Bcl-2 Independence of Flavopiridol-induced Apoptosis
MITOCHONDRIAL DEPOLARIZATION IN THE ABSENCE OF CYTOCHROME c RELEASE*

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Tatjana V. Achenbach, Rolf Müller‡, and Emily P. Slater
From the Institute of Molecular Biology and Tumor Research, Philipps-University, Emil-Mannkopff-Strasse 2, 35033 Marburg, Germany

The new chemotherapeutic agent, flavopiridol, presently in clinical trials, has been extensively studied yet little is known about its mechanism of action. In this study we show that the induction of apoptosis by flavopiridol is largely independent of Bcl-2. This is indicated by the observation that neither overexpression nor the antisense oligonucleotide-mediated down-regulation of Bcl-2 had any effect on flavopiridol-induced cell killing. Our results suggest that flavopiridol can induce apoptosis through different pathways of caspase activation with caspase 8 playing a pivotal role. In human lung carcinoma cells, which contain high levels of endogenous Bcl-2 and lack procaspase 8, flavopiridol treatment leads to mitochondrial depolarization in the absence of cytochrome c release, followed by the activation of caspase 3 and cell death. These results clearly differ from observations made with other anti-tumor drugs and might explain, at least in part, the unusual anti-tumor properties of flavopiridol.

Malignant growth results not only from enhanced cell proliferation, but also from decreased programmed cell death (1–3). Defects of the apoptotic machinery are also a major obstacle in cytotoxic chemotherapy, which is believed to kill malignant cells mainly through the induction of programmed cell death, including apoptosis (4). The molecular mechanisms underlying drug induced-apoptosis are still ill defined, but p53-mediated mitochondrial damage seems to play a major role in this process (4). Caspases, a growing family of cysteine proteases that cleave specific substrates at aspartic acid residues, have also been identified as major components of this pathway (5, 6). All caspases are synthesized as inactive proenzymes that must be activated by proteolytic cleavage at specific aspartate residues. A well characterized pathway of caspase activation involves release of cytochrome c from the mitochondria that together with APAF-1 and ATP leads to the proteolytic cleavage and activation of procaspase 9 (5, 6). The downstream effector proteases include caspase 3, whose activation leads to the typical hallmarks of apoptosis, such as chromatin condensation and membrane blebbing. The cytochrome c-triggered pathway is modulated by pro-apoptotic as well as anti-apoptotic Bcl-2 family members (7–9). These proteins act at mitochondria where they are believed to regulate the PTP1 and the release of cytochrome c (9, 10).

Cell surface receptor molecules such as CD95 (APO-1/Fas) are also involved in drug-induced apoptosis (11). Thus, DNA-damaging agents can induce expression of the CD95/Fas ligand system (12–14) or effect clustering of CD95 in the plasma membrane through other mechanisms (15). This triggers the recruitment of procaspase 8 to the receptor complex leading to its autocatalytic cleavage (16). Active caspase 8 in turn effects the activation of the executioner caspsases, such as caspase 3, followed by the proteolysis of a plethora of target proteins, and ultimately cell death. Caspase 8 can also activate the pro-apoptotic Bcl-2 family member Bid through proteolytic cleavage (17, 18). Bid has been shown to be able to trigger the release of cytochrome c from mitochondria, which may involve the interaction with the pro-apoptotic Bax protein followed by the induction of an altered conformation or the oligomerization of Bax (19, 20). Thus, Bid links the death receptor and mitochondrial pathways.

The synthetic flavone flavopiridol has shown promising results in preclinical studies (21, 22) as well as clinical trials (23, 24) as an anti-neoplastic agent. Flavopiridol inhibits the activity of multiple cyclin-dependent kinases, and this inhibition leads to an arrest of the cell cycle (25–29). In addition, flavopiridol has been shown to be an efficient inducer of apoptosis in a variety of tumor cells (28–35), although the precise molecular mechanisms remain largely obscure. Intriguingly, flavopiridol-induced apoptosis is refractory to various genetic alterations commonly found in human tumors, for example loss of p53 (29) or overexpression of multi-drug resistance genes, such as mrp-1 (36).

