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Chalcone HTMC causes in vitro selective cytotoxicity, cell-cycle G_1 phase arrest through p53-dependent pathway in human lung adenocarcinoma A549 cells, and in vivo tumor growth suppression

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ABSTRACT

The present Letter identified 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC) as a potent in vitro cytotoxic agent with selective activity against cell lines derived from human lung cancer. In A549 lung adenocarcinoma cells, HTMC caused G1 phase cell-cycle arrest. HTMC treatment also led to an inhibition of cell-cycle regulatory proteins phosphorylation of cdc2 (Tyr^{15} and Tyr^{161}) and Rb (Ser^{795} and $Ser^{807/811}$), which was accompanied by the accumulation of tumor suppresser genes p53 and p21. In addition, in vivo data demonstrated that HTMC act as a tumor growth suppressing agent.

Lung cancer is the most common cause of cancer-related death in men and women worldwide including Taiwan.[1] In addition, the incidence of adenocarcinoma, a subtype of non-small cell lung cancer is one of the most lethal cancers because of its high incidence and mortality.[2] The survival rate of patients with lung adenocarcinoma is poor even when treatment is given in the early stage of the disease, because most patients already have disease that has metastasized.[2] Novel chemotherapeutic agents have been developed against lung cancer, include camptothecin (CPT), however, it is not effective against lung adenocarcinoma due to tolerance of the cancer cells toward this drug.[3] Several CPT derivatives including irinotecan and topotecan have also been introduced to lung cancer therapy.[3] However, the response rates to these drugs remain low, and the overall survival rate has not been much improved. Furthermore, chemotherapy consisting of CPT derivatives usually encounters serious side effects, which hampers the continuous administration of these drugs to treat the disease.[3] Therefore, the development of novel chemotherapeutics with selective activity and alternative mode of action to treat lung adenocarcinoma is desired.

Chalcones are precursors of flavonoids and isoflavonoids, which are abundant in edible plants. The chemical structure of chalcones (1,3-diphenyl-2-propen-1-ones) consists of two aromatic rings joined by a three-carbon α,β-unsaturated carbonyl system. Previous studies have indicated that flavonoids, chalcones, and their derivatives demonstrate anticancer activity in various tumor cells.[4] However, the exact mechanisms by which chalcone compounds exert their cytotoxic effects in cancer cells remains unclear. Through screening of chalcone compounds that inhibit cancer cell proliferation, we previously reported that 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC, Fig. 1), a natural chalcone from a medicinal plant *Caesalpinia pulcherrima*,[5] has significant cytotoxic

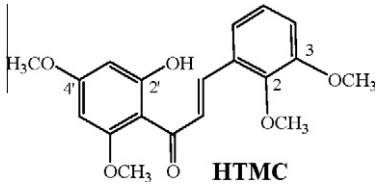

Figure 1. Chemical structure of 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC).

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activity against Jurkat (human lymphocytic) and U937 (human monocytic) cells,[6] however, the molecular mechanisms of its anti-proliferative action on malignant cell growth are not clear. On the other hand, a polymethoxyphenyl moiety is frequently met in the number of naturally occurring anticancer agents such as colchicine and combretastatin A.[7] In an effort to contribute to develop potential anticancer agents, and as part of a subsequent study to further explore the mechanism of HTMC anticancer activity,[6] we disclose here its effects on human lung adenocarcinoma cells.

HTMC (Fig. 1), was prepared by the Claisen–Schmidt condensation of 2-hydroxy-4,6-dimethoxyacetophenone with 2,3,-dimethoxybenzaldehyde in the presence of KOH (50%) following the procedure reported previously.[6,8] In this Letter, in an initial approach to analyze the effects of HTMC on human lung cancer cell lines, we performed MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] cytotoxicity assay,[8,9] using A549, H1299, and H1355 tumor cell lines in comparison to normal cell lines MRC-5, SV-40 transfected Beas-2B, and WI-38 cells. Cells seeded in a 96-well plate were treated with HTMC at concentrations of 6.25, 12.5, 25.0, and 50.0 μM. Cells treated with solvent (DMSO) were used as controls. After 24 h treatment with HTMC, the proliferation of three lung cancer cell lines were significantly reduced in a wide range of doses, with A549 cells being slightly sensitive than H1299 and H1355 cells (Fig. 2A). A549 cells treated with 50 μM HTMC for 24 h reduced the proliferation by 59%, and the calculated IC_{50} values were 47, 48, and 76 μM against A549, H1355, and H1299, respectively. In contrast, normal cell lines MRC-5 and Beas-2B cells were not affected with 50 μM HTMC, the highest concentration tested. To further examine the cell-specific anti-proliferation effect of HTMC, the lung cancer cells A549 and another normal cell line WI-38 cells were treated with HTMC for 24 h and subjected to apoptotic cell morphology observation by phase contrast microscopy (Fig. 2B). The apoptotic cells were dose-dependently increased in cancer cell line A549 cells as compared with normal cell line WI-38 cells (Fig. 2B). The arrows indicated the apoptotic cells induced by HTMC in A549 cells (Fig. 2B, *arrows*). These results indicated that HTMC apparently showed selective cytotoxicity against cancer-derived cells. Among the three lungs cancer cell lines examined, HTMC was more sensitive to A549 cells than H1299 and H1355, subsequently led to further examination on the mechanism of the anti-proliferative effect of HTMC in A549 cells.

