Potentiation of Apoptosis by Flavopiridol in Mitomycin-C-treated Gastric and Breast Cancer Cells

Gary K. Schwartz, Kian Farsi, Peter Maslak, David P. Kelsen, and David Spriggs
Division of Solid Tumor Oncology, Gastrointestinal Oncology Research Laboratory, Gastrointestinal Oncology Section [G. K. S., D. P. K.], Gastrointestinal Oncology Research Laboratory [K. F.], Division of Hematological Oncology, Department of Medicine [P. M.], and Division of Solid Tumor Oncology, Developmental Chemotherapy Section [D. S.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Abstract

Flavopiridol (L86–8275) is a synthetic flavone currently undergoing Phase I clinical trials. It is active against a series of human cancer cell lines and has been shown to inhibit a broad range of protein kinases, including cyclin-dependent kinases and protein kinase C (PKC). Previous studies have shown that the PKC-specific inhibitor safingol significantly enhances the induction of apoptosis by mitomycin-C (MMC) in gastric cancer cells. Because flavopiridol can potentially inhibit PKC, we elected to determine the extent to which flavopiridol would promote MMC-induced apoptosis in both gastric and breast cancer cells. For these studies, MKN-74 gastric cancer cells and MDA-MB-468 breast cancer cells were exposed to either no drug, 1 μg/ml MMC alone, 300 nm flavopiridol alone, or a combination of chemotherapy with flavopiridol for 24 h. Sequence specificity was also examined by first exposing cells to MMC for 24 h followed by flavopiridol for 24 h or to the same drugs in the reverse order. Apoptosis was measured by quantitative fluorescence microscopy of nuclear chromatin condensation in cells stained with the dye, bisbenzimide trihydrochloride. Exposure of MKN-74 cells to flavopiridol alone induced apoptosis in 12 ± 1% of the cells, and exposure to MMC alone induced apoptosis in 10 ± 1%. However, the combination of flavopiridol and MMC increased the induction of apoptosis to 55 ± 3% of the cells (P < 0.005 for the drug combination versus flavopiridol alone). Pretreatment with the PKC activator 3-phorbol 12-myristate 13-acetate only partially reversed this effect (43 ± 1%; P < 0.025). In MDA-MB-468 cells, flavopiridol alone induced apoptosis in 17 ± 1% of the cells, and MMC alone induced apoptosis in 10 ± 1% of the cells. The combination of flavopiridol and MMC increased the percentage of MDA-MB-468 cells undergoing apoptosis to 58 ± 4% (P < 0.005 for the drug combination versus flavopiridol alone). Sequential treatment with MMC followed by flavopiridol induced apoptosis in 63 ± 2% of the MKN-74 cells (P < 0.05 versus the concomitant drug combination) and in 76 ± 2% of the MDA-MB-468 cells (P < 0.025 versus the concomitant drug combination), whereas flavopiridol followed by MMC did not increase the induction of apoptosis in either cell line. As determined by the terminal deoxynucleotidyl transferase labeling of the 3' ends of DNA fragments produced in apoptotic cells, the induction of apoptosis with the combination of flavopiridol and MMC occurred to MKN-74 cells in all phases of the cell cycle (i.e., G0-G1, S, and G2-M). These results indicate that flavopiridol potentiates the cytotoxic effect of the chemotherapeutic agent MMC by promoting drug-induced apoptosis in tumor cells. Sequencing studies suggest that MMC followed by flavopiridol or simultaneous treatment is superior to flavopiridol followed by MMC. The enhancement of MMC-induced apoptosis by flavopiridol may be partially PKC dependent and is not associated with one specific region of the cell cycle.