Another frequent mechanism of resistance to chemotherapy is the overexpression of Bcl-2 (7), but its role in flavopiridol-induced cell killing is unclear. Bcl-2 blocks the release of cytochrome c, thereby preventing the activation of caspase 9 and its downstream caspases, which usually renders the cell insensitive to drug-induced apoptosis. Flavopiridol has been reported to down-regulate the expression of Bcl-2 in human cancer cell lines (31), but this finding could not be confirmed by others (28, 33) or in our own laboratory.2 The role of Bcl-2 in flavopiridol-triggered apoptosis is therefore unclear.

In the present study, we present compelling evidence that flavopiridol is able to kill tumor cells that are normally resistant to chemotherapeutic agents due to Bcl-2 overexpression or the absence of caspase 3 or 8. We also show that Bcl-2 blocks

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‡ To whom requests for reprints should be addressed. Tel.: 49-6421-28-66236; Fax: 49-6421-28-68923; E-mail: mueller@imt.uni-marburg.de.

1 The abbreviations used are: PTP, mitochondrial permeability transition pore; A5, antisense; DEVD, Ac-Asp-Glu-Thr-Asp-CHO; IETD, Ac-Ile-Glu-Thr-Asp-CHO; ODN, oligonucleotide; PARP, poly(ADP-ribose) polymerase; zVAD, benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

2 T. V. Achenbach and E. P. Slater, unpublished observation.
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flavopirdol-triggered cytochrome c release, but not mitochondrial depolarization and subsequent caspase 3 activation. These observations suggest that flavopiridol can induce apoptosis through multiple pathways, which appear to include novel mechanisms, and might provide an explanation for the unusual anti-tumor potency of flavopiridol.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium, and HL-60 and SW2 cells were cultured in RPMI 1640 medium each supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO2 in a humidified chamber.

Chemotherapeutics—Flavopiridol, camptothecin (Sigma), cisplatin (Sigma), and the caspase inhibitors (Biomol, Stratagene) (37) were dissolved in Me2SO and added to the culture medium at the indicated concentrations. The concentration of Me2SO in the medium was less than 1% (v/v). Cells were incubated at 37 °C for the indicated times and harvested.

Morphological Evaluation of Apoptosis—Cells were stained with Hoechst 33342 (10 μM) and propidium iodide (10 μM) for 10 min and analyzed under a fluorescence microscope (Leitz Aristoplan) with excitation at 360 nm. Because Hoechst 33342 stains all nuclei and propidium iodide stains nuclei of cells with a disrupted plasma membrane, nuclei of viable, necrotic, and apoptotic cells were observed as blue round nuclei, pink round nuclei, and fragmented blue or pink nuclei, respectively, under a fluorescent microscope (38).

Preparation of Cell Extracts—Cells from a 10-cm dish were harvested, pelleted, and washed twice in phosphate-buffered saline. After the final wash, the cell pellet was resuspended in an equal volume of buffer containing 20 mM HEPES, pH 7.8, 450 mM NaCl, and 0.2 mM EDTA, 0.5% glycerol, 5 μM dithioreitol, 5 μM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 5 μg/ml aprotinin. The cells were incubated for 5 min on ice and then lysed by freezing in liquid nitrogen and thawing in a 30 °C water bath three times. The lysate was centrifuged at 13,000 × g for 10 min at 4 °C and then transferred to a new tube. This preparation was stored at −70 °C.

Preparation of Cytosolic Extracts (39)—HeLa/SW2 cells were collected by centrifugation at 800 rpm for 5 min at 4 °C. The cells were washed twice with ice-cold PBS, pH 7.4, followed by centrifugation. The cell pellet was resuspended in 400 μl of extraction buffer, containing 288 mM sucrose, 50 mM Pipes KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM dithiothreitol, and protease inhibitors (see “Preparation of Cytosolic Extracts”). After 30 min of incubation on ice, cells were homogenized with a glass Dounce homogenizer and a B pestle (40 strokes). Cell homogenates were spun at 12,000 rpm for 15 min, and supernatants were removed and stored at −70 °C until analysis by gel electrophoresis.