Induction of apoptosis and/or inhibition of cell proliferation are highly correlated with the activation of variety of intracellular signaling pathways leading to arrest of cell cycle in the G1, S, or G2/M phase.[10] Cell-cycle regulation and its modulation by various plant-derived agents are gaining widespread attention in recent years.[11] A large number of phytochemicals have been shown to inhibit cell-cycle progression of various cancer cells.[12] To understand the mechanism involved in the anti-proliferative effects of HTMC, we performed the A549 cells cell-cycle phase distribution analysis. A549 cells were exposed with HTMC in the concentration range 6.25–50 μM for 24 h and stained with propidium iodide and analyzed by flow cytometry to determine the distribution of the total cells population in the different phases (G1, S, and G2/M). As shown in Fig. 3A, in the HTMC treated A549 cells the G1-phase population maximally increased together with a decreased S phase population (p <0.05). After 24 h of 12.5 μM HTMC treatment, the G1 population was increased from 62.9% to 83.4% and the S phase

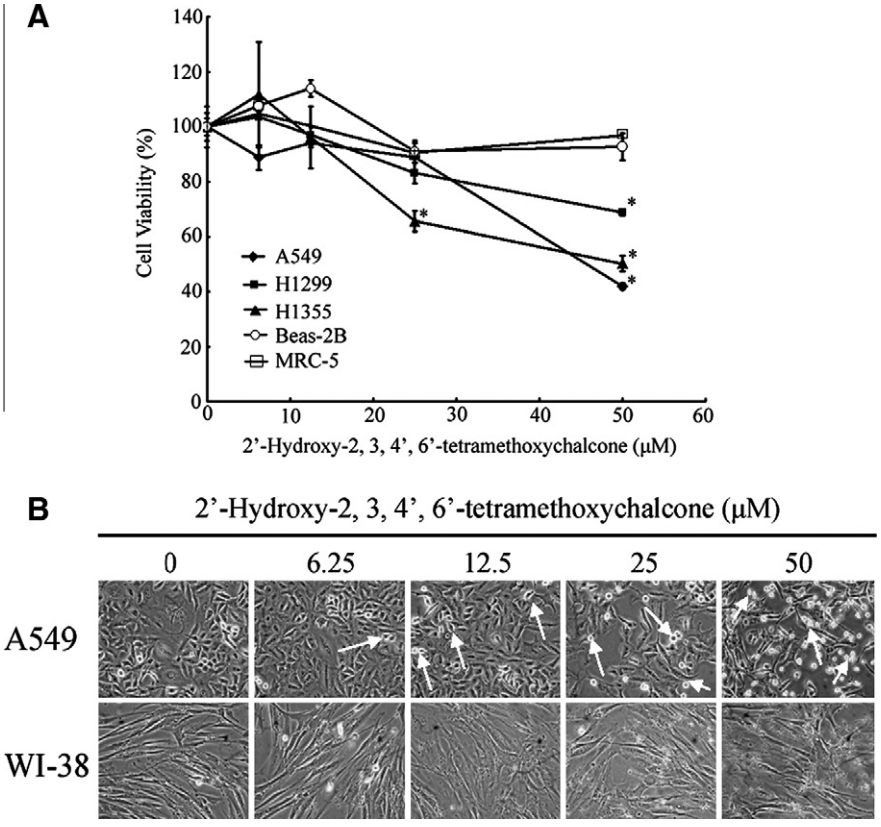
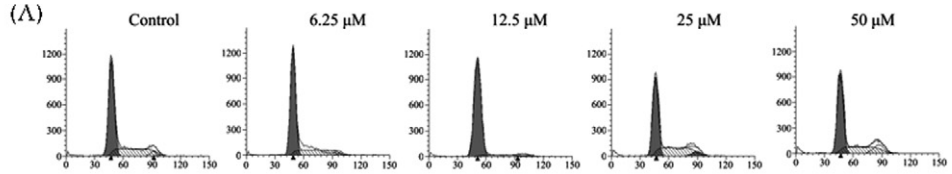
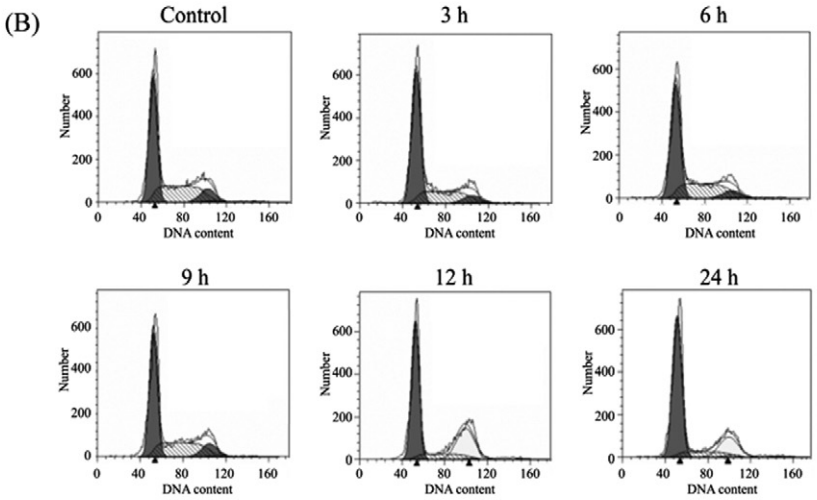