Introduction

Flavopiridol (L86–8275) is a synthetic flavone currently undergoing Phase I clinical trials under the supervision of the National Cancer Institute (1). It can be prepared by total synthesis or by derivation from a natural product obtained from Dysosyllum binectariferum, a plant indigenous to India (2). Single-agent antitumor activity has been observed in a variety of preclinical models (3). It has been reported to induce apoptosis in low nanomolar concentrations (i.e., 100 nm) in SUDHL-4 lymphoma cells, but a concentration of at least 1000 nm was required to produce a similar effect in PC-3 prostate cancer cells (4). However, in vitro and in vivo activity has been reported in other prostate cancer cell lines, as well as in prostate cancer xenografts (5). In the 250–300 nm range, flavopiridol also inhibits the colony formation of a series of human cancer cell lines that includes A549 (non-small cell lung cancer), HCT8 (ileocecal adenocarcinoma), T986 (glioblastoma), MCF-7 (breast adenocarcinoma), and HL-60 (promyelocytic leukemia; Ref. 6). However, except in the case of HL-60, the inhibition of colony formation was not associated with the induction of apoptosis. In fact, necrosis, as determined by trypan blue uptake, was only noted at 1,000–10,000 nm flavopiridol (6).

Although its exact mechanism of action remains unknown, flavopiridol has been reported to inhibit PKC3 enzyme activity by 50% (IC50) at a concentration of 6 μM (7). This inhibition is
more selective than its inhibition of cyclic AMP-dependent protein kinase (IC_{50} = 145 \mu M) or epidermal growth factor-receptor kinase (IC_{50} = 25 \mu M). Inhibition of CDKs, including CDK2 and CDK4, has been reported with an IC_{50} in the 100 nM range (8). Flavopiridol directly inhibits CDK1 enzymatic activity with an IC_{50} of 400 nM and also inhibits CDK1 phosphorylation (7–9).

Recent investigations indicate that the activation of the phosphoinositide-PKC pathway mediates antiapoptotic signals and that inhibition of this pathway by PKC-specific inhibitors induces proapoptotic signals (10–14). Consequently, inhibition of PKC may represent a novel approach for anticancer therapy. We have previously shown that exposure of gastric cancer cells to the PKC-specific inhibitor safingol (l-threo-dihydrosphingosine) in itself was not sufficient to induce apoptosis (15). However, when the cells were treated with a combination of safingol and a nontoxic concentration of the chemotherapeutic agent MMC, there was a significant induction of apoptosis. This effect was blocked by activation of PKC with the phorbol ester PMA, supporting a role for PKC in this process. In view of the fact that flavopiridol could potentially inhibit PKC, among a group of many kinase, we elected to determine whether the cytotoxicity of flavopiridol could also be enhanced by combining it with MMC against gastric and breast cancer cells.

Materials and Methods

Cell Culture. The human gastric cancer cell line MKN-74 was graciously supplied by Dr. E. Tahara (Hiroshima University, Hiroshima, Japan). They were maintained in Eagle’s MEM supplemented with 20% normal calf serum. The human breast cancer cell line MDA-MB-468 was obtained from the laboratory of Dr. John Mendelsohn (Memorial Sloan-Kettering Cancer Center) and was maintained in DMEM supplemented with 10% FBS, 0.45% glucose, and Ham’s F-12 nutrient mixture. The cell lines were kept in a humidified 5% CO_{2} atmosphere at 37°C; the cultures tested negative for Mycoplasma species.

Determination of Apoptosis. Apoptosis was measured by two different methods: (a) QFM of nuclear changes induced by apoptosis, as determined by bisbenzimide tetrachloride staining of nuclear chromatin (16); and (b) a TdT assay that labels the 3’-OH ends of DNA fragments produced in apoptotic cells as measured by flow cytometry (17). Cells were treated according to one of several protocols: (a) no drug (control) for 24 h; (b) 300 nM flavopiridol (graciously supplied by Dr. Edward Sausville, National Cancer Institute, Bethesda, MD) alone for 24 h; (c) 1 \mu g/ml MMC (Bristol-Myers Squibb, Princeton, NJ) alone for 24 h; (d) a combination of 300 nM flavopiridol and 1 \mu g/ml MMC for 24 h; (e) 300 nM flavopiridol for 24 h followed by drug washout with 1 X PBS and then 1 \mu g/ml MMC for 24 h; or (f) 1 \mu g/ml MMC for 24 h followed by drug washout with 1 X PBS and then 300 nM flavopiridol for 24 h. For these sequential studies, control cells were treated with either 300 nM flavopiridol or 1 \mu g/ml MMC for 24 h followed by drug washout with 1 X PBS and then exposed to medium containing serum (i.e., no drug) for 24 h, or cells were treated with the same sequential therapies but in just the reverse order before analysis by QFM.

