Methylated chrysin reduced cell proliferation, but antagonized cytotoxicity of other anticancer drugs in acute lymphoblastic leukemia

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The efficacy of 5,7-dimethoxyflavone (DMF), a methylated analog of chrysin, as a therapeutic agent to treat acute lymphoblastic leukemia (ALL) was investigated. Using a panel of ALL cell lines, the IC_{50} (half-maximal inhibitory concentration) of DMF varied between 2.8 and 7.0 μg/ml. DMF induced G_0/G_1 cell cycle arrest, concomitant with a decreased expression of phosphorylated retinoblastoma-associated protein 1. DMF increased the rate of apoptosis, although it was apparent only after a long period of exposure (96 h). The accumulation of oxidative stress was not involved in the growth-inhibitory effects of DMF. As DMF reduced the intracellular levels of glutathione, the combination effects of DMF with other anticancer drugs were evaluated using the improved Isobologram and the combination index method. In the simultaneous drug combination assay, DMF antagonized the cytotoxicity of 4-hydroperoxy-cyclophosphamide, cytarabine, vincristine, and L-asparaginase in all tested ALL cells. This study demonstrated that DMF, a methylated flavone, was an effective chemotherapy agent that could inhibit cell cycle arrest and induce apoptosis in ALL cell lines. However, combination therapy with DMF and other anticancer drugs is not recommended. *Anti-Cancer Drugs* **23:417–425**

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Introduction

Flavonoids are a group of natural polyphenolic compounds found ubiquitously in plants. They are commonly found in many ordinary foods, and are involved in diverse biological systems as they have anti-inflammatory [1], antioxidant, antiproliferative [2], and anticancer properties [3]. Among such effects, several flavonoids have been found to deplete intracellular glutathione (GSH) levels in cancer cells [4,5]. As cellular GSH constitutes an antioxidative defense mechanism, GSH depletion by flavonoids has been reported to induce oxidative stress-mediated apoptosis in some tumor cells [4]. Chrysin, one of the natural flavonoids abundant in honey and propolis, has recently attracted interest because of its potential anticancer effects, including GSH depletion [6–9]. 5,7-Dimethoxyflavone (DMF) is the methylated analog of chrysin, which was shown to overcome the poor oral bioavailability of chrysin [10]. In comparison with chrysin, DMF induced a significantly more potent antiproliferative effect against squamous cell carcinoma cells [10].

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer. Childhood ALL is a curable disease when treated with chemotherapy; however, treatment failure still occurs in some patients with ALL. Drug resistance of leukemic cells is one of the causes of treatment failure [11]. Specific to ALL, a poor response to glucocorticoid treatment is a clinically reliable indicator of a poor chemotherapy treatment outcome [12]. To improve the survival of those patients with drug-resistant ALL, the development of new molecular-targeting chemotherapy agents is essential. We have previously shown that GSH depletion by buthionine sulfoximine caused the accumulation of oxidative stress in ALL cells and subsequently induced apoptosis. This suggests that GSH could be a therapeutic molecular target in ALL [13]. However, we also identified buthionine sulfoximine-resistant ALL cells, where the oxidative stress induced by GSH depletion failed to induce mitochondrial death-signal activation.

Chrysin has been reported to have a more potent antileukemic effect than other flavonoids [14]. In this study, we evaluated the ability of DMF, the bioavailable derivative of chrysin, to induce apoptosis of ALL cells, especially against GSH-dependent and GSH-independent ALL cells.

Materials and methods

Cell lines

A panel of ALL cell lines, the YCUB series, has been previously described [15]. YCUB-8 is a newly established cell line derived from a 13-year-old female patient with *t*(1;19) positive-B cell-precursor ALL. All cells were

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grown in RPMI-1640 supplemented with 10% fetal bovine serum at 37°C and in a 5% CO_2 atmosphere. In our previous study [13], YCUB-2 and YCUB-4 cells were identified as GSH-dependent cells, whereas YCUB-5 was GSH independent.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay

Cells were seeded into a 96-well microplate at a density of 5×10^4 cells/well in 100 µl complete medium, containing DMF or other drugs at the indicated concentrations. After 4 days, the culture medium from the wells was discarded and replaced with 100 µl of a serum-free RPMI-1640 medium containing 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) at 0.5 mg/ml. After 4 h of incubation at 37°C, the formazan granules generated by live cells were dissolved in dimethyl sulfoxide. The absorbance was measured at 570 nm using a microplate reader, and the survival fraction of each well was calculated as the ratio of A570 (treated well/untreated well). The assay was performed using replications in six wells.

To evaluate the effect of the environmental oxygen concentration on DMF activity, YCUB-2 or YCUB-5 cells were precultured at a 1 or a 21% O_2 atmosphere for 24 h. To avoid long exposure to normal environmental atmosphere, complete medium containing each drug at the indicated concentration was prepared on a 96-well plate in advance. Cells were seeded onto the plate at a density of 2×10^4 cells/well. After 3 days, the survival of the cells was measured using the MTT assay.