Figure 2. (A) Cytotoxic effect of 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC) in A549, H1299, H1355 lung cancer cells, and in normal cell MRC-5 and SV-40 transfected Beas-2B. Cells were plated and incubated with indicated concentrations of HTMC (0, 6.25, 12.5, 25, and 50 μM) or control DMSO, after 24 h of incubation the viable cells were measured by MTS assay. Data represent the means ± SEM of triplicate experiments (*) indicated a p <0.05 with student t-test, as compared with DMSO treated cells. (B) Morphological changes of A549 and WI-38 after treatment with HTMC for 24 h.



2'-Hydroxy-2, 3, 4', 6'-tetramethoxychalcone (μM)

	Control	6.25	12.5	25	50
G1 (%)	62.9 ± 2.9	68.8 ± 9.6	83.4 ± 12.3	59.6 ± 1.8	53.74 ± 15.3
S (%)	31.34 ± 2.4	28.9 ± 5.9	13.3 ± 10.4	25.1 ± 13.1	26.2 ± 2.3
G2/M (%)	5.8 ± 1.6	4.5 ± 0.4	3.2 ± 1.9	15.3 ± 11.3	7.1 ± 3.8



2'-Hydroxy-2, 3, 4', 6'-tetramethoxychalcone (12.5 μM)

	Control	3	6	9	12	24 (h)
G1 (%)	59.6 + 6.3	56.2 + 6.6	50.1 + 7.0	56.1 + 1.9	65.9 + 11.8	80.0 + 5.3
S (%)	28.8 ± 7.5	35.0 ± 5.9	38.8 + 4.4	32.1 ± 3.2	17.6 ± 4.2	13.2 ± 4.4
G2/M (%)	11.5 ±1.9	8.7 ± 1.9	10.9 ± 3.6	11.7 ± 1.7	16.4 ± 14.6	9.4 ± 8.8

Figure 3. Effect of 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC) on A549 cell-cycle distributions. (A) Exponentially growing A549 cells were left untreated or were treated with indicated concentrations of HTMC (0, 6.25, 12.5, 25, and 50 μM) for 24 h and subjected to flow cytometric cell-cycle analysis. (B) A549 cells were left untreated or were treated with 12.5 μM HTMC, and cell-cycle distributions analyzed as indicated time periods. Cell-cycle distributions are shown by histogram of DNA content and cell number determined by flow cytometry. Data represent the means ± SEM of triplicate experiments and compared with DMSO treated cells (p <0.05).

population was decreased from 31.3% to 13.3%. The cell population in the G2/M phase was not changed significantly by 12.5 μM HTMC treatment (p <0.05). Noticeably, there was no significant effect (p <0.05) on the cell-cycle population when the HTMC concentration increased from 12.5 μM to 25 and 50 μM (Fig. 3A). Furthermore, A549 cells treated with 12.5 μM HTMC for 12 h and 24 h also increased the G1 phase population in a time-dependent fashion with concomitant decrease in the S phase (Fig. 3B). Interestingly, A549 cells treated with 12.5 μM HTMC for 6 h temporarily led to decreased (p <0.05) G1 phase population with concomitant increase in the S phase (Fig. 3B). This indicated that proliferation and S-phase progression was adversely affected within 6 h of treatment, while a block of cell-cycle progression in G1 in this non-synchronized culture unfolded within 12 h of treatment. These results indicated that the cell-cycle arrest at G1 phase was the cause of HTMC anti-proliferation effect in A549 cells.