Under conditions that activate PKC in this cell system, MKN-74 cells were preexposed to 20 nM PMA (Sigma Chemical Co.) for 2 h, followed by drug washout with 1 X PBS and exposure to 1 \mu g/ml MMC in combination with 300 nM flavopiridol for 24 h (18). Stock solutions of MMC (1 mg/ml) and flavopiridol (0.3 mg/ml) were constituted in water, stored at 4°C, and subsequently diluted in medium for each experiment. The doses of MMC and flavopiridol used were based on cytotoxicity studies with [\textsuperscript{3}H]thymidine and trypan blue, indicating \pm 20% inhibition of cell proliferation at these concentrations (15).

For QFM determinations, cells attached to the plates were released by trypsinization and were harvested with the floating cells. The cells were fixed in 3% paraformaldehyde and incubated at room temperature for 10 min. The fixative was removed and the cells were washed with 1 X PBS, resuspended in 30–50 \mu l of 1 X PBS containing only 24 \mu g/ml bisbenzimide tetrachloride, and incubated at room temperature for 15 min. Ten-\mu l aliquots of the cells were placed on glass slides coated with 3-amino-propyl-triethoxysilane, and duplicate samples of 400 cells each were counted and scored for the incidence of apoptotic chromatin condensation using an Olympus BH-2 fluorescence microscope equipped with a BH2-DM2U2UV Dichromic Mirror Cube filter (Olympus, Lake Success, NY). For cell viability assays, the trypsinized cells were combined with nonadherent cells, treated with 0.4% trypan blue, and counted using a hemocytometer.

For the TdT assays, the ApopTag Kit (Oncor, Gaithersburg, MD) was used. This method uses a fluorescein-labeled antidigoxigenin antibody directed against nucleotides of d-dUTP, which are catalytically added to the 3’-OH ends of fragmented DNA by TdT. Briefly, 1 \times 10^{6} to 2 \times 10^{6} cells were washed and fixed with 1% paraformaldehyde and 70% ethanol. The fixed cells were incubated in a reaction mixture containing TdT and d-dUTP for 30 min at 37°C. Stop/wash buffer was added, and the cells were resuspended in 100 \mu l of fluorescein-labeled antidigoxigenin antibody for 30 min at room temperature. The cells were then washed with 0.1% Triton X-100 and counterstained with PI solution. Green (d-dUTP labeled DNA strand breaks) and red (PI staining for total DNA content) fluorescence of individual cells were measured on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The resulting bivariate plots enabled the detection of apoptotic events within the cell cycle. In each experiment, the control population was gated so that the R_{1} cursor detected fewer than 2% of the measured cells as having increased green fluorescence. This was thought to represent the basal level of apoptosis. Cells with green fluorescence above the gated R_{1} threshold were considered apoptotic in subsequent measurements. The data from 10,000 cells were collected in list mode and analyzed using CellFit and LYSYS software (Becton Dickinson). Quantitative determinations of apoptosis represented the percentage of the population measured.

Statistical Analysis. All experiments were done in duplicate and were repeated at least three times unless otherwise

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4 Unpublished data.
Fig. 1 Photomicrographs of representative fields of MKN-74 cells stained with bisbenzimide trihydrochloride to evaluate nuclear chromatin condensation (i.e., apoptosis) after treatment for 24 h with no drug (A), 1 μg/ml MMC alone (B), 300 nm flavopiridol alone (C), or the combination of 1 μg/ml MMC and 300 nm flavopiridol (D).

