(S)-3-(3-Fluoro-4-Methoxybenzyl)-5,6,7-Trimethoxychroman-4-One Suppresses the Proliferation of Huh7 Cells by Up-regulating P21 and Inducing G₂/M Phase Arrest

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Abstract. Background/Aim: Hepatocellular carcinoma (HCC) is a prevalent type of cancer worldwide. Although sorafenib is the only chemotherapy agent used for HCC, there is a need to discover a more potent anticancer agent with reduced side-effects. The compound, (S)-3-(3-fluoro-4-methoxybenzyl)-5,6,7-trimethoxychroman-4-one (FMTC), was designed to inhibit tubulin assembly but its specific mechanisms of action have not been previously investigated. Herein, we investigated the regulation mechanisms by which FMTC affects the proliferation of the HCC cell line, Huh7.

Materials and Methods: The effects of FMTC on cell viability and growth were analyzed in the HCC cell line, Huh7. Cell cycle and apoptosis regulated by FMTC were analyzed using flow cytometry. To verify the regulation of mRNA and protein expression of cell proliferation-related factors by FMTC in Huh7 cells, RT-qPCR and western blot analyses were employed. Results: FMTC suppressed cell division dose-dependently by triggering cell cycle arrest at the G₂/M phase via p21 up-regulation. The increased phosphorylation of histone H3 on Ser-10 and the condensation of chromatin in FMTC-treated cells indicated mitotic arrest. Prolonged FMTC-induced cell cycle arrest triggered apoptosis. Conclusion: FMTC inhibits the proliferation of human liver cancer cells by up-regulating p21, thereby inducing cell cycle arrest at the G₂/M phase.

These findings highlight FMTC as a novel agent for HCC treatment.

Liver cancer is one of the most common cancers worldwide and hepatocellular carcinoma (HCC) accounts for 85%–90% of all primary liver cancers (1). Numerous studies indicate that alcohol abuse and smoking contribute to liver cancer development (2). HCC can also result from continuous liver cell damage caused by hepatitis B and C virus infection and exposure to aflatoxin (2). Although HCC is mainly treated through chemotheraphy and surgical resection, treatment is frequently ineffective because of rapid HCC proliferation and development of drug resistance (2). Currently, sorafenib is the only liver cancer drug approved by the US Food and Drug Administration (3). However, side-effects are common and include drug resistance and sensory neuropathy (2). Thus, more effective HCC treatments are urgently needed.

The cell cycle is regulated by checkpoint mechanisms, which respond to DNA damage, DNA replication, and spindle formation (4). The dysregulation of checkpoint mechanisms promotes cancer development and alters the sensitivity of tumor cells to chemotherapy, thereby negatively impacting cancer treatment (4). The cell cycle is regulated by cyclins and cyclin-dependent kinases (CDKs) (5). Activation of cyclin/CDK complexes promotes entry into each cell cycle phase (G₁, S, G₂, and M) (4). Cyclin E regulates the completion of the G₁ phase by binding to CDK1, whereas cyclin A and B1 induce entry into mitosis (the M phase) by binding to CDK1 (4). p21Waf1/Cip1/Sdi1, a CDK inhibitor belonging to the CIP/KIP family, binds to CDK1 and CDK2 complexes, thereby inhibiting the cyclin/CDK complex (6). p21 regulates the cell cycle via p53-dependent or independent pathways and has been reported to be involved in apoptosis and aging (6). Past studies have reported chemicals that exhibit anticancer effects by up-regulating p21 expression, thereby inducing cell cycle arrest and apoptosis (7). Diallyl disulfide and...
cinobufagin are reported to be effective against esophageal cancer by up-regulating p21, thereby inducing G2/M phase arrest (8, 9). Microtubule-interfering agents (MIAs) inhibit the cell cycle by interfering with mitotic spindle formation and inducing M phase arrest (10). Most MIAs, such as paclitaxel and colchicine, trigger apoptosis by phosphorylating Bcl-2 and activating JNK signaling (11, 12). (S)-3-(3-fluoro-4-methoxybenzyl)-5,6,7-trimethoxychroman-4-one (FMTC) is a novel compound that contains a trimethoxyphenyl group and structurally resembles colchicine. Because the trimethoxyphenyl group binds to the colchicine binding site on tubulin, FMTC may inhibit tubulin polymerization (13, 14). However, studies on FMTC, its potential antitumor effects, and the underlying molecular mechanisms are insufficient.

