The cyclin-dependent kinase inhibitor flavopiridol sensitizes human hepatocellular carcinoma cells to TRAIL-induced apoptosis

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Received March 1, 2006; Accepted May 2, 2006

Abstract. Flavopiridol was one of the first cyclin-dependent kinase inhibitors demonstrated to have an antitumor effect in several cancer types. Here, we investigated the effects of flavopiridol on TNF-related apoptosis-inducing ligand (TRAIL) in the human hepatocellular carcinoma (HCC) cell lines HLE and HepG2, and evaluated the role of flavopiridol in apoptosis. To better understand the mechanism of increased TRAIL sensitivity in HCC cells, we determined the effect of flavopiridol on cell surface expression of TRAIL and TRAIL receptors using flow cytometry analysis. The levels of survivin, FLIP, Bcl-xL and X-chromosome-linked IAP (XIAP) in treated and untreated cells was also determined. Flavopiridol decreased cell viability in a dose-dependent manner in the two HCC cell lines tested. The pan-caspase inhibitor z-VAD-FMK did not inhibit the effect. However, subtoxic levels of flavopiridol dramatically enhanced TRAIL-induced apoptosis in both cells. Flavopiridol up-regulated TRAIL, TRAIL-R1 and TRAIL-R2 in both cell lines. In addition, flavopiridol down-regulated expression of survivin in both cell lines, and expression of FLIP and Bcl-xL were down-regulated in HLE cells. In summary, flavopiridol augmented TRAIL sensitivity by up-regulation of TRAIL receptors and down-regulation of survivin, FLIP and Bcl-xL. Thus, combining flavopiridol with a TRAIL agonist may prove to be an effective new strategy for treatment of HCC.

Introduction

Human hepatocellular carcinoma (HCC) is a rapidly progressive tumor. In HCC, the balance between cell proliferation and cell death may play a particularly important role (1).

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Key words: cyclin-dependent kinase inhibitor, flavopiridol, hepatocellular carcinoma, TNF-related apoptosis-inducing ligand, survivin, FLIP, Bcl-xL.
In a recent study, expression of the TNF family proteins TNF-α and TNF-related apoptosis-inducing ligand (TRAIL) was shown to correlate with lethality of flavopiridol in some cell lines (10,15). TRAIL selectively induces apoptosis in various transformed cell lines but not more normal tissues (18). TRAIL can induce apoptosis by interaction with two receptors, referred to as TRAIL-R1 and -R2 (or DR4 and DR5, respectively, for death receptor 4 and 5). Each of these receptors has a death domain that mediates cellular apoptosis. TRAIL-R1 and -R2 are expressed in malignant cells as well as in normal tissues. We previously reported that treatment with the conventional chemotherapeutic agents doxorubicin and camptothecin dramatically augmented TRAIL-induced cytotoxicity in most HCC cells (18). However, the anti-HCC mechanisms of flavopiridol and the effect of the combination of flavopiridol and TRAIL on HCC cells have not been completely elucidated. Therefore, we investigated the effects of flavopiridol on human HCC cells and evaluated the cytotoxic mechanism, especially as it relates to the apoptotic pathway.

Materials and methods

Materials. Flavopiridol was a kind gift from Aventis Pharmaceuticals (Bridgewater, NJ). A stock solution of flavopiridol was made at a concentration of 10 mM in DMSO and stored at -20°C.

Cell lines. The HCC cell line HLE was purchased from the Health Science Research Resources Bank (Osaka, Japan). The human HCC cell line HepG2 was purchased from the American Type Culture Collection. HCC cell lines were cultured in Dulbecco’s modified Eagle’s medium at 37°C. All media were supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 10% heat-inactivated fetal calf serum (Invitrogen).