Results

Effect of Flavopiridol and MMC on the Induction of Apoptosis in MKN-74 and MDA-MB-468 Cells. MKN-74 and MDA-MB-468 cells were treated with flavopiridol in the presence or absence of a fixed dose of MMC for 24 h. The results of the QFM analysis for the MKN-74 cells are shown in Fig. 1. MMC alone for 24 h (Fig. 1B) induced apoptosis in 10 ± 1% of the MKN-74 cells, which was greater than untreated controls (Fig. 1A; 1 ± 1%). However, the combination of flavopiridol and MMC significantly increased the percentage of cells undergoing apoptosis from 12 ± 1% with flavopiridol alone (Fig. 1C) to 55 ± 3% with MMC and flavopiridol in combination (Fig. 1D; *P* < 0.005). This effect was observed with flavopiridol concentrations as low as 30 nm (data not shown). With the 2-h pretreatment with PMA, the percentage of cells undergoing apoptosis decreased to 43 ± 1% (*P* < 0.025 versus flavopiridol and MMC together).

To assess cell viability, MKN-74 cells were treated with MMC, flavopiridol, or the combination therapy for 24 h under conditions that were identical to the QFM and examined for the uptake of trypan blue. The percentage of viable cells was as follows: untreated control cells, 92 ± 1%; MMC alone, 84 ± 1%; flavopiridol alone, 88 ± 1%; flavopiridol and MMC in combination, 71 ± 3%. Thus, over this 24-h period there appears to be a statistically significant decrease in cell viability with the combination therapy when compared to either MMC (*P* < 0.001) or flavopiridol (*P* < 0.0005) alone.

The effect of flavopiridol on potentiating MMC-induced apoptosis was not limited to gastric cancer cells (Fig. 2). Treatment of MDA-MB-468 breast cancer cells with MMC alone induced apoptosis in 10 ± 1% of the cells, which was significantly greater than untreated controls (1 ± 1%; *P* < 0.01). Flavopiridol alone significantly increased the percentage of cells undergoing apoptosis to 17 ± 2% (*P* < 0.025 versus MMC alone). However, when flavopiridol was combined with MMC, a further significant increase in the apoptotic fraction (58 ± 4%) was observed (*P* < 0.005 versus flavopiridol alone).

Effect of the Relative Sequence of Flavopiridol and MMC on the Induction of Apoptosis in MKN-74 and MDA-MB-468 Cells. On the basis of the sequence-specific interactions of PKC inhibitors with cytotoxic agents, we elected to explore the sequence specificity of MMC and flavopiridol. Sequential treatment of MKN-74 cells with MMC for 24 h followed by flavopiridol for 24 h induced apoptosis in 63 ± 2% of the cells (Fig. 3A). This was significantly greater than that observed with treatment with MMC alone for 24 h followed by no drug for 24 h (27 ± 1%; *P* < 0.001), by no drug followed by flavopiridol (4 ± 1%; *P* < 0.005), and by the two drugs given together continuously for 24 h (55 ± 3%; *P* < 0.05). In contrast, sequential treatment with flavopiridol followed by MMC
induced apoptosis in only 17 ± 1% of the MKN-74 cells, which was significantly less than the same two drugs given either sequentially in the reverse order (63 ± 2%; \( P < 0.001 \)) or continuously in combination for 24 h (55 ± 3%; \( P < 0.005 \)).

Sequential treatment of MDA-MB-468 cells with MMC for the first 24 h followed by flavopiridol for the subsequent 24 h induced apoptosis in 76 ± 2% of the cells (Fig. 3B). This was significantly greater than was induced by MMC alone followed by no drug (28 ± 1%; \( P < 0.001 \)), by no drug followed by flavopiridol (29 ± 1%; \( P < 0.001 \)), and by the two drugs given together continuously for 24 h (58 ± 4%; \( P < 0.025 \)). However, treatment with flavopiridol followed by MMC induced apoptosis in only 22 ± 1% of the MDA-MB-468 cells, which was significantly less than the same two drugs given either sequentially in the reverse order (76 ± 2%; \( P < 0.0005 \)) or continuously in combination for 24 h (58 ± 4%; \( P < 0.005 \)).