The combination effects of 5,7-dimethoxyflavone with other drugs

The combination effects of DMF and 4-hydroperoxycyclophosphamide (4HO-CY), cytarabine (AraC), L-asparaginase (L-asp), or vincristine (VCR) were evaluated using the improved Isobologram method described by Steel and Peckham [16] and by the combination index (CI). The theoretical basis of the improved Isobologram has been documented by Kano *et al.* [17]. In the improved Isobologram, three isoeffect lines illustrate the theoretical additivity of two drugs and are derived from the dose–response curve of each drug. The mode I line was based on the hypothesis that two drugs act nonexclusively in totally different modes of action (hetero-additive). In contrast, two mode II lines assume that two drugs act exclusively in the same mode of action (homo-additive). In this study, the concentration to induce 80% growth inhibition (IC_{80}) was expressed as 1.0, and the isoeffect lines to determine the IC_{80} by the drug combination were constructed. If the observed data points, which indicate the concentrations of two drugs in combination to induce the IC_{80}, fall in the area surrounded by the isoeffect lines (envelope of additivity), the combination effect is considered as additive. When the data points appear above or below the area, the effect is considered to be antagonistic or synergetic.

The CI was calculated using the following equation:

$$CI = (D)_a / (D_x)_a + (D)_b / (D_x)_b$$

In the equation $(D)_a$ and $(D)_b$ represent the dose of drug A or B, which yields the target effect (the IC_{80}) when used in combination, and $(D_x)_a$ or $(D_x)_b$ is the dose of drug A or B that yields the target effect as a single agent. When the CI = 1.0, this demonstrates an additive effect between the drugs, whereas a CI of greater than or less than 1 indicates an antagonistic or a synergistic effect.

In this study, the drug combination assay was assessed using a constant-ratio design. The drug mixture, containing the two drugs to be tested, was prepared at the indicated concentration ratio, and a serial dilution of the mixture was tested for its ability to inhibit the growth of ALL cell lines.

Cellular glutathione levels

5×10^5 Cells were seeded into 12-well plates and incubated for 48 h with or without DMF at the indicated concentration. Cells were collected, washed with PBS, and lysed in 200 µl of 0.1% Triton-X (v/v) in 10 mmol/l HCl. The total cellular GSH level was measured using the 5,5′-dithio-bis-(2-nitrobenzoic acid)-glutathione disulfide reductase method as previously reported [18].

GSH levels were measured in triplicate for each sample and standardized against the protein level in the same sample. The levels of cellular GSH after DMF exposure were expressed as a percentage of the untreated control. The assay was performed in triplicate.

Apoptosis

The rate of apoptosis was measured by flow cytometry using the PE-conjugated Annexin V Apoptosis Detection Kit 1 (BD Pharmingen, Franklin Lakes, New Jersey, USA). YCUB-2 cells seeded at 1×10^5 cells/ml were incubated with or without DMF at 20 µg/ml for 2 or 4 days. Cells were harvested, washed with PBS, and resuspended in the binding buffer. Anti-Annexin V-PE antibody and 7-amino-actinomycin were added according to the manufacturer's instructions. After 15 min of incubation at room temperature, the Annexin V-positive apoptotic cells were measured. Each condition was tested in replicates of three wells.

Cell cycle analysis

YCUB-2 or YCUB-5 cells were seeded to a 24-well plate at a density of 1×10^5 cells/500 µl/ with or without 20 µg/ml DMF for 24–96 h. Cells were harvested and fixed overnight in ice-cold 70% ethanol. Cells were washed in PBS and resuspended in 500 µl PBS containing propidium iodide at a final concentration of 10 µg/ml and RNase A at 10 µg/ml. After 15 min of incubation at room temperature,

the DNA content per single cell was measured by flow cytometry. Cell cycle analysis was performed in triplicate.

Reactive oxygen species

YCUB-2 or YCUB-5 cells were seeded to a 24-well plate at a density of 1×10^5 cells/500 µl with or without 5 or 20 µg/ml DMF for 24 h. Cells were washed with PBS and incubated in PBS containing dihydrorhodamine-123 (DHR; Sigma-Aldrich, St Louis, Missouri, USA) at 1 µmol/l for 30 min at 37°C. Cells were washed and resuspended in PBS. The fluorescent rhodamine 123, the oxidized form of DHR induced by cellular reactive oxygen species (ROS), was measured by flow cytometry.

Protein phosphorylation screening

To investigate the molecular pathways of DMF-induced cell cycle arrest, a global proteomics phospho-antibody array-based approach was performed by the Kinetworks Phospho-Site Screen (KPSS)-10.1 (Kinexus, Vancouver, BC, Canada) as described on the Kinexus website (*http://www.kinexus.ca*). This screen detects 44 different phospho-sites in 21 cell cycle-relating proteins by phospho-site-specific antibodies. The expression levels of phospho-proteins in DMF-treated cells were expressed as the ratio of the intensity of the chemiluminescence signal for each band on the immunoblot to the corresponding band signal of the untreated control.

Reagents

DMF was purchased from Indofine Chemical Company (Hillsborough, New Jersey, USA). DMF was dissolved in dimethyl sulfoxide and stored at −20°C. AraC was purchased from Sigma-Aldrich Japan (Tokyo, Japan), dissolved in sterile distilled water, and stored at −20°C. 4HO-CY or Asp was kindly provided by Shionogi & Co., Ltd (Osaka, Japan), or Kyowa (Tokyo, Japan).

Statistics

Data were expressed as means ± SD. The comparison of data was performed using a Student's two-tailed *t*-test, and the significance was indicated when the *P*-value was lower than 0.05.