Detection of apoptosis-related proteins by immunoblotting. Expression of survivin, FLIP, XIAP and Bcl-xL in HCC cell lines were analyzed by immunoblotting. Briefly, cells were harvested after stimulation with flavopiridol (0-500 nM). Cells were then lysed on ice in lysis buffer (50 mM/l Tris-HCl pH 8.0, 150 mM/l NaCl, 5 mM/l ethylenediaminetetraacetic acid, 1% NP40, 1 mM phenylmethylsulfonyl fluoride). After centrifugation, supernatants were collected and protein content was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein from each extract were separated by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Toyo Roshi, Tokyo, Japan) using the Bio-Rad electrotransfer system (Bio-Rad Laboratories). Blots were blocked by incubation in 5% milk with Tris-HCl pH 7.5 and 0.1% Tween-20 for 2 h at room temperature and probed overnight at 4°C with mouse anti-survivin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-FLIP monoclonal antibody (MBL, Nagoya, Japan), rabbit anti-Bcl-xL polyclonal antibody (Transduction; Lexington, KY) or mouse anti-XIAP monoclonal antibody (Transduction). Antibodies were diluted 1:1000 with 5% milk in Tris-HCl (pH 7.5) and 0.1% Tween-20. The immunoblots were then probed with horseradish peroxidase-conjugated anti-mouse IgG or horseradish peroxidase-conjugated anti-rabbit IgG (diluted 1:2000 with 5% milk in Tris-HCl pH 7.5). After the final wash, the signal was detected with an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Detection of apoptosis. A total of 5x10^4 HLE or HepG2 cells were cultured in 35-mm culture dishes for 24 h, followed by the addition of 10 ng/ml recombinant human TRAIL (R&D Systems, Minneapolis, MN) and/or 100 nM flavopiridol. After incubation for 24 h, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO) and observed under a fluorescence microscope (Zeiss, Göttingen, Germany). To assess the viability of HCC cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed. The HCC cells were plated at a density of 1.0x10^4 cells/well in 96-well flat-bottom microtiter plates (Corning Glass Works, Corning, NY) and each plate was incubated for 24 h at 37°C in 5% CO₂. Next, flavopiridol 0-500 nM was added in the absence or presence of 0-100 ng/ml TRAIL (R&D Systems), 0-100 ng/ml TNF-α (Cosmo Bio Co., Tokyo, Japan), or 0-100 ng/ml anti-Fas agonistic antibody (MBL), and the plates were incubated for 24 h. Then, the live cell count was assayed with a Cell Titer 96 assay kit (Promega, Madison, Wisconsin) according to the manufacturer’s instructions. The absorbance of each well was measured with a microtiter plate reader (Bio-Rad) at 570 nM.
Flow cytometric analysis. Approximately 5x10^6 cells of each cell line cultured with or without 100 nM flavopiridol for 48 h were washed twice with 1% bovine serum albumin in phosphate-buffered-saline (PBS) and incubated for 2 h on ice with 5 μg/ml PE anti-human TRAIL, PE-conjugated anti-human TRAIL-R1, PE-conjugated anti-human TRAIL-R2 or PE-conjugated mouse IgG3 isotype control (eBioscience, San Diego, CA). After two washes with PBS the cells were analyzed for TRAIL, TRAIL-1 and TRAIL-2 expression by FACScan using CellQuest software (Becton Dickinson, Tokyo, Japan).

*zVAD inhibition*. An MTT assay was performed to assess the influence of flavopiridol after treatment with z-VAD-FMK, a general caspase inhibitor. The HCC cells were plated at a density of 1x10^5 cells/well in 96-well flat-bottom microtiter plates, and each plate was incubated for 24 h at 37°C in 5% CO₂. We treated cells with 100 nM flavopiridol, followed by 20 μM z-VAD-FMK and the plates were incubated for 24 h. Finally, the live cell count was assayed using a Cell Titer 96 assay kit (Promega, Madison, Wisconsin) according to the manufacturer’s instructions. The absorbance of each well was measured on a microtiter plate reader (Bio-Rad) at 570 nM.

Results

Flavopiridol reduced viability of HCC cells. To investigate change in viability in response to flavopiridol, HCC cells were incubated with various concentrations of flavopiridol for 24 h. Cell viability was assessed by the MTT assay. Flavopiridol decreased cell viability in the HCC cell lines HLE and HepG2 in a concentration-dependent manner (Fig. 1). When these cells were incubated with 500 nM of flavopiridol, the percentage of viable cells was 48% for HLE and 40% for HepG2 cells.

