (488 nm) using the WinMDI (version 2.8) software. Compound **12** was examined at 0.5, 2, 5, 10 and 20 μM on HCT 116 cells and at 0.5 and 2 μM on CCL 186 cells. Compounds **22** and **27** were tested on HCT 116 cells at concentrations of 3, 6, 10 and 20 μM (**22**) and at 5, 10, 20 and 50 μM (**27**).

### 5.6. Protein extraction and Western blot analysis

Human colon carcinoma cells (HCT 116) were grown to confluence and maintained in this state for a minimum of 5 days. The cells were then trypsinized and subcultured at 8000 cells/$cm^2$ in growth media (McCoy 5A) containing 10% fetal calf serum. The test compound was added at the specified concentration and incubated with the cells for up to 24 h. Control or treated cells were rinsed with ice-cold PBS and transferred to Eppendorf tubes. The contents of each tube were suspended in 100 μl lysis buffer (Phosphatase™ extraction buffer, Novagen) in the presence of protease inhibitors (15 μl, protease inhibitor cocktail, Roche). The cell suspension was incubated for 20 min (4 °C) and centrifuged (20 min, 10 K rpm) and the clear supernatants were stored in aliquots at −80 °C. Protein concentrations were measured with protein assay reagent (Dye Reagent Concentrate, Bio-Rad). For Western blot analysis, the protein extract was diluted with Laemmli buffer (4×) and analyzed by SDS gel electrophoresis on appropriate polyacrylamide gels (6–12%) with 20 μg protein loaded on each lane. The proteins on the gels were then transferred to polyvinylidene diflouride (PVDF) membranes (Bio-Rad) by a semi-dry transfer method. After blocking with Tris-buffered saline containing 5% low-fat milk and 0.1% Tween 20, the membranes were probed with

antibodies against the following proteins: cyclin D1, cyclin B, CDK 4, Rb, E2F-1 and β-actin using standard techniques. The membrane blots previously probed with protein specific primary antibodies were reacted (1 h) with secondary antibodies (horseradish peroxidase anti-mouse IgG or horseradish peroxidase anti-rabbit IgG), washed extensively with Tris-buffered saline (0.1% Tween 20) and submerged (5 min) in a mixture of horseradish peroxidase substrate (luminol). Membranes were lightly hand-blotted dry, exposed for varying periods (10 s to 5 min) to autoradiography film and developed to visualize the antibody–antigen complexes.

## Acknowledgments

X. L. Liu gratefully acknowledges research scholarship support from the National University of Singapore. This work was supported by Grant RP140000042112 from National University of Singapore.

## References and notes

1. Dimmock, J. R.; Elias, D. W.; Beazely, M. A.; Kandepu, N,M. *Curr. Med. Chem.* **1999**, *6*, 1125.
2. Go, M. L.; Wu, X.; Liu, X. L. *Curr. Med. Chem.* **2005**, *12*, 483.
3. De Vincenzo, R.; Scambia, G.; Benedetti, P. P.; Ranelletti, F. O.; Bonanno, G.; Ercoli, A.; Belle Monache, F.; Ferrari, F.; Piantelli, M.; Mancuso, S. *Anticancer Drug Des.* **1995**, *10*, 481.
4. De Vincenzo, R.; Ferlini, C.; Distefano, M.; Gaggini, C.; Riva, A.; Bombardelli, E.; Morazzoni, P.; Valenti, P.; Belluti, F.; Ranelletti, F. O.; Mancuso, S.; Scambia, G. *Cancer Chemother. Pharmacol.* **2000**, *46*, 305.
5. Park, E. J.; Park, H. R.; Lee, J. S.; Kim, J. *Planta Med.* **1998**, *64*, 464.
6. Yamamoto, S.; Aizu, E.; Jiang, H.; Nakadate, T.; Kiyoto, I.; Wang, J. C.; Kato, R. *Carcinogenesis* **1991**, *12*, 317.
7. Nguyen-Hai, N.; Kim, Y.; You, Y. J.; Hong, D. H.; Kim, H. M.; Ahn, B. Z. *Eur. J. Med. Chem.* **2003**, *38*, 179.
8. Stoll, R.; Renner, C.; Hansen, S.; Palme, S.; Klein, C.; Belling, A.; Zeslawski, W.; Kamionka, M.; Rehm, T.; Muhlhahn, P.; Schumacher, R.; Hesse, F.; Kaluza, B.; Voelter, W.; Engh, R. A.; Holak, T. A. *Biochemistry* **2001**, *40*, 336.
9. Kumar, S. K.; Hager, E.; Pettit, C.; Gurulingappa, H.; Davidson, N. E.; Khan, S. R. *J. Med. Chem.* **2003**, *46*, 2813.
10. Sabzevari, O.; Galati, G.; Moridani, M. Y.; Siraki, A.; O'Brien, P. J. *Chem. Biol. Interact.* **2004**, *148*, 57.
11. Lawrence, N. J.; McGown, A. T.; Ducki, S.; Hadfield, J. A. *Anti-Cancer Drug Des.* **2000**, *15*, 135–141.
12. Fu, Y.; Hsieh, T. C.; Guo, J.; Kunicki, J.; Lee, M. Y. W. T.; Darzynkiewicz, Z.; Wu, J. M. *Biochem. Biophys. Res. Commun.* **2004**, *322*, 263.
13. Won, S. J.; Cheng, C. T.; Tsao, L. T.; Weng, J. R.; Ko, H. H.; Wang, J. P.; Lin, C. N. *Eur. J. Med. Chem.* **2005**, *40*, 103.
14. Edwards, M. L.; Stemerick, D. M.; Sunkara, P. S. *J. Med. Chem.* **1990**, *33*, 1948.
15. Ducki, S.; Forrest, R.; Hadfield, J. A.; Kendall, A.; Lawrence, N. J.; McGown, A. T.; Rennison, D. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1051.
16. Lawrence, N. J.; Rennison, D.; McGown, A. T.; Hadfield, J. A. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3759.
17. Xia, Y.; Yang, Z. Y.; Xia, P.; Bastow, K. F.; Nakanishi, Y.; Lee, K. H. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 699.
18. Nielsen, S. F.; Larsens, M.; Boesen, T.; Schenning, K.; Kromann, H. *J. Med. Chem.* **2005**, *48*, 2667.
19. Schoepfer, J.; Fretz, H.; Chaudhuri, B.; Muller, L.; Seeber, E.; Meijer, L.; Lozach, O.; Vangrevelinghe, E.; Furet, P. *J. Med. Chem.* **2002**, *45*, 1741.
20. Alley, M. C.; Scudiero, D. A.; Monks, A.; Hursey, M. L.; Czerwinski, M. J.; Fine, D. L.; Abbott, B. J.; Mayo, J. G.; Shoemaker, R. H.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 589.
21. Liu, M. Ph.D. Thesis, National University of Singapore, Singapore, 2003.