Results

5,7-Dimethoxyflavone inhibited the growth and reduced the cellular glutathione of acute lymphoblastic leukemia cells

DMF reduced the growth and the cellular content of GSH in ALL cells in a dose-dependent manner (Fig. 1). In a panel of ALL cell lines, the IC_{50} or IC_{80} values of DMF after 4 days of incubation ranged from 2.8 to 7.0 µg/ml or from 3.9 to 11.0 µg/ml, respectively. The levels of cellular GSH after DMF exposure at 5 µg/ml were reduced in all cells in the range of 31.8–68.2% of untreated controls (Table 1). In our previous study [13], YCUB-2 and YCUB-4 cells were characterized as GSH-dependent cells, whereas YCUB-5 cells were GSH independent. Contrary to our previous data, there was no significant difference in the DMF sensitivity among these cells in this study. To assess the role of GSH depletion in the growth-inhibitory effect of DMF, the toxicity of DMF was tested in YCUB-2 cells, in the presence of 250 or 500 µmol/l *N*-acetyl-L-cysteine (NAC), which is known to increase cellular GSH [19]. However, NAC did not alter the DMF toxicity in YCUB-2 cells (data not shown).

5,7-Dimethoxyflavone induced G_0/G_1 cell cycle arrest and apoptosis in YCUB-2 cells

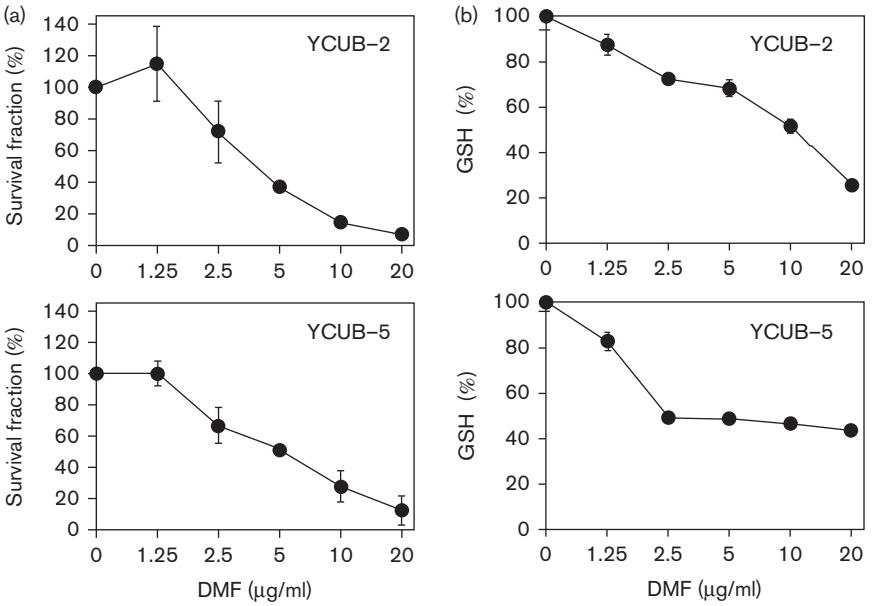
DMF induced cell cycle arrest at G_0/G_1 in YCUB-2 cells (Fig. 2). YCUB-2 cells were cultured with or without DMF at 20 µg/ml for 24, 48, or 96 h, and the number of cells at each cell cycle phase was measured by flow cytometry. After 24 h, the number of YCUB-2 cells with G_0/G_1 was significantly increased to 47.4 ± 2.1% in DMF-treated cells compared with 39.3 ± 0.8% in the untreated control ($P = 0.013$). In contrast, the number of YCUB-2 cells in the S phase decreased to 13.8 ± 0.3% after DMF exposure, compared with 31.7 ± 0.5% in the untreated control ($P < 0.01$). After 48 h, the number of cells at G_0/G_1 increased to 64.7 ± 4.0% in treated cells in contrast to 49.0 ± 1.9% in the untreated control ($P = 0.01$). Cells in the S or the G_2/M phase after 48 h were significantly decreased in treated cells compared with untreated controls ($P < 0.01$). The number of cells with sub G_0/G_1 DNA content was slightly increased in DMF-treated cells after 48 h ($P = 0.07$), and the difference was significant after 96 h ($P < 0.01$). Similar to YCUB-2 cells, the number of YCUB-5 cells in the S or the G_2/M phase was decreased after 48 h of DMF exposure, although a significant difference was only observed in the S phase (6.6 vs. 1.9%, $P < 0.01$). In YCUB-5 cells, the number of cells in the sub G_0/G_1 phase was 24.1 ± 1.2% after 48 h of DMF exposure, which was significantly higher than 16.9 ± 0.4% in the untreated control ($P < 0.01$).

DMF increased the number of apoptotic cells as measured by Annexin V-positive YCUB-2 cells. The number of Annexin V-positive apoptotic cells after a 48-h treatment of 20 µg/ml DMF was 8.8 ± 0.8%, which was only slightly higher than 3.9 ± 0.8% in the untreated control ($P < 0.01$, Fig. 3). The number of apoptotic cells significantly increased to 18.7 ± 1.4% after 96 h of treatment compared with 2.6 ± 0.6% in untreated controls ($P < 0.01$). These data suggested that DMF inhibited the growth of YCUB-2 cells primarily by cell cycle arrest and induced apoptosis only after a long duration of exposure.