In order to ask if flavopiridol can induce apoptosis in HCC cells, we next assessed DAPI staining 24 h after treatment with 100 nM flavopiridol. Flavopiridol-treated HLE cells did not show features typical of apoptosis (Fig. 6b); the same result was observed for HepG2 cells (data not shown). Despite this, reduced viability in these cell lines prompted us to analyze apoptosis-related factors in these cells more rigorously. Thus, we next examined the effect of a caspase inhibitor on flavopiridol-treated cells. In order to determine whether or not the caspase pathway is related to the apoptosis of HCC cells induced by flavopiridol, we examined the changes in viability of HCC cells treated with flavopiridol in the absence of a subtoxic level of flavopiridol (100 nM; •). Cell viability decreased in response to TRAIL in a dose-dependent manner. In combination, flavopiridol and TRAIL caused a synergistic reduction in cell viability in both cell lines tested. Cell viability was assessed using the MTT assay. The data shown are the mean ± SD of six independent experiments.

Flavopiridol augmented TRAIL, TNF-α and anti-Fas agonistic antibody-induced apoptosis. It is well known that TRAIL
induces apoptosis in HCC cells via a caspase cascade. To examine the relationship between flavopiridol and TRAIL-induced apoptosis, HCC cells were incubated with various concentrations of TRAIL in the presence or absence of subtoxic levels of flavopiridol (100 nM) for 24 h. Cell viability decreased in response to TRAIL in a dose-dependent manner in both cell lines (Fig. 3). Moreover, treatment with both TRAIL and flavopiridol had a synergistic effect on reduction of cell viability (Fig. 3).

To examine whether, when combined, TRAIL and flavopiridol induce apoptosis in HCC cells, we next assessed DAPI staining 24 h after treatment with TRAIL (10 ng/ml) and flavopiridol (100 nM). We did not observe induction of apoptosis in TRAIL-treated HLE cells (Fig. 6a) or in HepG2 cells (data not shown). However, cells treated with both TRAIL and flavopiridol showed features typical of apoptosis, including nuclear condensation and nuclear fragmentation (Fig. 6c and d). These results indicate that flavopiridol may affect the TRAIL-induced apoptosis pathway in HCC cells.

Next, we examined the effect of flavopiridol on TNF-α and anti-Fas agonistic antibody-induced apoptosis. Flavopiridol synergistically augmented TNF-α-induced apoptosis in HLE cells (Fig. 4a) but not in HepG2 cells (Fig. 4b). In addition, flavopiridol enhanced anti-Fas agonistic antibody-induced apoptosis in both cell types (Fig. 5a and b).

Flavopiridol up-regulated expression of TRAIL, TRAIL-R1 and TRAIL-R2. In order to examine the mechanisms of increased TRAIL susceptibility in flavopiridol-treated HCC cells, we looked at the cell surface expression of TRAIL and TRAIL receptors in HCC cells (HLE and HepG2) using flow cytometric analysis. After 48 h treatment with 100 nM flavopiridol, expression of TRAIL, TRAIL-R1 and TRAIL-R2 was significantly up-regulated in both cell types (Fig. 7).

Flavopiridol suppressed the apoptosis-related proteins survivin, FLIP and Bcl-xL. Next we investigated the effects of flavopiridol on the levels of anti-apoptotic proteins, since these proteins play a major role in controlling apoptotic pathways (19-22). To determine the relative levels of a select group of apoptosis-related proteins, we used immunoblotting to detect survivin, FLIP and XIAP, which are direct inhibitors of cell-death proteases, and Bcl-xL, which is an apoptosis inhibitory protein. In both cell types, detectable levels of survivin were reduced in response to flavopiridol treatment in a concentration-dependent manner (Fig. 8). Moreover, FLIP and Bcl-xL levels were reduced in a concentration-dependent manner in HLE cells but not in HepG2 cells (Fig. 8). Expression of XIAP was not influenced by flavopiridol treatment in either cell line (Fig. 8).

