Z-FA-fmk Inhibits Effector Caspases but not Initiator Caspases 8 and 10, and Demonstrates That Novel Anticancer Retinoid-related Molecules Induce Apoptosis via the Intrinsic Pathway

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Abstract
Synthetic retinoid-related molecules (RRMs) have been described that show strong antiproliferative activity and induce apoptosis in cancer cells. These RRM\textsuperscript{s} induce caspase activity independently of the retinoid receptors in Jurkat T cells. We observed that the inhibitor of cathepsins B and L Z-FA-fmk blocks the induction of DEVDase activity, DNA fragmentation, and externalization of phosphatidylserine by selective RRM\textsuperscript{s}. Z-FA-fmk can inhibit caspase activity \textit{in vitro} and selectively inhibits recombinant effector caspases 2, -3, -6, and -7. In contrast, purified initiator caspases 8 and 10 are not affected, whereas the apoptosome-associated caspase 9 is only partially inhibited by Z-FA-fmk \textit{in vitro}. These data correlate with the covalent binding of biotinylated Z-FA-fmk to the active large subunit of effector caspases. This selective targeting of effector caspases is also observed in Jurkat cells and has been used to demonstrate that RRM\textsuperscript{s} induce apoptosis through the mitochondrial pathway and activate caspase 8 in a Z-FA-fmk-sensitive manner. Thus, Z-FA-fmk fails to inhibit Fas-mediated activation of caspase 8, but completely inhibits RRM-induced processing of caspase 8. Z-FA-fmk does not prevent the autoproteolytic cleavage of caspase 9 in Jurkat cells and partially inhibits the processing and full maturation of effector caspases induced by the RRM\textsuperscript{s}. Moreover, Z-VAD-fmk and Z-FA-fmk have no effect on the release of cytochrome c induced by the RRM\textsuperscript{s}. Other cathepsin inhibitors elicit no effect on RRM-induced apoptosis in Jurkat cells, suggesting that caspases are the major effectors of RRM action.

Introduction
Vitamin A derivatives (retinoids) regulate important biological functions and modulate the growth and differentiation of cancer cells. Because of this latter property, retinoids have shown promise as anticancer agents. Currently available retinoids have failed in the clinic because of limited efficacy and considerable toxicity. Certain natural and synthetic retinoids, such as anhydroretinol and 4-HPR,\textsuperscript{3} have been shown to induce apoptosis through the generation of reactive oxygen species (1, 2). A different class of synthetic RRM\textsuperscript{s}, which includes the RAR\textgamma-selective compounds CD437 and MX2870-1 (3, 4) and the antagonist MX781 (5), are also potent inducers of apoptosis. RRM-induced apoptosis does not require transcription/translation and is independent of the retinoid receptors (4, 6–8), although CD437 also induces apoptosis in ovarian carcinoma cells in a RAR-dependent manner (9). The mechanism of RRM-induced apoptosis is largely unknown, but activation of stress kinases JNK and p38 by the RAR\textgamma-selective RRM\textsuperscript{s} has been shown to be necessary for the release of cytochrome c and subsequent activation of caspases (10).

Initiation and execution of apoptosis relies on a complex network of cysteine proteases named caspases, which cleave their targets in an aspartate-directed manner and which exist as inactive proenzymes that are activated by limited proteolysis (11). Caspases involved in apoptosis are divided into two main groups: initiator and effector caspases. Caspases 8 and 10 initiate the extrinsic pathway, which is activated on ligation of cell surface death receptors (Fas/CD95, TNF receptor 1; Refs. 12–14). Caspase 9 is the apical caspase in the intrinsic or mitochondria-initiated apoptosis pathway, which is normally triggered by stress and chemotherapeutic drugs and which requires the release of cytochrome c from the mitochondria and interactions with Apaf-1 (15–19). Activation of upstream caspases leads to the proteolysis and activation of the effector caspases 3, -6, and -7 (16, 20–23), which are involved in the cleavage of specific cellular proteins causing the appearance of characteristic apoptotic phenotypes (24, 25). Caspase 2 contains a large prodomain and can be activated by caspase 3 during the mitochondria-initiated pathway (23). In addition, caspase 2 also can induce the release of mitochondrial proteins (26, 27) and is required for mitochondrial permeabilization in stress-induced apoptosis (28).