The role of oxidative stress in the growth-inhibitory effect of 5,7-dimethoxyflavone

GSH plays a crucial role in cellular antioxidant defense. Depression of cellular GSH has been reported to allow the accumulation of oxidative stress in cells. To determine whether oxidative stress is associated with the

Fig. 1



5,7-Dimethoxyflavone (DMF) reduced cell proliferation and cellular glutathione (GSH) levels in a dose-dependent manner in acute lymphoblastic leukemia cells. (a) YCUB-2 or YCUB-5 cells were grown in the presence or absence of DMF at 1.25–20 μg/ml in a 96-well plate for 4 days. The surviving cells were quantified using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, and the survival fraction was calculated as a ratio compared with the untreated control well. (b) YCUB-2 or YCUB-5 cells were cultured with or without DMF at the concentration indicated in the figure for 48 h. Cellular GSH was measured using the 5,5′-dithio-bis-(2-nitrobenzoic acid)–glutathione disulfide reductase method. The levels of cellular GSH after DMF exposure were expressed as a percentage of the untreated control.

Table 1 IC_{50}, and IC_{80} values of 5,7-dimethoxyflavone and glutathione suppression by 5,7-dimethoxyflavone in a panel of acute lymphoblastic leukemia cell lines

	Growth inhibition		
	IC_{50} (μg/ml)	IC_{80} (μg/ml)	GSH suppression[a] (%)
YCUB-2	6.4 ± 1.9	9.3 ± 1.0	68.2 ± 3.6
YCUB-4	3.0 ± 0.3	5.9 ± 2.0	31.8 ± 5.5
YCUB-5	7.0 ± 2.6	10.5 ± 1.7	48.6 ± 2.1
YCUB-6	3.6 ± 2.7	5.6 ± 2.7	44.2 ± 4.8
YUCB-8	2.8 ± 3.5	3.9 ± 4.3	33.6 ± 5.5

DMF, 5,7-dimethoxyflavone; GSH, glutathione; IC_{50}, IC_{80}, the concentration to induce 50 or 80% growth inhibition.
[a]The ratio of cellular GSH after 24 h of treatment by 5 μg/ml DMF compared with untreated control.

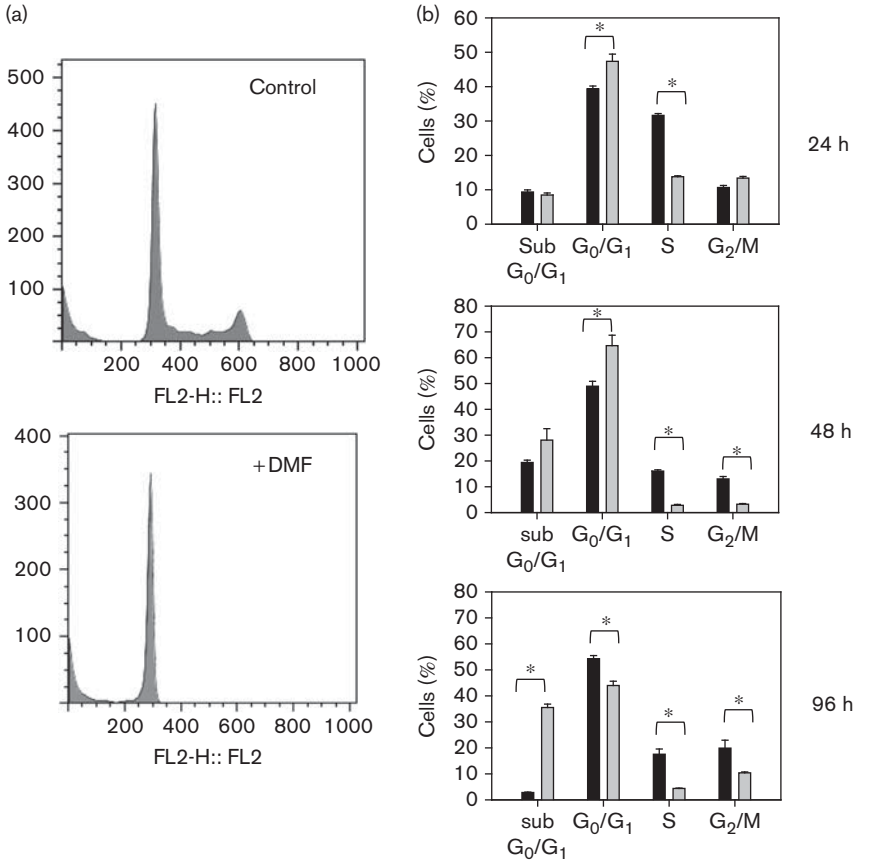
growth-inhibitory effect of DMF, the effects of DMF were tested both in a 21% and in a 1% O_2 atmosphere. As shown in Fig. 4, the survival of YCUB-2 cells after 5 or 20 μg/ml DMF exposure at 1% O_2 was not significantly different compared with the survival at 21% O_2. In contrast, the toxicity of 4HO-CY was significantly reduced under the 1% O_2 condition (Fig. 4).

YCUB-2 and YCUB-5 cells were treated with DMF at 5 or 20 μg/ml for 24 h, and the accumulation of ROS was measured using the ROS probe, DHR-123. As shown in Fig. 5, the accumulation of ROS, indicated by rhodamine-123 fluorescence, was observed in YCUB-5 cells, but not in YCUB-2 cells. These results suggested that DMF inhibited the growth of YCUB-2 cells through an oxidative stress-independent mechanism.