The control of the cell cycle is a highly regulated process, which involves a complex cascade of events. The modulation of cell-cycle regulatory proteins, include the cyclins, cyclin-dependent kinases (Cdk's), and Cdk inhibitors (Cki's) that drive cell-cycle progression through the phosphorylation and dephosphorylation of cell-cycle checkpoints at G1/S and G2/M.[13] To understand the mechanism underlying G1 arrest in HTMC-treated A549 cells, we next determined the effect of HTMC on cell-cycle regulatory molecules including cell division cycle 2 kinase (cdc2) and retinoblastoma protein (Rb). A549 cells were treated with different concentrations (0, 6.25, 12.5, and 25 μM) of HTMC for 24 h and then harvested for Western blot analysis.[8] The cdc2 phosphorylation were examined

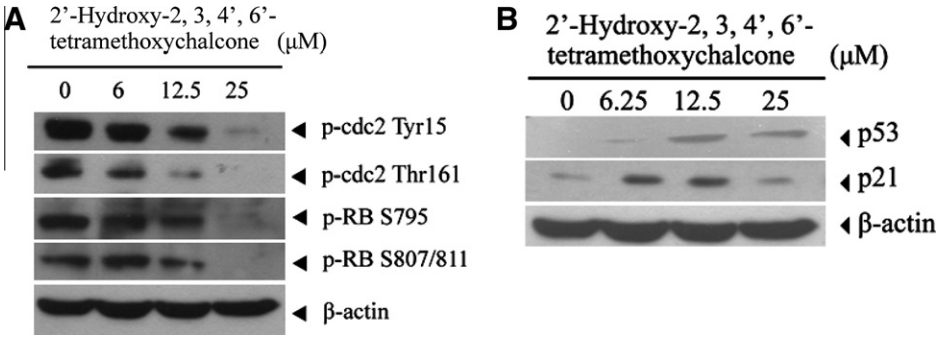


Figure 4. Effect of 2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (HTMC) on cell-cycle regulatory proteins. (A) Western blot analysis of phosphorylation status of cdc2 and Rb in 24 h HTMC treated A549 cells. (B) Expression of p53 and p21 protein in A549 cells after treatment with HTMC for 24 h.

by immunoblot cellular homogenates with anti-phospho cdc2 polyclonal antibodies that detect cdc2 when it is phosphorylated at Thr^{161} or Tyr^{15}, two sites known to be important for full activation of the kinase.[13] As shown in Fig. 4A, HTMC caused a dose-dependent de-phosphorylation of cdc2 at Thr^{161} and at Tyr^{15}. On the other hand, the protein Rb acts as a critical regulator for the G1–S phase progression of the cell cycle by trapping E2F family, an essential transcriptional factor required for the expression of cell proliferation-associated genes.[14] We subsequently determined the phosphorylation status of Rb following exposure of exponentially growing A549 cells to HTMC by immunoblotting using specific antibodies against the phosphorylation sites in Rb.[8] As shown in Fig. 4A, the degrees of Rb phosphorylation at Ser^{795} and $Ser^{807/811}$ were down-regulated by HTMC in a dose-dependent manner. It is known that interaction between pRb and E2F family controls over G1–S transition of the cell cycle. In early G1, pRb is phosphorylated to a limited extent by cdk4 and cdk6 in association with D-type cyclins, this hypophosphorylated pRb forms a complex with E2F transcription family and preventing E2F from DNA, therefore causing cell cycle to arrest in G1.[14] As reported previously, phosphorylation of Ser^{795}, $Ser^{807/811}$ initiates the process of cdk-mediated pRb inactivation and the deliberation of E2F.[14] In this letter, Western blot analysis of pRb expression showed phosphorylation of pRb was inhibited by HTMC (Fig. 4A), in combination with flow cytometry analysis in Figure 3A showed treatment of HTMC increased G1 arrest by 20% at 12.5 μM. In Figure 3B, a further time-course treatment of HTMC (12.5 μM) yielded the same result that cells arrest in G1 increased by 20%, indicating an inhibitory effect of HTMC on A549 cell growth by decreased pRb phosphorylation at Ser^{795} and $Ser^{807/811}$. These results indicated that reduced expression of phospho-cdc2 and phosphor-Rb may be involved in HTMC-induced G1 phase arrest and led to A549 cell growth inhibition.