Western Blot Analysis—Protein samples from treated cells were subjected to SDS-polyacrylamide gel electrophoresis (12%). The proteins were transferred onto nitrocellulose paper by electrophoresis using a semi-dry blotting chamber. The membrane was blocked with 5% nonfat milk for 2 h and incubated with the primary antibody (caspase 3, Bcl-2, actin, and PARP, Transduction Laboratories; caspase-8, Santa Cruz; cytochrome c, 65981 A PharMingen; or actin, Roche Molecular Biochemicals) for 2 h at room temperature. Unbound antibody was washed five times with PBS. The membrane was then incubated with the secondary antibody (alkaline phosphatase conjugate; Santa Cruz) for 2 h at room temperature, washed, and the enzyme expression was detected upon addition of ECL (Amersham Pharmacia Biotech).

Synthesis of Oligonucleotides—Oligonucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry on a 392 DNA/RNA Synthesizer (Applied Biosystems GmbH, Weiterstadt, Germany) and purified by preparative reverse-phase high performance liquid chromatography. The sequences of ODN 2009 (Bcl-2 AS) and the control (sense) have been described previously (40).

Transfections—ODN transfections were carried out with Lipofectin (Life Technologies, Inc.) and 250 nM ODN for 6 h. The cells were treated with chemotherapeutics and harvested after 18 h. Bcl-2 transfections in HeLa cells were carried out with Superfect (according to the manufacturer’s protocol) protocol (Qiagen) for 3 h. The mouse Bcl-2 cDNA (41) kindly provided by Prof. T. Mörs (Essen, Germany), was digested with NotI and XhoI and cloned into pcDNA3.

Transfection of cDNA Expression Vector—The insert encoding cDNA was excised from pCI (42) using XhoI and EcoRI and ligated into pcDNA3 (Invitrogen, Groningen, Netherlands). Cells were co-transfected with a pEGFP-C1 vector to be able to score for transfected cells microscopically. Tumor necrosis factor-α (TNF-α) was purchased from Roche Molecular Biochemicals.

Mitochondrial Depolarization—The kit DePsipher™ (5,5,6,6-tetraethylrhodamine-1,3,3′,3′-tetraethylbenzimidazol-carboxyanine iodide) was used according to the manufacturer’s protocol (R&D Systems).

Confocal Microscopy—HeLa cells were grown on coverslips, and SW2 cells were centrifuged onto microscope slides. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, rinsed, and permeabilized in ice-cold acetone for 10 min. After washing with PBS, the cells were blocked with casein blocking reagent (Pierce) for 60 min at 37 °C. After washing with PBS, the cells were incubated with Cy3-conjugated goat anti-mouse antibody (1:200; Dianova) for 60 min at 37 °C, washed, and mounted. Samples were analyzed on a Leitz DM RXE confocal microscope.

Measurement of ATP/ADP Ratio—The ADP/ATP ratio was measured by the luciferin/luciferase method using an AppGlow™ Adenylate Nucleotide Ratio Assay (AMS Biotechnology). Luciferase-lysate reagent (100 μl) was automatically injected into 100 μl of suspension cells (10,000 cells: SW2, H69), and the luminescence for ATP was analyzed immediately with a 10-s integration on an AutoLumat (Berthold) (A). To measure ADP, the ADP in the extract was converted to ATP by adding the ADP Converting reagent (100 μl) to the lysate after 10 min and another 10-s integration was measured immediately (B) and after an additional 5 min (C). The ADP/ATP ratio is calculated from measurements A, B, and C as follows: (C − B)/A. A higher ratio relative to control is an indication of apoptosis.

RESULTS

Flavopiridol-induced Apoptosis Is Refractory to Bcl-2 Expression—To assess the relevance of endogenous Bcl-2 expression for flavopiridol-induced apoptosis, several tumor cell lines expressing varying levels of the protein were compared with respect to their response to flavopiridol. HeLa cells (cervical carcinoma) contain barely detectable levels of Bcl-2 protein, expression in HL-60 cells (promyelocytic leukemia) is also low albeit readily detectable, whereas the abundance of Bcl-2 protein in SW2 cells (small cell lung carcinoma) is very high (Fig. 1A). All lines contain only low levels of the pro-apoptotic Bax protein (data not shown). The cells were treated with 500 nM flavopiridol for 18 h, stained with Hoechst 33342 and propidium iodide, and scored microscopically for apoptotic nuclei and dead cells, respectively. The term “cell killing” used in this study refers to the fraction of apoptotic cells plus the fraction of dead cells, with the latter representing usually less than 20% of the sum. Whereas HeLa cells responded to both camptothecin and flavopiridol by undergoing apoptosis, the SW2 were killed to a significant extent only by flavopiridol (Fig. 1B). The delayed cell death in SW2 cells in response to flavopiridol (see Fig. 7 and “Discussion”) might be due to a prolonged cell cycle caused by the high level of Bcl-2 expression (43). It has previously been shown that flavopiridol-induced apoptosis of tumor cells can be cell cycle-dependent (32).