Given the widespread occurrence of HCC and the limited efficacy of current standard systemic treatments, there is a pressing need for development of more potent therapeutic agents. Herein, we have studied the potential of FMTC as a cancer growth inhibitor, an alternative therapeutic strategy for liver cancer.

Materials and Methods

Chemical synthesis and preparation. FMTC was prepared as previously described (15). The characteristics of FMTC are as shown below. Optical rotation representing the property of chiral substances; [α]D25 =+38 (c 0.08, CH3OH). 1H and 13C NMR spectra were recorded using a Bruker 600 MHz spectrometer as solution; 1H NMR (600 MHz, CDCl3) δ 6.97 (dd, 1H, J=8.4, 1.9 Hz), 6.93 (dd, 1H, J=12.0, 4.2 Hz), 4.08 (dd, 1H, J=11.3, 7.7 Hz), 3.93 (s, 3H), 3.88 (s, 3H), 3.87 (s, 3H), 3.81 (s, 3H), 3.18 (dd, 1H, J=14.0, 4.5 Hz), 2.74 (m, 1H), 2.63 (dd, 1H, J=14.0, 10.4 Hz). 13C NMR (150 MHz, CDCl3) δ 191.1, 159.8, 159.5, 154.6, 151.6, 146.5, 137.7, 131.6, 124.9, 117.0, 113.7, 108.8, 96.1, 69.2, 61.7, 61.5, 56.5, 56.2, 48.3, 32.0. The results of analysis using low-resolution mass spectra (LRMS) electrospray ionization (ESI) are as follows; LRMS (ESI) calculated for C20H21FO6 [M+H+] 377.14, found 377.22. A stock FMTC solution (10 mM) was prepared in dimethyl sulfoxide (Sigma–Aldrich, St. Louis, MO, USA) and stored at -20°C.

Cell culture. Huh7 and Hep3B cells were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in RPMI 1640 (WELGENE Inc., Gyeongongsangbuk-do, Republic of Korea) and DMEM (WELGENE Inc.), respectively, supplemented, with 10% fetal bovine serum (FBS; WELGENE Inc.), 50 μM penicillin, and 50 μg/ml streptomycin (Gibco Brl, NY, USA). Cells were cultured in a humidified incubator at 37°C, 5% CO2.

Cell viability and growth assays. Cell viability was evaluated using the Ez-Cytox solution (Daecil Lab, Seoul, Republic of Korea) according to manufacturer’s instructions. For cell viability assays, Huh7 cells were seeded in 96-well plates at a density of 4x10^4 cells/well in replicates of three. For cell growth assays, they were seeded in 96-well plates at a density of 1.5x10^3 cells/well. They were then treated with FMTC in 10% FBS media. The optical densities of the supernatants were read at 450 nm on a Synergy H1 Microplate Reader (BioTek Instruments, Winooski, VT, USA). Cell viability and growth assays were repeated three times.

Flow cytometry. Huh7 cells (1x10^6) were treated with FMTC in 10% FBS medium for 24 h. For cell cycle analysis, cells were then harvested using trypsin-EDTA and then fixed with 70% cold ethanol for 3 h at -20°C. They were then washed with PBS 1X and stained with a Muse® Cell Cycle Kit (MCH100106, Luminex Corporation, Austin, TX, USA) for 30 min at room temperature, in the dark. Apoptosis rates were determined using an Muse® Annexin V & Dead Cell Kit (MCH100105, Luminex Corporation). Cells were harvested under the same conditions as for cell cycle analysis and then washed twice with ice-cold PBS. Annexin V-labeled cell lines were stained and then incubated at room temperature for 10 min. They were then washed with PBS, followed by fluorescence imaging on a Zeiss LSM 780 confocal microscope. Images were visualized using the Zeiss Zen software. The immunofluorescence staining data provided are representative and have been collected from three independent experiments.