The combination effects of 5,7-dimethoxyflavone with other anticancer drugs

DMF-induced GSH depression was hypothesized to enhance the cytotoxic effects of other anticancer drugs, because GSH has been shown to confer drug resistance to cancer cells [20]. YCUB-2, YCUB-5, and YCUB-6 cells were treated either singly or as a combination of DMF and other anticancer drugs, including 4HO-CY, AraC, L-asp, or VCR. The combination effects were evaluated using the improved Isobologram method. Drugs were added simultaneously and several drug ratios were tested by the constant-ratio design. The results are summarized in Table 2, and the representative Isobolograms are shown in Fig. 6. In YCUB-2 cells, DMF had additive effects with 4HO-CY at the ratios of 10 : 1, 80 : 1, or 160 : 1 (DMF : 4HO-CY) and with VCR at all concentration ratios. However, DMF demonstrated antagonistic effects when combined with AraC, and L-asp at any dose ratio, and with 4HO-CY at 20 : 1 and 40 : 1. Similarly, DMF combined with four anticancer drugs showed antagonistic effects in YCUB-5 and YCUB-6 cells, although there were several exceptional additive effects with VCR in YCUB-5 and with 4HO-CY in YCUB-6. The CI values in these experiments are also summarized in Table 2. Supporting the results by the improved

Fig. 2



5,7-Dimethoxyflavone (DMF)-induced cell cycle arrest in YCUB-2 cells. YCUB-2 cells were incubated with DMF at 20 μg/ml for 24, 48, or 96 h, and cell cycle analysis was performed by flow cytometry. The representative results after 48 h of incubation are shown in (a). In (b), the black bar represents the mean number of untreated control cells at each phase of the cell cycle. The gray bar represents DMF-treated cells. The error bars show the SD. After 24 h, the number of DMF-treated cells in the G_0/G_1 phase was significantly increased, concomitant with a significant decrease in cells in the S phase. After 48 h, the numbers of cells in the S and G_2/M phases were significantly decreased. The number of cells with sub G_0/G_1 DNA content was significantly increased in DMF-treated cells after 96 h. Because of the increased proportion of cells at sub G_0/G_1, the rate of cells at the G_0/G_1 phase became relatively lower in DMF-treated cells compared with the untreated control at 96 h. $^*P<0.05$.

Isobologram, the CI values were above 1.0 in most experiments using any drug combinations.

Protein phosphorylation screening

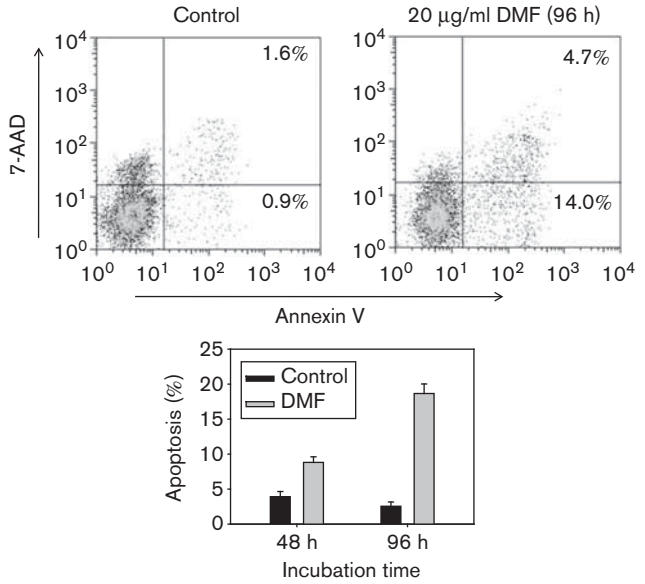
The molecular pathway included in DMF-induced cell cycle arrest was investigated by KPSS-10.1. YCUB-2 cells were treated with DMF at 20 μg/ml for 24 h, and the cell lysate was applied onto the proteomics array. The relative expression levels of phospho-proteins in DMF-treated cells compared with the expression in untreated controls are summarized in Table 3. The proteins that could not be detected in either treated or control cells were not shown. Among a panel of phospho-proteins relating to cell cycle signaling, a significant increase in expression (> 150% change) was seen in nucleophosmin, extracellular regulated protein-serine kinase 1 (Erk1), glycogen synthase-serine kinase 3 alpha and beta (GSK3a, b), and ribosomal S6 protein-serine kinase 1/2 (RSK1/2). A significant decrease (< −50% change) was observed in breast cancer type 1 susceptibility protein (BRCA1) and retinoblastoma-associated protein 1 (Rb). The expression level of phosphorylated cyclin-dependent protein-serine kinase 1/2 (CDK1/2) was not altered by DMF.

Discussion

Considerable research has been undertaken on the biological effects of flavonoids, which are present in our normal diets. Some flavonoids are known to possess anticancer or anticarcinogenic effects [1–3]. Through a screening test of 22 flavonoids, chrysin has been shown to be the most potent flavonoid to induce apoptosis in U937 leukemic cells [14]. In this study, we focused on the antileukemic effects of DMF, the methylated analog of chrysin, because of its significantly increased bioavailability compared with the parental compound [10].