1 Supported by grants from NIH (CA 75033) and the California Cancer Research Program (00-0778V-20253) to F. J. P.
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3 The abbreviations used are: 4-HPR, 4-hydroxyphenyl-retinamide; AFC, 7-amino-trifluoromethyl coumarin; fmk, fluoromethylketone; JNK, c-Jun NH\textsubscript{2}-terminal kinase; PI, propidium iodide; RAR, retinoic acid receptor; RRM, retinoid-related molecule; TNF, tumor necrosis factor; ICM, intracellular-like medium.
Mitochondria have been well recognized as initial sensors of apoptosis signals other than membrane receptor-mediated responses (29). In addition to cytochrome c, several other proteins are released from the mitochondria during apoptosis. These include the apoptosis inducing factor, Smac/DIABLO, endonuclease G, and the serine protease Htr2A/Omi, which can play different roles in the execution of the cell death program independently of caspases (30–34). Accordingly, apoptosis can also occur in the absence of measurable caspase activity (35), suggesting that other proteases, including granzymes, calpains, and cathepsins, may participate in the induction of apoptosis (reviewed in Ref. 36). For example, cathepsins B and D are apparently necessary for the induction of apoptosis by TNFα and oxidative stress, respectively (37–40).

The use of specific caspase inhibitors has proven an essential role for caspases in apoptosis. Synthetic peptides such as Z-DEVD-fmk and Z-VAD-fmk have been widely used to block apoptosis (e.g., Refs. 41–43). However, the pan-caspase inhibitor, Z-VAD-fmk, can also inhibit other cysteine proteases in vitro, including calpain and cathepsin B (44, 45). Similarly, the inhibitor of cathepsins B and L Z-FA-fmk has been shown to partially prevent p53-dependent apoptosis (46) and to inhibit certain caspases in vitro (40, 47). To gain insight into the mechanism of RRM-induced apoptosis, we decided to examine the effect of Z-FA-fmk and other protease inhibitors on the induction of apoptosis by RRMs and to characterize in more detail the inhibition of recombinant caspases by the cathepsin inhibitor Z-FA-fmk in vitro. We found that Z-FA-fmk efficiently inhibited the activity of purified recombinant effector caspases 2, -3, -6, and -7 in vitro. In contrast, the activity of caspases 8 and 10 was unaffected, whereas caspase 9 was only partially inhibited by Z-FA-fmk. The selectivity of Z-FA-fmk toward effector caspases was also observed in intact cells and has been useful to demonstrate that RRM-induced apoptosis occurs via the mitochondrial (intrinsic) pathway.

**Materials and Methods**

**Cells and Reagents.** Jurkat cells were grown in RPMI containing 10% heat-inactivated fetal bovine serum, glutamine, nonessential amino acids, penicillin, and streptomycin. Cells were incubated with RRMs in the presence of medium containing 0.5% fetal bovine serum. Caspase inhibitors and substrates were purchased from Enzyme System Products (Dublin, CA). Other protease inhibitors were obtained from Peptides International (Louisville, KY) or Calbiochem (San Diego, CA). Purified recombinant caspases 3, -6, and -7 were a generous gift of Dr. Guy Salvesen (The Burnham Institute, La Jolla, CA; Ref. 48). Caspases 2, -8, -9, and -10 were purchased from Bioworld. Selective RRMs were obtained from Maxia Pharmaceuticals Inc. and prepared in DMSO as 10-mM stock solutions. Antibodies against Bid and cleaved caspases 3 and 9 (Asp-315 and Asp-330) were obtained from Cell Signaling (Beverly, MA). Monoclonal antibody against cytochrome c (7H8.2C12) and antibodies against caspases 2 and 7 were purchased from Pharmigen (San Diego, CA). Caspase 8 antibody was from R&D Systems (Minneapolis, MN).