Cellular extracts were prepared using RIPA lysis buffer and analyzed by western blot. Cell extracts were boiled for 5 min in 5× sample buffer (62.5 mM Tris–HCl pH 6.8, 1% β-mercaptoethanol, 10% glycerol, 2% SDS, 0.2% bromophenol blue, and 5% glycerol) at 100°C for 5 min. They were then loaded onto SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were then blocked with 5% nonfat, skimmed milk for 1 h, at room temperature. Primary antibodies were diluted at 1:1000 in 3% bovine serum albumin (BSA) in 1X TBST (Tris-buffered saline with 0.02% sodium dodecyl sulfate (SDS), 0.02% bromophenol blue, and 5% glycerol) at 100°C for 5 min. They were then washed with PBS, followed by fluorescence imaging on a Zeiss LSM 780 confocal microscope. Images were visualized using the Zeiss Zen software. The immunofluorescence staining data provided are representative and have been collected from three independent experiments.
Antibodies. Antibodies against Histone H3 (9715), Phospho-(p)-Histone H3 (Ser-10, #9701), Caspase 3 (#9662), cleaved-Caspase 3 (#9661), PARP (#9532), and cleaved-PARP (#5625) were purchased from Cell signaling technology, Inc. Antibodies against p21 (sc-6246), Cyclin B1 (sc-166210), Cyclin E1 (sc-377100), BAX (sc-7480), and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology, Inc. HRP-conjugated polyclonal anti-mouse IgG (HRP, GTX213111-01, GeneTex, Inc., Irvine, CA, USA) and HRP-conjugated polyclonal anti-rabbit IgG (LF-SA8002, Young In Frontier Co., Ltd., Seoul, Republic of Korea) were used at 1:5,000.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis for CDKN1A gene expression. To evaluate the expression of the gene CDKN1A encoding p21, RNA was isolated from Huh7 cells using a Labopass total RNA extraction solution (COSMO Genetech, Seoul, Republic of Korea) according to the manufacturer’s instructions. The RNA was then reverse transcribed into cDNA using Oligo(dt) primers and a TOScript cDNA synthesis kit (ENzynomics, Daejeon, Republic of Korea). Gene expression levels were normalized to the reference gene, GAPDH. The sequences of the primers used were as follows: GAPDH (sense: 5'-gtctcaccacagtgaag-3', antisense: 5'-tgcttcaacccatgg-3'); CDKN1A (sense: 5'-taacctgagttcagcagac-3', antisense: 5'-tgaggtgtagaatctgtcatgct-3'). RT-qPCR analysis was performed in a three independent experiments and the figures shown are representative of these experiments.

Luciferase reporter assay. Hep3B cells (5×10⁵ cells/well) were seeded in 100 mm dishes and co-transfected with a pAP1-Luc reporter plasmid (Agilent Technologies Inc., Santa Clara, CA, USA) containing the AP1 promoter and the gWIZ-GFP plasmid. The transfected cells (1×10⁵ cells/well) were then seeded onto a 12-well plate, incubated overnight, treated with FMTC, and then cultured for an additional 24 h. The cells were then lysed using a cell culture lysis reagent (Promega Corporation, Fitchburg, WI, USA), and the luciferase activity measured according to manufacturer instructions (Promega, Madison, WI, USA). Luciferase activity was normalized to gWIZ-GFP. Luciferase reporter assay was repeated 3 times as described above.

Statistical analyses. All data were expressed as mean±standard deviation (SD). Statistical analysis of data was performed with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). One-way ANOVA and Dunnett’s multiple comparisons were used to calculate significance between different experimental groups. *p<0.05, **p<0.005, and ***p<0.001, respectively.

Results

**FMTC alters cell morphology and inhibits cell growth.** To determine the antiproliferative effects of FMTC (Figure 1a), Huh7 cells were treated with FMTC at 100, 200, 500, and 1000 nM for 24 or 48 h (Figure 1b). Cell viability analysis revealed the proliferation of Huh7 cells was unaffected by treatment with FMTC for 24 h at concentrations of 100, 200, or 500 nM. However, treatment of Huh7 cells with FMTC for 48 h tended to decrease cell viability at 500 nM and triggered significant cell death at 1000 nM. Treating Huh7 cells with FMTC triggered dose-dependent morphological changes when compared to DMSO-treated control cells (Figure 1c). FMTC induced the rounding up (shrinkage) of Huh7 cells, thereby phenocopying the effect of nocodazole, a representative MIA. Moreover, FMTC inhibited cell proliferation in Huh7 cells even at low concentrations (Figure 1d). Interestingly, cell growth inhibition was initially observed at a concentration of 100 nM FMTC, the same concentration at which the morphology was altered. These data suggest that FMTC effectively inhibits the growth of Huh7 cells.

