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5-Methoxyflavanone induces cell cycle arrest at the G2/M phase, apoptosis and autophagy in HCT116 human colon cancer cells☆

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ABSTRACT

Natural flavonoids have diverse pharmacological activities, including anti-oxidative, anti-inflammatory, and anti-cancer activities. In this study, we investigated the molecular mechanism underlying the action of 5-methoxyflavanone (5-MF) which has a strong bioavailability and metabolic stability. Our results show that 5-MF inhibited the growth and clonogenicity of HCT116 human colon cancer cells, and that it activated DNA damage responses, as revealed by the accumulation of p53 and the phosphorylation of DNA damage-sensitive proteins, including ataxia–telangiectasia mutated (ATM) at Ser1981, checkpoint kinase 2 (Chk2) at Thr68, and histone H2AX at Ser139. 5-MF-induced DNA damage was confirmed in a comet tail assay. We also found that 5-MF increased the cleavage of caspase-2 and -7, leading to the induction of apoptosis. Pretreatment with the ATM inhibitor KU55933 enhanced 5-MF-induced γ-H2AX formation and caspase-7 cleavage. HCT116 cells lacking p53 ($p53^{-/-}$) or p21 ($p21^{-/-}$) exhibited increased sensitivity to 5-MF compared to wild-type cells. 5-MF further induced autophagy via an ERK signaling pathway. Blockage of autophagy with the MEK inhibitor U0126 potentiated 5-MF-induced γ-H2AX formation and caspase-2 activation. These results suggest that a caspase-2 cascade mediates 5-MF-induced anti-tumor activity, while an ATM/Chk2/p53/p21 checkpoint pathway and ERK-mediated autophagy act as a survival program to block caspase-2-mediated apoptosis induced by 5-MF.

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Introduction

Colon cancer is one of the most common human tumors in developed and developing countries. The development of cancer-preventive and -therapeutic agents from natural products is an area of considerable interest. Flavonoids are a class of more than 4000 phenylbenzopyrones that are widely distributed in edible plants. They have diverse pharmacological activities, including anti-oxidative, anti-inflammatory, and anti-cancer activities (Harborne and Williams, 2000; Pietta, 2000; Yao et al., 2004). Furthermore, dietary flavonoids reduce the risk of some malignant neoplasms and suppress tumor cell growth (Knekt et al., 1997; Prasad et al., 2010). Although dietary flavonoids have been shown to exert beneficial effects in cell culture experiments, most attempts to apply them to *in vivo* experiments have been unsuccessful (Walle et al., 2007). This is mainly due to the poor oral bioavailability of polyphenols, whose free hydroxyl groups render them susceptible to "rapid intestinal/hepatic conjugation by glucuronidation and/or sulfation and excretion" (Ross and Kasum, 2002; Walle et al., 2007). It has been suggested experimentally that the replacement of these hydroxyl groups with a methoxyl (OCH_3) group could overcome the low bioavailability and poor metabolic stability of the compounds, increasing their potential for therapeutic application (Wen and Walle, 2006; Walle, 2007). Of the methoxylated flavones, 5,7-dimethoxyflavanone and 5-methoxyflavanone (5-MF) are highly resistant to hepatic metabolism and exhibit greater intestinal absorption than other polymethoxy flavones (Walle and Walle, 2007). Despite these findings, the cellular and molecular mechanisms underlying the anti-tumor effects of methoxylated flavones remain poorly understood, primarily because their biological activities are largely dependent on their individual flavonoid structures.

Programmed cell death (PCD) is a genetically regulated form of cell death, which is essential for various biological events such as morphogenesis and the elimination of potentially harmful cells. PCD includes two morphologically distinct processes: apoptosis (PCD

Abbreviations: 5-MF, 5-methoxyflavone; ATM, ataxia–telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; Chk, checkpoint kinase; PCD, programmed cell death.

☆ The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/gCjqsf.

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type-I) and autophagic cell death (PCD type-II). Apoptosis is regulated by Bcl-2 family proteins and caspases (a group of cysteine proteases), which have been intensively investigated. Autophagy is an evolutionarily conserved and self-destructive process that is activated by various stimuli, including nutrient depletion and genotoxic stress (Levine and Klionsky, 2004; Maiuri et al., 2007; Rubinsztein et al., 2007). Autophagy functions as a means of eliminating intracellular parasites, harmful aggregated proteins, and excess or damaged organelles within characteristic vacuoles known as autophagosomes, which fuse with lysosomes to form autolysosomes. Autophagy serves as an adaptive response that is essential for long-term survival during growth factor withdrawal or cellular stress (Boya et al., 2005; Lum et al., 2005; Mizushima and Klionsky, 2007). During cancer progression, autophagy protects cancer cells from diverse cellular stresses, including hypoxia and nutrient starvation (Levine and Kroemer, 2008; Wang and Levine, 2010). In certain cancer cells, autophagy induced by chemotherapy or radiotherapy may prevent cells from undergoing apoptosis, producing unfavorable conditions after anti-cancer therapy (Boya et al., 2005). On the contrary, several studies have reported that autophagy triggers tumor cell death in response to various anti-cancer agents (Kuo et al., 2006; Aoki et al., 2007; Turcotte et al., 2008; Salazar et al., 2009). Thus, autophagy is also implicated in the promotion of cellular suicide, presumably due to the large-scale, irreversible destruction of cellular contents or the activation of death signaling pathways (Baehrecke, 2005; Scott et al., 2007). There is also evidence that autophagy and apoptosis are coordinated (Tormo et al., 2009). However, the relationship between these processes varies according to cell type and the source of stress.