To obtain direct evidence that the Bcl-2 expression does not prevent cell killing by flavopiridol, HeLa cells were transiently transfected with a Bcl-2 expression vector prior to treatment with chemotherapeutics. The successfully transfected cells were identified by co-expression of enhanced green fluorescent protein. When comparing the total cell population to those cells transfected with enhanced green fluorescent protein and Bcl-2 cDNA, there was no apparent difference in the level of cell killing after treatment with flavopiridol. In contrast, cells treated with camptothecin under the same experimental conditions displayed a ~60% decrease in the extent of cell death compared to transfection with Bcl-2 (Fig. 2). Similar results were obtained with the prostate carcinoma cell line PC-3 (data not shown). These observations strongly suggest that flavopiridol-induced apoptosis is indeed refractory to the overexpression of Bcl-2.

To confirm and extend these observations, SW2 cells were treated with an AS ODN directed against the Bcl-2 mRNA (40).
levels of Bcl-2, and SW2 cells, which show a high abundance of the protein (see Fig. 1A). This analysis was carried out by immunoblotting using antibodies that are specific for the pro-forms of caspases 3, 8, and 9, or for the caspase 3 substrate PARP (44). In addition, we determined cytoplasmic cytochrome c, which is required for the induction of caspase 9 (10). The results of this study are shown in Figs. 4–6 and can be summarized as follows.

(i) As expected, none of the procaspases investigated was cleaved in SW2 cells in response to camptothecin, which is unable to induce apoptosis in these cells due to their high content in Bcl-2.

(ii) Both cell lines contain procaspase 9 (Figs. 4 and 5), but cleavage was seen only in HeLa cells, with camptothecin being the more rapid and more efficient inducer (Fig. 4). In agreement with this finding, cytochrome c release was detected only in HeLa cells both by immunoblotting (Fig. 4) and immunocytochemistry (Fig. 6).

(iii) In HeLa cells, cleavage of procaspase 8 was seen after treatment with either drug, albeit with different kinetics (Fig. 4). At 6 h there was cleavage of procaspase 8 after exposure to flavopiridol, increasing at 12 h, and by 18 h no pro-form was detectable. After treatment with camptothecin, there was only a slight cleavage after 12 h, which increased at 18 h, but at 24 h there was still some pro-form observable (Fig. 4). Since SW2 cells were found to lack procaspase 8 (Fig. 5), we also analyzed another small cell lung carcinoma cell line, H69, which contains similarly high levels of Bcl-2 but is not deficient in procaspase 8 (data not shown). In these cells, flavopiridol triggered caspase 8 activation (data not shown), indicating that this step is not blocked by Bcl-2.

Transfection of the SW2 cells with 250 nm AS ODN led to a ~90% decrease in the level of Bcl-2 protein at 48 h (Fig. 3A). As expected, after transfection of the AS ODN, the cells showed a clearly enhanced response both to camptothecin and cisplatin (Fig. 3B), whereas a control sense ODN did not chemosensitize the cells. In contrast, AS ODN treatment did not alter the cells’ responsiveness to flavopiridol.

Caspase Activation and Cytochrome c Release in Flavopiridol-induced Apoptosis: Evidence for Multiple Pathways—To elucidate the pathways involved in flavopiridol-induced apoptosis, we determined the activation of defined caspases in comparison to camptothecin-triggered cell killing using two different cell systems, i.e. HeLa cells, which contain negligible

![Fig. 1. Endogenous levels of Bcl-2.](image1)

![Fig. 2. Effect of ectopically expressed Bcl-2.](image2)

![Fig. 3. Bcl-2 antisense ODN treatment of SW2 cells.](image3)
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The goal of the present study was to elucidate the pathways that convey the cytotoxic signal induced by flavopiridol to the presence of these inhibitors are presented in Fig. 7.

(i) As expected, zVAD blocked the induction of apoptosis by either drug in HeLa cells (Fig. 7A) and by flavopiridol in SW2 cells (Fig. 7B), confirming that caspases are instrumental in the process.