**FMTC arrests the cell cycle in the G2/M phase and elevates p21 protein levels.** To further investigate the inhibitory effect of FMTC on the proliferation of Huh7 cells, we carried out cell cycle analysis using flow cytometry (Figure 2a). This analysis revealed that treatment with FMTC at concentrations >200 nM for 24 h significantly increased the proportion of cells in the G2/M phase, indicating that FMTC inhibits mitotic progression in Huh7 cells. Because histone H3 phosphorylation on Ser-10 (H3S10P) plays an important role in mitotic chromosome condensation, it is used as a marker of mitotic arrest (16). Thus, we examined H3S10P levels after treating cells with FMTC (Figure 2b). This analysis revealed that FMTC elevated H3S10P levels at 200 nM, the same concentration at which it triggered G2/M-phase cycle arrest in Huh7 cells, suggesting that FMTC induced chromosome condensation. Next, we used Hoechst 33342 nuclear DNA labeling to determine the extent of FMTC-induced chromosomal aggregation (Figure 2c). This analysis revealed that the nuclei of DMSO-treated Huh7 cells appeared blue, with an evenly distributed staining pattern. However, the nuclei of nocodazole-treated cells were bright blue or aggregated blue. Interestingly, the nuclei of the FMTC-treated cells exhibited a staining pattern like that of nocodazole-treated cells. These data suggest that FMTC induces chromosome condensation or multinucleation in Huh7 cells, which may lead to mitotic arrest.

p21 arrests cell cycle progression during G2/M by inhibiting CDK2/cyclin E activity through accumulation from late G2 to early mitosis (17). Thus, we investigated whether FMTC induces the accumulation of p21 and found that treatment with FMTC for 24 h elevated p21 protein levels dose-dependently, whereas p21 mRNA levels remained unchanged (Figure 2d). This observation suggests that FMTC may regulate p21 expression post-translationally. To confirm that FMTC mediates cell cycle arrest in the G2/M phase, we examined the levels of cyclin B1, which is known to be up-regulated in mitosis. This analysis revealed that the levels of cyclin B1 were elevated upon treatment with FMTC, while those of cyclin E1, an S-phase regulator, decreased. Together, these results imply that FMTC induces G2/M arrest by up-regulating p21.

**FMTC induces apoptosis through sustained cell cycle arrest.** Cell cycle arrest occurs when cells need to undergo DNA...
repair. However, prolonged cell cycle arrest activates apoptosis (18). Because prolonged mitotic arrest activates caspase-dependent apoptosis (19), we evaluated the effect of FMTC on apoptosis in Huh7 cells using the Annexin V/7-AAD assay (Figure 3a). This analysis showed that treating Huh7 cells with FMTC at 500 nM triggered a significant increase in apoptosis. To confirm that indeed, FMTC induces apoptosis, we used western blot analysis to assess the levels of apoptotic proteins (Figure 3b). This analysis revealed that FMTC elevates the expression of BAX and enhances the cleavage of apoptosis markers Caspase 3 and PARP.

Because MIAs are reported to induce apoptosis by activating JNK (12), we investigated whether FMTC also activates JNK and observed that FMTC dose-dependently increased JNK phosphorylation on Thr-183 and Tyr-185 (Figure 4a). To determine whether JNK activation by FMTC...
affected downstream signaling pathways, we carried out luciferase analysis using Hep3B cells bearing an AP-1 reporter plasmid containing the AP-1 promoter. This analysis found that treating the cells with FMTC at concentrations of 0, 200, and 500 nM for 24 h increased AP-1 reporter activity dose-dependently (Figure 4b). Treating Huh7 cells with FMTC also increased the phosphorylation of c-Jun at Ser-63, a JNK target phosphorylation site (Figure 4c). These data
indicate that FMTC can activate JNK signaling, which promotes FMTC-induced apoptosis.