Tumor suppressor gene p53 is a key element in the induction of cell-cycle arrest and apoptosis following DNA damage or cellular stress in human cells.[15] The p53-dependent cell-cycle arrest requires trans-activation of $p21^{WAF1/Cip1}$ or other cell-cycle-related factors.[15] The induction of $p21^{WAF1/Cip1}$ causes subsequent arrest in the G1 or G2/M phase of the cell cycle via its ability to inhibit cdc2.[16] We therefore, next determined the effect of HTMC on the cytoplasmic levels of p21 and p53. As shown by immunoblot analysis in Fig. 4B, HTMC dose-dependently upregulated the expression of tumor suppressor protein p53 and its downstream target molecule p21. The induction of p21 protein expression was p53-dependent, because incubation of A549 cells with HTMC did not cause any significant change in p21 protein expression (data not shown). It is unclear at the present time whether HTMC could also cause DNA damage and triggers p53 activation and accumulation of p21 and remain to be further elucidated. Taken together, the upregulation of p21, p53, and the downregulation of phospho-cdc2 and phosphor-Rb may be one of the molecular mechanisms by which HTMC inhibited A549 cells growth and induced G1 cell-cycle arrest.

We also examined the in vivo antitumor activity of HTMC.[8] For this purpose, 1×10^7 A549 cells injected sc into the flank of Balb/c mice rapidly gave rise to exponentially growing tumors. Small solid tumors were observed 7 days following cell inoculation. The tumor volumes were monitored every 3 days. As shown in Figure 5, the results showed that after 30 days the mean volume of tumors in mice treated with HTMC (1 mg/kg body weight) was reduced by 33% as compared with the vehicle-treated mice (control group mean, $1654.4 \pm 124.5\ mm^3$; HTMC group mean, $1234.0 \pm 204.7\ mm^3$; $p < 0.05$). The mean volume of tumors in positive control sesame oil treated mice was reduced to $731.8 \pm 140.3\ mm^3$ (Fig. 5). HTMC was well tolerated by the mice, and the animals did not show any differences in the body weights and health or behavioral problems. To our knowledge, studies described in this Letter represent the first systematic characterization of both in vitro and in vivo anticancer activities of HTMC.

In summary, this study demonstrated for the first time that HTMC was a selective cytotoxic agent and induced G1 cell-cycle arrest in human lung cancer cells, and the upregulation of p21, p53 and the downregulation of cdc2, Rb may be one of its molecular mechanisms. In addition, in vivo data demonstrated that HTMC act as a tumor growth suppressing agent. Therefore, the potential

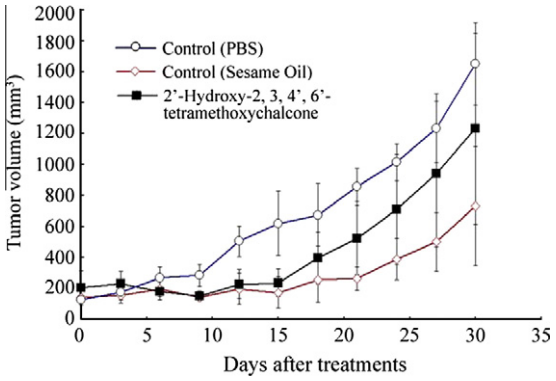


Figure 5. Effect of HTMC on tumor growth in vivo. Approximately 1×10^7 A549 cells were sc injected into each flank of the studied mice to initiate tumor growth. Twenty-four hours after cell implantation, the group 1 continued to receive sterilized PBS, whereas animals in groups 2 and 3 received sesame oil and HTMC (1 mg/kg body weight, $N = 5$, respectively). Sesame oil and HTMC were ip injected every week. Once tumors started to grow, their sizes were measured every 3 days and the tumor volume was calculated. The quantitative data are shown as means ± SD of average tumor volume for each treatment group.

of HTMC to inhibit lung cancer cells and the chemical nature of this low-molecular-mass compound that translates to lower cost of synthesis suggested that it may be developed as a potential therapeutic drug to treat lung cancer.

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Supplementary data

Supplementary data (experimental procedure for synthesis of HTMC and its ^{1}H, ^{13}C NMR and ESI-MS spectral data; and experimental procedures for biological testing are included) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.056.

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