(ii) Likewise, IETD clearly delayed killing of HeLa cells by flavopiridol, whereas it only marginally affected camptothecin-induced killing (Fig. 7A). In agreement with this observation, the viral caspase 8 inhibitor crmA (46) reduced flavopiridol-triggered apoptosis to a much greater extent than camptothecin-induced cell death (~60% versus ~30%; Fig. 7D). These observations suggest that caspase 3 is involved in flavopiridol-induced killing in HeLa cells, but not in SW2 cells, which, as shown above, lack caspase 8.

(iii) In HeLa cells, DEVD efficiently inhibited camptothecin-induced killing (from ~75% in the absence of the inhibitor to ~30% in DEVD-treated cells at 20 h), whereas it led only to a small decrease in flavopiridol-induced cell killing (from 75% to 65%) (Fig. 7A). This suggests that, in HeLa cells, caspase 3 is essential for camptothecin-induced apoptosis, but not for flavopiridol-triggered cell death. Since PARP cleavage was observed 24 h after flavopiridol treatment even in the presence of DEVD (Fig. 7C), the involvement of a caspase that is different from, but functionally related to, caspase 3 seems likely. Such a caspase is not activated in camptothecin-treated cells, since in these cells PARP cleavage was inhibited by DEVD (Fig. 7C).

In SW2 cells, DEVD had a dramatic effect on flavopiridol-induced apoptosis, indicating that in these cells caspase 3 is essential (Fig. 7B).

Flavopiridol Induces Mitochondrial Membrane Depolarization—To address the question as to whether mitochondria are involved in flavopiridol-induced apoptosis despite the lack of cytochrome c release, we determined the depolarization of mitochondria in response to the drug. For this analysis, we used the two small cell lung carcinoma cell lines, SW2 and H69, which contain similarly high levels of Bcl-2. The membrane potential-sensitive dye DePsiphertm was added to cultures of control and treated cells, and the fraction of cells with depolarized mitochondria was determined microscopically. Both cell lines showed a rapid depolarization affecting 30% of the cell population as early as 6 h (Fig. 8). This loss of mitochondrial potential increased as a function of time. Camptothecin treatment led only to a slight increase in the fraction of cells with depolarized mitochondria and only at late time points (24 h).

Interestingly, an inhibitor of the PTP, bongkrekic acid, was unable to prevent mitochondrial depolarization in flavopiridol-treated SW2 and was unable to rescue these cells from apoptosis (Fig. 9). In contrast, bongkrekic acid significantly reduced mitochondrial depolarization in camptothecin-treated HeLa cells (Fig. 9).

Since ATP synthesis in respiring mitochondria is dependent on an intact membrane potential a prediction from these observations would be an increase in the ADP/ATP ratio in flavopiridol-treated SW2 and H69 cells. The data in Fig. 10 confirm this prediction. After treatment with flavopiridol a progressive and clear increase in the intracellular ADP/ATP ratio was observed. In agreement with the results on mitochondrial depolarization, the ADP/ATP ratio was affected by camptothecin to a considerably smaller extent and this effect occurred later.

These results clearly suggest an involvement of the mitochondria in flavopiridol-mediated apoptosis that does not include the release of cytochrome c.

DISCUSSION

(iv) In HeLa cells, treatment with camptothecin led to a rapid cleavage of procaspase 3 at 6 h (Fig. 4). In contrast, treatment with flavopiridol resulted in a slight activation of caspase 3 at 12 h, which increased at 18 h (Fig. 4). Likewise, cleavage of procaspase 3 by flavopiridol occurred relatively late in SW2 cells (Fig. 5).

These kinetics support the hypothesis that caspases 9 and 3 play a primary role in camptothecin-induced apoptosis in HeLa cells, while caspase 8 is a major player in flavopiridol-triggered death. Since SW2 cells lack procaspase 8, the pathway of flavopiridol-induced apoptosis appears to be substantially different in these cells.