Discussion

In this study, we analyzed the cytotoxic effects of flavopiridol on human hepatocellular carcinoma (HCC) cells. Flavopiridol
decreased cell viability in HCC cells in a concentration-dependent manner. When these cells were incubated with 500 nM of flavopiridol, viability of both HLE and HepG2 cells decreased. This effect was addressed further by asking if caspases play a role in the reduction of cell viability observed after flavopiridol treatment. We found that the change in HCC cell viability in response to flavopiridol was not suppressed by z-VAD-FMK, a strong pan-caspase inhibitor. These results suggest that the caspase-mediated pathway is not involved in flavopiridol-mediated cytotoxicity.

Flavopiridol is a semi-synthetic flavonoid and the first cyclin-dependent kinase (Cdk) inhibitor to enter clinical trials. It strongly inhibits Cdk1, Cdk2, Cdk4 and Cdk7 and causes cytostatic or cytotoxic effect on various human cancer cell lines (8,10). It also broadly suppresses the transcription of genes, including cyclin D1, and binds to DNA. By inhibiting Cdns, flavopiridol arrests the cell cycle in G1-S and G2-M boundary phase (14,23). In a large proportion of solid tumor cell lines, concentrations of 100-300 nM flavopiridol cause arrest at both the G1 and G2 phase of the cell cycle, consistent with inhibition of Cdns 2, 4, 6, and 1. Flavopiridol induces apoptosis in a p53-independent manner, cell death usually follows cell cycle arrest and is delayed, maximally occurring at 72 h after initiation of treatment and requiring concentrations higher than those required to inhibit Cdk activity (24). A recent study revealed that flavopiridol causes cells to accumulate in the G1 phase of the cell cycle (8,25). In addition, the study showed that flavopiridol induces apoptosis in a manner that is associated with down-regulation of cyclins D1 and B1, as well as inhibition of Cdk1, Cdk2 and Cdk4 (8). Moreover, the effect is accompanied by a conformational change and mitochondrial localization of Bax in MB-468 cells, which are a human breast cancer-derived cell type (8). Treatment with flavopiridol also attenuated the mRNA and protein levels of the anti-apoptotic proteins XIAP, cIAP-2, Mcl-1, Bcl-xL, and survivin in some cancer cell lines (8,13,15). Despite these
extensive studies on flavopiridol functions, the exact mechanism of the anticancer activity associated with flavopiridol remains poorly understood.

In the current study, flavopiridol augmented TRAIL, TNF-α and anti-Fas antibody-induced apoptosis synergistically (10,15,25). These results indicate that flavopiridol can modulate sensitivity to the TNF receptor signaling pathway. We have been focusing on a TRAIL-oriented strategy for cancer therapy because TRAIL selectively induces apoptosis in various transformed cell lines but not in near-normal tissues (18). TRAIL is regulated by two receptors, TRAIL-F1 and TRAIL-F2, which are members of the so-called death receptor family. Each of these receptors contains a death domain that mediates cellular apoptosis. Two additional death receptors were identified recently, TRAIL-R3 (or DcR1) and TRAIL-R4 (or DcR2). These receptors inhibit apoptosis by acting as decoy receptors; although they show similarity to TRAIL-R1 and -R2 in other regions, TRAIL-R3 and -R4 do not contain a cytoplasmic death domain. These decoy receptors are expressed at high levels in normal tissues and their expression levels are substantially lower in malignant cells, indicating a low cytotoxic effect in normal cells (18). Therefore, TRAIL may be a promising cytotoxic cytokine for induction of apoptosis. However, most HCC cells are resistant to TRAIL and co-treatment with chemotherapeutic agents or irradiation sensitized TRAIL-resistant cells to TRAIL. We have found that treatment with sub-cytotoxic levels of flavopiridol dramatically sensitizes HCC cells to TRAIL-induced apoptosis. Sensitization of