**Identifying Gastric Cancer Cells Undergoing Apoptosis by the TdT Assay.** Combining the TdT assay with PI staining enables detection of apoptotic events within the cell cycle. MKN-74 cells were treated in the same manner as the QFM determinations and analyzed by combining the TdT assay and flow cytometry. With this approach, apoptotic cells were identified as those with increasing green fluorescence shifting above the R4 cursor of the contour plot. Fig. 4 shows that treatment of MKN-74 cells with MMC (Fig. 4B) or flavopiridol (Fig. 4C) alone for 24 h resulted in some cells exhibiting increased green fluorescence (above the R4 cursor), indicating DNA strand breaks characteristic of apoptosis. This was greater than the levels of green fluorescence observed for the untreated control cells (Fig. 4A), i.e., essentially no spontaneous apoptosis. However, continuous exposure of the cells to the combination of MMC and flavopiridol for 24 h (Fig. 4D) resulted in a large increase in cells exhibiting elevated green fluorescence. This was associated with a relative depletion of cells in G0-G1 and G2-M and an increase in those in S (data not shown). However, the effect of the combination of flavopiridol and MMC does not appear to be cell cycle specific, as the enhancement of apoptosis appeared to occur to cells in all phases of the cell cycle.

**Discussion**

The present study shows that the combination of the bifunctional alkylating agent MMC and flavopiridol potentiates the apoptotic response 2–3-fold in gastric and breast cancer cells. This effect of flavopiridol on enhancing chemotherapy-induced apoptosis is more potent than that reported for the PKC-specific inhibitor safingol (15). As a single agent at high micromolar concentrations, safingol did not induce a significant degree of apoptosis in the MKN-74 cells. Significant apoptosis was achieved only when micromolar concentrations of safingol were combined with MMC. In contrast, even at nanomolar concentrations, flavopiridol alone induced a modest degree of
apoptosis in both cell lines, an effect that was significantly enhanced when nanomolar concentrations of flavopiridol were combined with MMC. In addition, in this current study, we used 1 \( \mu \)g/ml of MMC with flavopiridol. This combination induced apoptosis in 60% of the MKN-74 cells. In the safingol studies it was necessary to use 5 \( \mu \)g/ml of MMC to achieve a comparable degree of apoptosis (3). Therefore, when compared to safingol, flavopiridol has more single-agent activity, and even at nanomolar concentrations, it requires less MMC to achieve an equally toxic effect.

There are several possible explanations for these differences. Although flavopiridol is a more specific inhibitor for PKC than it is for cyclic AMP-dependent protein kinase or epidermal growth factor-receptor kinase, it still inhibits all of these kinases in comparable micromolar concentrations (3). However, the effects that we observed with flavopiridol were in the nanomolar range. Whereas the effect of safingol on cell cycle-specific events is unknown, flavopiridol in nanomolar concentrations inhibits CDKs, including CDK1, CDK2, and CDK4 (7–9). This may account for some of the cytotoxicity observed with flavopiridol in the MKN-74 and the MDA-MB-468 cells. Nevertheless, the effect of flavopiridol on enhancing MMC-induced apoptosis was partially reversed by pretreating with the PKC activator PMA under conditions associated with activation of PKC in the MKN-74 cells (18). Because PMA activation alone can partially reverse the induction of apoptosis by MMC alone (data not shown), it is possible that the partial reversal of the enhancement of MMC-induced apoptosis by flavopiridol is independent of PKC. These results would suggest that at least some of the effect of flavopiridol on enhancing MMC-induced apoptosis may be due to PKC inhibition, yet inhibition of other protein kinases, including the CDKs, would appear to play a more dominant role. The contribution of PKC to the potentiation of MMC-induced apoptosis by flavopiridol is being further evaluated with studies using antisense against the PKC isoform, PKCa, which is expressed by the MKN-74 cells (18).