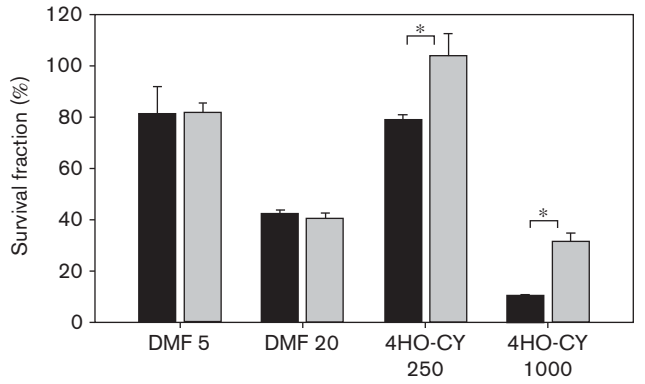
To our knowledge, no previous study has evaluated the antileukemic effects of DMF; however, in this study, it

Fig. 3



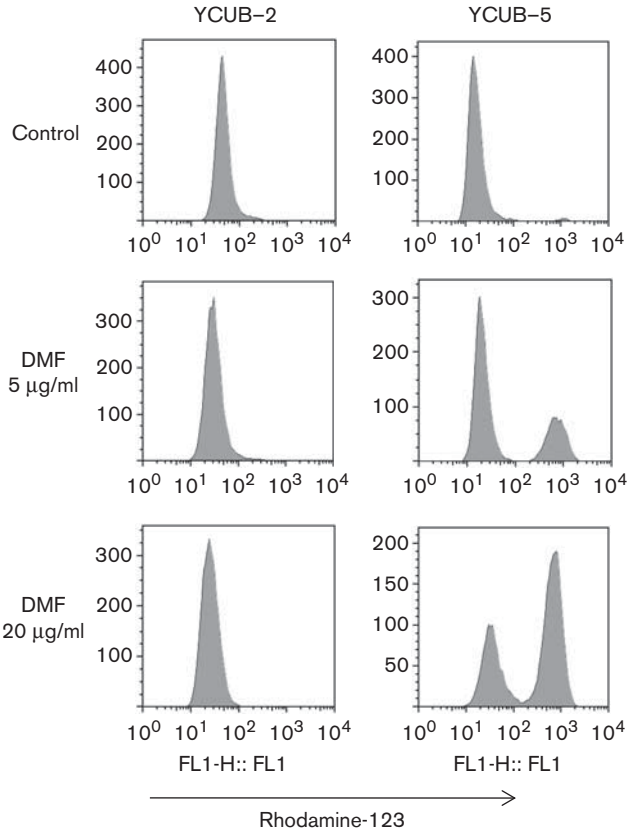
5,7-Dimethoxyflavone (DMF) increased the rate of apoptosis in YCUB-2 cells. The rate of apoptosis was measured by Annexin V staining after 48 or 96 h of exposure to DMF at 20 μg/ml. The black or gray bars and error bars represent the mean and SD of rates of Annexin V-positive apoptotic cells in untreated control or DMF-treated cells, respectively. The number of apoptotic cells after 48 h was 3.9 ± 0.8% in untreated controls and 8.8 ± 0.8% in DMF-treated cells, respectively ($P < 0.01$). The rate of apoptosis significantly increased to 18.7 ± 1.4% after 96 h of treatment compared with 2.6 ± 0.6% in untreated controls ($P < 0.01$).

Fig. 4



The growth inhibition by 5,7-dimethoxyflavone (DMF) was not affected by the environmental oxygen concentration. YCUB-2 cells were preincubated for 24 h in a 21 or a 1% O_2 atmosphere. Cells were seeded into a 96-well plate, where the culture medium contained DMF at 5 or 20 μg/ml or 4-hydroperoxy-cyclophosphamide (4HO-CY) at 250 or 1000 μg/ml. Cells were cultured for 3 days under 21 or 1% O_2, and cell survival was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Black or gray bars and error bars represent the mean % survival fraction and SD at 21 or 1% O_2, respectively. The cytotoxicity of 4HO-CY was significantly reduced in 1% O_2 compared with 21% O_2 atmosphere, whereas the cytotoxicity of DMF was not altered by the O_2 concentration. *$P < 0.05$.

Fig. 5



The accumulation of reactive oxygen species (ROS) by 5,7-dimethoxyflavone (DMF) exposure. YCUB-2 or YCUB-5 cells were treated with DMF at 5 or 20 μg/ml for 24 h. The accumulation of ROS was measured by flow cytometry with the ROS probe, dihydrorhodamine-123. The accumulation of ROS, indicated by green fluorescence of rhodamine-123, was observed in YCUB-5 cells, but not in YCUB-2 cells.

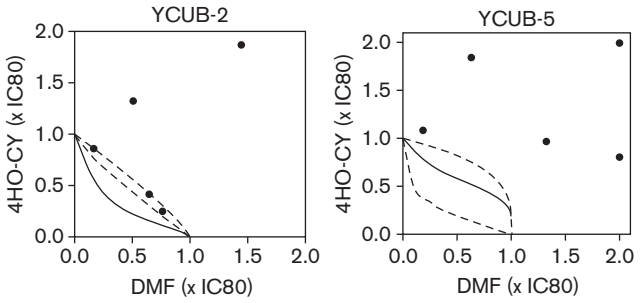
was clearly demonstrated that DMF inhibited the growth of all tested ALL cells. As DMF has been reported to be less toxic to noncancer cells [10], the clinical application of DMF in ALL therapy is conceivable. In-vivo experiments using rats demonstrated that the DMF concentration in plasma or liver tissue after oral administration at 5 mg/kg reached 2.5 or 16.5 μmol/l (0.7 or 4.7 μg/ml, respectively) without affecting any clinical symptoms including body weight [21]. In this study, the IC_{50} values of DMF in ALL cells varied between 2.8 and 7.0 μg/ml, which were considered to be in the range of biologically achievable concentrations. In ALL cells, glucocorticoid resistance is a well-documented characteristic of a multidrug-resistant phenotype [12,22]. In our previous study, we identified YCUB-2 and YCUB-5 cells as prednisolone-resistant ALL cells, whereas YCUB-4 was prednisolone sensitive [15]. In this study, DMF sensitivity was not significantly different between YCUB-4 and YCUB-2 or YCUB-5 cells, suggesting that DMF is possibly effective