Figure 7. Influence of flavopiridol on TRAIL and TRAIL receptor expression in HCC cells. (a) HLE, (b) HepG2. HCC cells were incubated for 48 h in the absence or presence of flavopiridol (100 nM). HCC cells were stained using PE-anti-human TRAIL, TRAIL-R1 or TRAIL-R2 (black line, control; green line, no flavopiridol; red line, flavopiridol). Autofluorescence was determined using cells incubated with PE-control mouse IgG.
XIAP, cIAP-2, Mcl-1, Bcl-xL, and survivin on mRNA levels. Previous reports indicated that flavopiridol decreases the expression of these proteins. We found that the expression of survivin was down-regulated in both cells in a concentration-dependent manner. The expression of FLIP and Bcl-xL was also significantly down-regulated in both cells in a concentration-dependent manner. We also looked at expression of apoptosis-related proteins. We found that the expression of survivin was down-regulated in HLE cells in a concentration-dependent manner. The expression of FLIP and Bcl-xL was significantly down-regulated in both cells in a concentration-dependent manner.

TRAIL sensitivity in response to flavopiridol treatment. Increased TRAIL-R1 and TRAIL-R2 expression may provide a mechanism to increase sensitivity of cells to the action of TNF family members, and TRAIL-R1 and -R2 are of particular importance in interpreting TRAIL-induced apoptosis that we observed for HCC cells.

In conclusion, we have shown that flavopiridol can augment sensitivity of cells to the action of TNF family members, and TRAIL in particular, by up-regulation of TRAIL receptors and down-regulation of survivin. These results suggest that combining flavopiridol with a TRAIL agonist may be prove to be an effective new strategy for treatment of HCC.

References

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Survivin is the most potent member of the IAP family and blocks the action of various caspases. Moreover, IAP expression correlates with abbreviated survival, an unfavorable prognosis, resistance to therapy and accelerated rates of recurrence (17,20). We previously showed that survivin is overexpressed in human HCC (19) and that it is a principal inhibitor of apoptosis via its ability to inhibit caspase-3 and -7. Survivin has also been implicated in regulating the assembly of microtubules: mouse embryos without survivin gene function lack a mitotic spindle and spindle midzone microtubules (17). We have found that survivin promotes transition from G1 to S phase and promotes cell proliferation by interacting with Cdk4 and releasing p21 from Cdk4 (19). These results indicate that down-regulation of survivin protein levels by flavopiridol contribute to the augmentation of TRAIL-induced apoptosis that we observed for HCC cells.

HCC cells to TRAIL-induced apoptosis was also independent of p53 status. p53 wild-type (HepG2) and p53 mutant-type (HLE) were similarly sensitized to TRAIL by flavopiridol, through TRAIL-R2 is transcriptionally induced by p53, which is activated by many chemotherapeutic agents (18).

These results indicate that flavopiridol modulates the apoptotic pathway in a way that increases sensitivity of cells to TRAIL, receptor-mediated signaling. In order to better understand the mechanism of this effect, we paid special attention to inhibitors of the TRAIL receptor-mediated apoptosis pathway. First, we looked at cell surface expression of TRAIL and its receptors in HCC cells. TRAIL, TRAIL-R1 and TRAIL-R2 expression was significantly up-regulated by flavopiridol treatment in both types of HCC cells. Activation of TRAIL-R1 and -R2 has been shown to increase TRAIL-induced apoptosis both in vitro and in vivo, indicating that TRAIL-R1 and -R2 are of particular importance in interpreting the TRAIL signal. Therefore, the finding that flavopiridol increased TRAIL-R1 and TRAIL-R2 expression may provide an important clue to understanding the mechanism of enhanced TRAIL sensitivity in response to flavopiridol treatment.

We next investigated the role of IAPs in the TRAIL signaling pathway, since these proteins play an important role in determining the sensitivity of apoptosis in HCC cells (21,26). We also looked at expression of apoptosis-related proteins. We found that the expression of survivin was significantly down-regulated in both cells in a concentration-dependent manner. The expression of FLIP and Bcl-xL was also down-regulated in HLE cells in a concentration-dependent manner. Previous reports indicated that flavopiridol decreases XIAP, cIAP-2, Mcl-1, Bcl-xL, and survivin on mRNA levels in human breast cancer cells (8,17). The mechanism by which flavopiridol down-regulates survivin, FLIP and Bcl-xL expression is not known with certainty but very likely involves flavopiridol-mediated inhibitory effects on transcription.