The aim of the present study was to clarify the mode of action of methoxylated flavones in the context of their anti-tumor activity, and to investigate the relationship between apoptosis and autophagy using 5-MF (Fig. 1A), which is known to have high bioavailability and metabolic stability (Walle, 2007; Walle et al., 2007). Our data show that 5-MF potently inhibited the growth and long-term clonogenicity of HCT116 human colon cancer cells. To identify the molecular mechanisms underlying these responses to 5-MF, we investigated the effects of 5-MF on cell cycle progression, DNA damage responses, apoptosis, and autophagy. We found that 5-MF caused p53 and phosphorylated forms of the DNA damage-sensitive proteins ataxia–telangiectasia mutated (ATM), checkpoint kinase 2 (Chk2), and H2AX to accumulate; it also reduced the phosphorylation of Chk1, and induced caspase-dependent apoptosis. Furthermore, it triggered

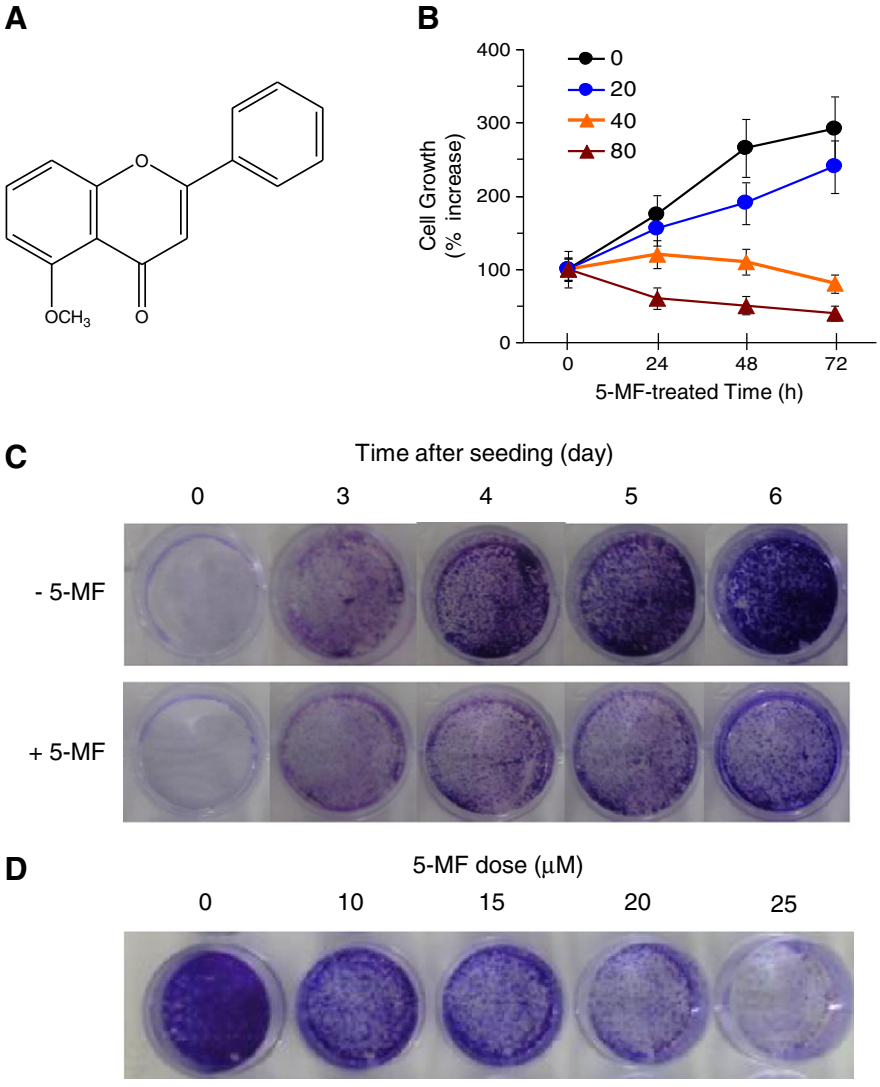

Fig. 1. Effect of 5-MF on antiproliferative activity. (A) Chemical structure of 5-methoxyflavone. (B) Cell proliferation assay. HCT116 cells were treated with different doses (0, 20, 40, or 80 μM) of 5-MF for varying lengths of time (24–72 h), and cell proliferation was measured using a Cell Counting Kit-8. The data shown represent the mean ± S.D. for one experiment performed in triplicate. (C) Time-dependent clonogenic survival assay. HCT116 cells (5×10^3) were seeded in the presence of 20 μM 5-MF for 3, 4, 5 and 6 days, and clonogenic assay was carried out. (D) Dose-dependent clonogenic Survival assay. HCT116 cells (5×10^3) were seeded and cultured for 7 days in the absence or presence of 5-MF (0, 10, 15, 20, and 25 μM). Similar results were obtained from two other independent experiments.

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