The trypan blue studies indicate a significant decrease in cell viability with the combination of flavopiridol and MMC when compared to either drug alone. However, the loss of cell viability with the drug combination is still less than the corresponding increase in apoptosis observed under the same conditions. An incongruity between apoptosis and reduction in cell viability has been previously reported (19). These disparate results may be due to the use of different cell lines and to different treatment conditions. For example, exposure of the human A549 human lung carcinoma cell line to micromolar concentrations of flavopiridol induced a maximum uptake of trypan blue at 72 h, but it did not lead to any evidence of apoptotic morphology (6). Another possible explanation may reside in what is actually being analyzed by QFM and the uptake of trypan blue. Necrotic death, in contrast to programmed cell death (apoptosis), is characterized by increased plasma membrane permeability and loss of cell membrane integrity (20). In contrast, apoptotic cells show

\[ \text{DNA Content (PI fluorescence)} \]

**Fig. 4** Contour density maps of DNA content versus labeling of DNA strand breaks with dUTP and fluoresceinated digoxigenin antibody in MKN-74 cells. Apoptotic cells are identified as those exhibiting increased green fluorescence emitted from d-dUTP:fluorescein isothiocyanate-labeled antibody complexes (dUTP incorporation (Incorp.) (FITC fluorescence)) above the R1 cursor compared to the control population, compared to cellular DNA content (PI fluorescence), as described in "Materials and Methods." MKN-74 cells were incubated for 24 h with no drug (A), 1 \( \mu \)g/ml MMC alone (B), 300 nm flavopiridol alone (C), or the combination of 1 \( \mu \)g/ml MMC and 300 nm flavopiridol (D). The G0-G1, S, and G2-M regions of the cell cycle are indicated in A.
distinctive changes in morphology with intact cell membranes, as shown by QFM, and an increase in DNA fragmentation, as indicated by the TdT labeling assays (20). Thus, by these definitions, necrotic cells should show an increased tendency to take up trypan blue. Our results would then indicate that the exposure of MKN-74 human gastric cancer cells to nanormolar concentrations of flavopiridol in combination with low micromolar concentrations of MMC for 24 h results in an increase in both apoptosis and necrosis. However, the increase in apoptosis exceeds that of necrosis in the same cell population treated under identical conditions.

The sequencing of flavopiridol relative to chemotherapy appears to affect the induction of apoptosis. Pretreatment of MKN-74 and MDA-MB-468 cells with flavopiridol before MMC induced a degree of apoptosis that was significantly less than MMC followed by flavopiridol or by the two drugs given together for 24 h. Treatment of the MKN-74 and the MDA-MB-468 cells with the sequence of MMC followed by flavopiridol resulted in the greatest percentage of induction of apoptosis in these cells. This represented more than an additive effect of either drug alone. The sequence of therapy with flavopiridol may then have a significant impact on the induction of apoptosis. These results are relevant in regard to the design of future clinical trials combining flavopiridol with MMC, as well as with other chemotherapeutic agents.

It is interesting that flavopiridol potentiates apoptosis in gastric and breast cancer cells with both wild-type (MKN-74) and mutated (MDA-MB-468) p53 (21, 22). These results suggest that the effect of flavopiridol on potentiating chemotherapy-induced apoptosis may be independent of the p53 status of the cells. This observation will require further investigation but is consistent with the report that flavopiridol function is not dependent on p53 status (8).

In addition to the effect of flavopiridol on MMC-induced apoptosis, we have observed a similar degree of potentiation with flavopiridol in combination with other chemotherapeutic agents, including paclitaxel and cisplatin (23). These studies indicate that flavopiridol is part of a new class of therapeutic agents that potentiate the cytotoxic effects of anticancer chemotherapy by promoting the induction of apoptosis. The demonstration that flavopiridol potentiates chemotherapy-induced apoptosis has important clinical implications and indicates new directions in the introduction of this agent into clinical trials in combination with chemotherapy.

References


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