Defined Caspases Are Instrumental in Flavopiridol-induced Apoptosis—Next we addressed the role of caspase activation in the induction of apoptosis by flavopiridol or camptothecin both in HeLa and SW2 cells. Three peptide inhibitors of caspases were tested for their ability to alter flavopiridol- and camptothecin-induced killing: zVAD, a general inhibitor of caspases; DEVD, a preferential inhibitor of caspase 3; and IETD, a preferential inhibitor of caspases 6 and 8 (45). The results of treating HeLa or SW2 cells with flavopiridol or camptothecin in the presence of these inhibitors are presented in Fig. 7.
death machinery and to investigate the influence of Bcl-2 on this process. Our data clearly show that caspases are instrumental in flavopiridol-induced apoptosis and that multiple pathways are used, allowing flavopiridol to escape from certain resistance mechanisms, such as Bcl-2 overexpression. Interestingly, flavopiridol not only uses “classical” pathways of drug-induced apoptosis, but also seems to trigger hitherto unidentified mechanisms.

**Apoptotic Pathways Involving Caspase 8**—In HeLa cells, which contain low levels of Bcl-2 and a normal set of procaspases, flavopiridol triggers the early cleavage of procaspase 8, the delayed activation of caspase 3, and a partial, late cleavage of caspase 9. The activation of caspase 3 in response to flavopiridol has also been reported by others (33, 47). Taken together with the inhibitor studies, i.e. inhibition of cell killing by IETD and crmA, it is likely that caspase 8 plays an essential role. Although the downstream caspase 3 is activated, it does not seem to be essential, since PARP cleavage and cell death continued to occur in the presence of DEVD. In agreement with this conclusion, flavopiridol efficiently induces cell death in the human breast carcinoma line MCF-7 (data not shown), which lacks caspase 3 (44). This suggests that another unidentified caspase (subsequently referred to as caspase X) transmits the signal from activated caspase 8 (or caspase 8 itself) to the apoptotic machinery, a mechanism that does not operate after camptothecin treatment. Even though the identity of caspase X is unclear, it is unlikely to be one of the recently described family members involved in apoptosis, such as caspase 2 (48) and caspase 12 (49), which are located in the Golgi complex and the endoplasmic reticulum, respectively. This assumption is also supported by the fact that caspase 12 is not inhibited by crmA (49).

The activation of caspase 9 in flavopiridol-treated HeLa cells is likely to occur via the caspase 8-Bid-mitochondria pathway, which leads to cytochrome c release, followed by cleavage of procaspase 9. It is unlikely that flavopiridol efficiently triggers a caspase 8-independent signaling pathway leading to the release of cytochrome c from mitochondria, since IETD had a
dramatic effect on the induction of apoptosis. In agreement with this notion is the observation that caspase 3, the only known effector caspase downstream of caspase 9, is largely dispensable in flavopiridol-induced apoptosis (as discussed above).

On the basis of these findings, it would seem that the caspase 8/caspase 3 or caspase X pathway is of particular relevance for the induction of cell death by flavopiridol (see model in Fig. 11), which contrasts with the observations made with camptothecin. Apoptosis induced by the latter drug seems to involve predominantly the caspase 8-independent release of cytochrome c. At present, we do not know how flavopiridol leads to the activation of the initiator caspase 8. In many other systems of drug-induced apoptosis, the CD95/Fas ligand system plays an important role in triggering this pathway. However, the implication of p53 in the drug-induced activation of CD95 (12, 15) could make this less likely in view of the p53 independence of flavopiridol-induced apoptosis (29). The inhibition of cyclin-dependent kinases by other means, such as p16 overexpression (50) or down-regulation of cyclin D1 by antisense (51) can also result in apoptosis, but the underlying mechanisms are also unknown.

Clearly, tumor cells usually overexpress numerous proteins that are endowed with potentially pro-apoptotic properties, such as Myc or E2F. These proteins can induce apoptosis when cell cycle progression is stalled, as for instance in response to DNA-damage or a metabolic blockade (2, 52). The fact that this apoptotic pathway involves the release of cytochrome c and the CD95/Fas ligand system has provided a first link between the...
cell cycle and one of the commonly used apoptotic pathways (53). In the case of flavopiridol, this could also explain in part the tumor cell specificity of the drug. It will be interesting to investigate whether defined oncoproteins or other altered regulators of the cell cycle play a role in flavopiridol-induced cell death.