Discussion

Since liver cancer remains hard to cure, the discovery of new effective treatments is urgently required and is a challenge for many researchers (2, 20). Herein, we showed that FMTC has potent antitumor effects against HCC cells. Our results showed that FMTC inhibited cell growth by inducing a G2/M cell cycle arrest in Huh7 cells. In FMTC-treated cells, chromosome condensation and increased H3S10P levels were also observed, suggesting that FMTC exerts antiproliferative effects through mitotic arrest. Furthermore, FMTC did not affect the induction of cyclin B1, which promotes the assembly of the mitotic apparatus and chromosome alignment in early mitosis. This suggests that FMTC-mediated mitotic arrest is not driven by cyclin B1 suppression. Treating Huh7 cells with FMTC elevated p21 protein levels without changing its mRNA levels, suggesting that p53, the transcriptional activator of p21, is not involved in the FMTC-induced antiproliferative mechanism. FMTC may stabilize the p21 protein through regulation of post-translational modifications, such as phosphorylation. The p21 protein has several putative phosphorylation sites (21). Both p38α and JNK1 activation by TGF-β1 increased the phosphorylation levels of Thr-57 and Ser-130 on p21, thereby mediating its stabilization (22). Additionally, phosphorylation of Thr-145 by Pim-1/2 enhances p21 stability (21). FMTC might also regulate USP11 (23), a deubiquitylase known to inhibit p21 proteolysis by the APC/CDC20 complex, thereby inducing degradation of the p21 protein in early prometaphase (24). Because FMTC is an anti-tubulin drug, it is expected to regulate the cell cycle by blocking spindle formation. Therefore, the mechanism of FMTC-mediated induction of
p21 expression for cell cycle arrest requires further investigation. Although p21 can induce cell cycle arrest in the G1/S and G2/M phases of the cell cycle (6), FMTC caused p21 to arrest only in the G2/M phase. This indicates that the p21-mediated G1/S and G2/M cell cycle arrests are regulated via different mechanisms.

Since antiproliferative agents and apoptosis inducers are widely used in cancer chemotherapy, both inhibition of proliferation and activation of apoptosis may increase the potential for cancer treatment (25-27). Prolonged FMTC-mediated cell cycle inhibition activates an apoptosis-dependent pathway. FMTC up-regulated the apoptotic factor, BAX, and activated Caspase 3. We found that FMTC elevated c-Jun phosphorylation and its transcriptional activity via JNK activation. Previous studies have reported that MIAs activate JNK through activation of various signaling pathways, such as Ras and the apoptosis signal-regulating kinase (12). Because activation of the JNK signaling pathway by FMTC is probably regulated by upstream regulators, the underlying FMTC mechanism of action needs further investigation.

Our study proposes FMTC, which exerts antiproliferative effects by elevating p21 protein levels and inducing mitotic arrest in Huh7 cells, as a new antitumor agent. However, because we only examined the antitumor effects of FMTC against HCC cells in vitro, these observations need to be validated through in vivo studies, such as using nude mouse hepatocarcinoma xenografts.
Conclusion

In this study, we investigated the effects of FMTC on cell growth inhibition and its regulatory mechanisms in Huh7 cells, an HCC cell line. We showed that FMTC inhibits the proliferation of Huh7 cells by inducing cell cycle arrest in the G2/M phase and initiating apoptosis (Figure 4d). Our data suggest that FMTC triggers mitotic arrest via p21 accumulation and impaired microtubule formation. Additionally, prolonged treatment of Huh7 cells with FMTC induced apoptosis. Although this study showed the effect of FMTC on the cell cycle and apoptosis, further studies are needed to understand the specific signaling pathways involved. In conclusion, our findings suggest FMTC as a novel antiproliferative agent that effectively inhibits the cell cycle in Huh7 cells.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors’ Contributions

Haelim Yoon wrote the original manuscript draft. Haelim Yoon and JunHo Lee performed the experiments and analyzed the data. Sayeon Cho designed the study, interpreted the data, and obtained final approval for the manuscript.

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