Table 2 The combination effects of 5,7-dimethoxyflavone with other anticancer drugs

DMF (μg/ml): 4HO-CY (μg/ml)	CI	Isobologram	DMF (μg/ml) : AraC (ng/ml)	CI	Isobologram	DMF (μg/ml) : L-asp (U/ml)	CI	Isobologram	DMF (μg/ml) : VCR (ng/ml)	CI	Isobologram
YCUB-2											
10 : 1	1.02	Additive	1 : 2	>2	Antagonistic	1 : 1	>2	Antagonistic	40 : 1	1.01	Additive
20 : 1	1.83	Antagonistic	1 : 4	>2	Antagonistic	1 : 2	NE	NE	80 : 1	1.11	Additive
40 : 1	>2	Antagonistic	1 : 6	>2	Antagonistic	2 : 1	NE	NE	160 : 1	0.97	Additive
80 : 1	1.06	Additive	1 : 8	>2	Antagonistic	4 : 1	>2	Antagonistic	320 : 1	0.86	Additive
160 : 1	1.01	Additive	1 : 16	>2	Antagonistic	8 : 1	>2	Antagonistic			
YCUB-5											
10 : 1	1.27	Antagonistic	1 : 2	NE	NE	1 : 1	>2	Antagonistic	40 : 1	1.38	Antagonistic
20 : 1	>2	Antagonistic	1 : 4	>2	Antagonistic	1 : 2	NE	NE	80 : 1	0.80	Additive
40 : 1	>2	Antagonistic	1 : 6	>2	Antagonistic	2 : 1	NE	NE	160 : 1	0.64	Additive
80 : 1	>2	Antagonistic	1 : 8	NE	NE	4 : 1	>2	Antagonistic	320 : 1	0.78	Additive
160 : 1	NE	NE	1 : 16	>2	Antagonistic	8 : 1	>2	Antagonistic			
YCUB-6											
10 : 1	1.16	Antagonistic	1 : 2	>2	Antagonistic	1 : 1	>2	Antagonistic	40 : 1	1.65	Antagonistic
20 : 1	1.59	Antagonistic	1 : 4	>2	Antagonistic	1 : 2	>2	Antagonistic	80 : 1	1.28	Antagonistic
40 : 1	0.79	Additive	1 : 6	NE	NE	2 : 1	NE	NE	160 : 1	1.46	Antagonistic
80 : 1	0.90	Additive	1 : 8	>2	Antagonistic	4 : 1	1.86	Antagonistic	320 : 1	1.16	Antagonistic
160 : 1	0.93	Additive	1 : 16	>2	Antagonistic	8 : 1	>2	Antagonistic			

CI, the combination index; NE, not evaluated.

Fig. 6



The improved Isobologram. The combination effects of 5,7-dimethoxyflavone (DMF) with other anticancer drugs were evaluated using the improved Isobologram. The mode I (solid) and mode II (dash) isoeffect lines, which represent the theoretical concentrations of tested drugs to achieve 80% growth inhibition (IC_{80}) when used in combination, were constructed from the dose–response curves of tested drugs used as a single agent (details in 'Materials and methods'). When the observed data, representing the concentrations of two tested drugs, achieved the IC_{80}, and were found in the area surrounded by the mode I and II lines, the combination effect was considered as being additive. When data points were found above or below the area, the effects were considered to be antagonistic or synergistic. In the representative figures, three data points were additive and two were antagonistic in YCUB-2 cells. In YCUB-5 cells, all data points showed antagonistic effects after DMF and 4-hydroperoxy-cyclophosphamide (4HO-CY) treatment.

against both glucocorticoid-sensitive and glucocorticoid-resistant ALL cells.

Tumor cells under hypoxic conditions have been shown to be resistant to anticancer drugs [23]. The bone marrow is known to provide a hypoxic microenvironment to leukemic cells [24], and it could potentially contribute to the treatment failure or relapse after chemotherapy. In this study, the toxicity of DMF was not affected by environmental oxygen concentrations. Therefore, DMF might be utilized to prevent ALL relapse in hypoxic microenvironments.

GSH depletion is suggested to be involved in the anticancer effect of chrysin and other flavonoids [4]. In this study, it was shown that DMF also decreased cellular GSH levels in all tested ALL cells. However, it was unlikely that GSH depletion played a significant role in the growth-inhibitory effect of DMF, as it suppressed the growth of GSH-dependent ALL cells, as well as the growth of GSH-independent YCUB-5 cells. NAC treatment, which can increase cellular GSH, did not alter the growth-inhibitory effect of DMF. Although cytotoxicity by GSH depletion is associated with increased oxidative stress, the accumulation of ROS was not observed in YCUB-2 cells after DMF exposure, and hypoxic conditions did not alter the growth-inhibitory effect of DMF.