**Apoptotic Pathways in Cells Expressing High Levels of Bcl-2 and Lacking Procaspase 8—**

SW2 cells express a very high level of Bcl-2, which blocks the release of cytochrome c and the subsequent activation of caspase 9. These cells also lack procaspase 8, but nevertheless caspase 3 is activated in response to flavopiridol. In contrast to the situation in HeLa cells, this activation of caspase 3 is essential. What is the mechanism leading to the caspase 3 activation? At present, we can only speculate, but the available information allows for the discussion of possible mechanisms.

It is unlikely that flavopiridol itself mediates procaspase 3 cleavage, since the caspase 8 inhibitors IETD and crmA block apoptosis by flavopiridol in HeLa cells. It seems more likely that a mitochondrial pathway is involved, even though cytochrome c is not released (see model in Fig. 11). This hypothesis is based on the observation that depolarization of the mitochondrial inner membrane is efficiently induced by flavopiridol and is an early event. This depolarization is followed by a clear increase in the ADP/ATP ratio, which presumably is a consequence of impaired ATP synthesis, since the mitochondrial ATP synthetase is dependent on the maintenance of an intact membrane potential. That cytochrome c release and depolarization are separable is conceivable in view of published data (54), although the precise molecular mechanisms are not clear. It has been suggested that cytochrome c release is mediated by Bax through the formation of pores in the outer mitochondrial membrane, but the role of the PTP in this process remains unclear (9, 10, 55). It is intriguing that bongkrekic acid, an inhibitor of the PTP (10), was unable to prevent mitochondrial depolarization in flavopiridol-treated cells, although it did inhibit depolarization and apoptosis in response to camptothecin. Therefore, flavopiridol either induces mitochondrial permeability not involving the PTP or it directly interferes with the function of PTP.

It has been reported previously that hypericin-photo-induced mitochondrial membrane depolarization is also refractory to the action of bongkrekic acid, which appears to be the result of direct drug damage of the mitochondrial membrane (56). This, however, seems to a specific property of hypericin, which in conjunction with light is a powerful generator of free radicals. Similar properties are not known for flavopiridol. On the other hand, chemical compounds affecting the PTP are known, such as cyclosporin and bongkrekic acid itself (10). In addition, different members of the Bcl-2 family, like Bax, Bcl-2, and BclXL, have been reported to interact with components of the PTP, i.e. the voltage-dependent anion channel and/or the adenine nucleotide translocase (10). It may be possible that flavopiridol acts at similar targets, and thereby contributes to the induction of apoptosis. In agreement with such a hypothesis would be the observation that Bcl-2 was unable to block flavopiridol-triggered mitochondrial membrane depolarization, which is an
Caspase 3 activity. The Bid-mediated cytochrome c, that is sufficient to trigger apoptosis in the absence of caspase 8 then activates caspase 3 and an unidentified caspase, termed case, procaspase 8 is activated by an unknown mechanism. Active caspase 3 or caspase 8, since it can utilize alternate pathways for the induction of cell death. These caspases are also lacking in certain human tumors, which could render them resistant to conventional chemotherapy. Taken together with the previously reported p53 independence and refractoriness to multi-drug resistance, flavopiridol could make an invaluable contribution to clinical oncology. In this context, the strong synergism of flavopiridol with other drugs, such as taxol (47), might be of particular importance.

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**FIG. 11. Putative pathways of flavopiridol-induced apoptosis.** Pathway A plays a prominent role in caspase 8-expressing cells. In this case, procaspase 8 is activated by an unknown mechanism. Active caspase 8 then activates caspase 3 and an unidentified caspase, termed caspase X, that is sufficient to trigger apoptosis in the absence of caspase 3 activity. The Bid-mediated cytochrome c release and procaspase 9 activation seems to play a minor role in flavopiridol-induced apoptosis. Pathway B represents a caspase 8-independent induction of the mitochondrial pathway leading to caspase 9 activation, as in the case of p53-mediated cell death, but this pathway does not seem to be of importance for flavopiridol-induced apoptosis. Pathway C deserves particular attention since it is triggered in the cells expressing high levels of Bcl-2 and lacking caspase 8. Here, flavopiridol triggers, through an unknown mechanism, the depolarization of mitochondria in the absence of cytochrome c release, followed by the activation of caspase 3, which in this case in functionally crucial. Release of protons into the cytoplasm may be potentially involved. However, at this point it cannot be ruled out that mitochondrial depolarization and activation of caspase 3 are separate events (pathway D), although this may be less likely.
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