DMF induced G_0/G_1 cell cycle arrest in ALL cells. DMF also increased the rate of apoptosis, although it was only observed after a long period of drug exposure. In the growth curve analysis of the DMF-treated YCUB-2 cells by the trypan-blue dye exclusion, the number of living cells did not change up to 6 days (data not shown). These data indicate that DMF is a cytostatic rather than a cytotoxic agent. The observed increase in the apoptotic rate after DMF exposure might represent a relative percentage increase of the spontaneous apoptotic cells that accumulated during culture. Chrysin, the parental unmethylated molecule of DMF, has been shown to induce cell cycle arrest at the G_2/M phase rather than the G_0/G_1 phase in cancer cells [10]. It has also been reported that the methylation status of flavonoids is associated with their apoptosis-inducing activity, and that chrysin, compared with its methylated analog, induced apoptosis more potently [25]. Except for bioavailability, several biological activities are suggested to be different between chrysin and DMF.

Analysis of the protein phosphorylation screening demonstrated that DMF could decrease Rb1 phosphorylation,

Table 3 Protein phosphorylation screening

Protein	Abbreviations	Epitope	% Signal intensity[a]
3-Phosphoinositide-dependent protein-serine kinase 1	PDK1	S241	58%
Nucleophosmin	B23 (NPM)	S4	65%
Nucleophosmin	**B23 (NPM)**	**T199**	**190%**
Nucleophosmin	B23 (NPM)	T234 + T237	74%
Breast cancer type 1 susceptibility protein	**BRCA1**	**S1497**	**−68%**
Cyclin-dependent protein-serine kinase 1/2	CDK1/2	T14 + Y15	−3%
Cyclin-dependent protein-serine kinase 1/2	CDK1/2	T161/T160	5%
Cyclin-dependent protein-serine kinase 1/2	CDK1/2	Y15	22%
Extracellular regulated protein-serine kinase 1	**Erk1**	**T202 + Y204**	**194%**
Glycogen synthase-serine kinase 3α	**GSK3a**	**S21**	**Increased[b]**
Glycogen synthase-serine kinase 3β	**GSK3b**	**S9**	**Increased[b]**
MAPK/ERK protein-serine kinase 1	MEK1	T292	40%
MAPK/ERK protein-serine kinase 2	MEK2	T394	−2%
Retinoblastoma-associated protein 1	Rb	S612	−49%
Retinoblastoma-associated protein 1	**Rb**	**S780**	**−74%**
Retinoblastoma-associated protein 1	Rb	S807	−42%
Retinoblastoma-associated protein 1	**Rb**	**S807 + S811**	**−55%**
Retinoblastoma-associated protein 1	**Rb**	**T356**	**−78%**
Retinoblastoma-associated protein 1	**Rb**	**T821**	**−75%**
Retinoblastoma-associated protein 1	**Rb**	**T826**	**−57%**
Ribosomal S6 protein-serine kinase 1/2	RSK1/2	S221/S227	32%
Ribosomal S6 protein-serine kinase 1/2	RSK1/2	S363/S369	−19%
Ribosomal S6 protein-serine kinase 1/2	RSK1/2	S363/S369	−17%
Ribosomal S6 protein-serine kinase 1/2	RSK1/2	S380/S386	36%
Ribosomal S6 protein-serine kinase 1/2	**RSK1/2**	**S380/S386**	**328%**
Ribosomal S6 protein-serine kinase 1/2	**RSK1/2**	**S380/S386**	**205%**
Src proto-oncogene-encoded protein-tyrosine kinase	Src	Y530	8%

DMF, 5,7-dimethoxyflavone.
[a]The relative signal intensity of each band in DMF-treated cells compared with untreated control.
[b]The signal was detected only in treated cells. Bold characters represent a significant change of >150% or < −50%.

consistent with the results from cell cycle analysis [26]. In this study, however, we could not identify a more precise mechanism of how DMF decreased phosphorylation of Rb1 and induced cell cycle arrest. Other significant changes in protein phosphorylation were not directly associated with cell cycle arrest. For instance, the increased phosphorylation at S4 or T199 of nucleophosmin has been reported in association with cell proliferation [27,28]. Future studies will be required to elucidate the molecular mechanisms of DMF relating to cell cycle regulation.

Considering novel clinical applications for ALL therapy, the combined use of DMF with other anticancer drugs is not recommended from our results. We had hypothesized that GSH depletion by DMF could enhance the cellular accumulation and toxicity of anticancer drugs. Studies show that high levels of intracellular GSH are associated with chemotherapy resistance in tumor cells and that the reduction of GSH enhanced the sensitivity to chemotherapy drugs [29–31]. Contrary to our expectations, DMF antagonized the cytotoxic effects by the four different anticancer drugs in ALL, although DMF actually reduced the intracellular GSH levels. The parental analog of DMF, chrysin, has also been shown to inhibit topotecan-induced apoptosis in an intestinal epithelial cell line, although chrysin enhanced cellular topotecan accumulation [32]. Cell cycle arrest might have conferred drug resistance to the chemotherapy drugs AraC or VCR, whose toxicities are known to be related to cell proliferation. In childhood ALL, it has been reported that proliferating cells were more sensitive to drugs including L-asp [33], whose toxicity is theoretically cell cycle independent. This study suggests that flavonoids such as chrysin or DMF, which exhibit anticancer effects primarily by cell cycle arrest, are better utilized as a single therapeutic agent and not in combination therapy.

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Conflicts of interest

There are no conflicts of interest.

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