**Fluorometric Determination of Caspase Activity.** Protein extracts were prepared from Jurkat cells in cytosol extraction buffer [25 mM PIPES (pH 7), 25 mM KCl, 5 mM EGTA, 1 mM DTT, 10 μM cytochalasin B, 0.5% NP40, and a mixture of protease inhibitors consisting of 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin] and cleared by centrifugation at 20,000 x g for 30 min at 4°C. Ten μg of protein were diluted in a total volume of 100 μl of caspase buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 0.1% 3-[3-cholamidopropyl]-dimethylammonio]-1-propane sulfonate, and 10% sucrose] containing 100 μM Ac-DEVD- AFC as described previously (48). Alternatively, extracts prepared in ICM buffer (see below) were used. For the inhibition studies, purified recombinant caspases were diluted in 90 μl of caspase buffer containing increasing amounts of fmk inhibitors. After 30 min of incubation at 37°C, 10 μl of the appropriate AFC substrate were added, and the reactions were further incubated at 37°C for 30 min. The release of AFC was measured every 3 min as emission at 510 nm on excitation at 390 nm using a Victor2 microplate reader (Perkin-Elmer). Caspase activity (pmol AFC min⁻¹ μg⁻¹) was calculated from the initial slope. Caspases 3 (0.03 pmol) and 7 (0.03 pmol) were incubated with 100 μM Ac-DEVD-AFC. Caspase 6 (1 pmol) activity was analyzed in the presence of 200 μM Ac-VEID-AFC. The activity of caspase 2 (0.35 pmol) was measured in the presence of 200 μM Ac-VDVAD-AFC, and caspase 9 (18.17 pmol) was assayed in the presence of 2 μM Ac-LHDAF. Caspases 8 (0.047 pmol) and 10 (1.21 pmol) were incubated with 200 μM and 1 mM Ac-IETD-AFC, respectively.

**Measurement of Cathepsin Activity.** Protein extracts were prepared from 2.5 × 10⁶ Jurkat cells in 50 μl of ICM buffer [120 mM KCl, 10 mM NaCl, 1 mM KH₂PO₄, 20 mM HEPES-Tris (pH 7.1), 2 mM succinate, 100 μM/digitonin, and 1 mM phenylmethylsulfonyl fluoride]. Protease inhibitor leupeptin was excluded from the ICM buffer because of its potent inhibition of cathepsins B and L. Ten μg of protein were diluted in a total volume of 100 μl of caspase buffer [0.1 2-[N-morpholino]ethane sulfonic acid buffer (pH 6.1), 1 mM EDTA, 2 mM DTT] or in cathepsin L buffer [20 mM sodium acetate (pH 5.0), 4 mM EDTA, 4 μM urea, and 8 mM DTT], containing 100 μM BOC-Leu-Arg-Arg-AFC, 20 μM Z-Phe-Arg-AFC, and 10 μM Ac-Leu-Arg-Arg-AFC. Fractions were analyzed in 2 μl of ICM buffer (see below) and in ICM buffer containing 100 μM cytochalasin B, 0.5% NP40, and 10 μM Ac-Leu-Arg-Arg-AFC. Fractions were analyzed in 2 μl of ICM buffer (see below) and in ICM buffer containing 100 μM cytochalasin B, 0.5% NP40, and 10 μM Ac-Leu-Arg-Arg-AFC.
15 min at room temperature in the dark. Cells were subsequently analyzed by flow cytometry (FACS Calibur) for apoptosis (fluorescein isothiocyanate) and viability (PI).

**Affinity-labeling of Purified Caspases with Biotinylated Peptides.** Purified recombinant caspases were diluted in 20 μl of caspase buffer and incubated for 30 min at 37°C with the indicated concentrations of biotinylated peptides in the absence or in the presence of unlabeled peptides. The reaction was stopped by the addition of Laemmli buffer, and proteins were analyzed by SDS-PAGE (20%) and transferred to Immobilon-P membrane (Millipore, Bedford, MA). Proteins were detected with a horseradish peroxidase conjugated to streptavidin followed by ECL (Amersham Pharmacia).

**Immunoblot Analysis of Caspase Activation and Cytochrome c Release.** To analyze caspase activation, cytosol extracts were prepared in either CE or ICM buffer. Lysates in ICM buffer were used to examine cytochrome c release. Twenty to 50 μg of protein extracts were separated in a 17.5% SDS-polyacrylamide gel, transferred to Immobilon-P membrane, and analyzed exactly as described (10).

**Results**

**Z-FA-fmk Inhibits the Induction of DEVDase-dependent Events by Selective RRMs.** Z-FA-fmk is an inhibitor of cathepsins B and L (49), which has been shown to partially prevent p53-dependent apoptosis in myeloid cells (46). Similarly, Jurkat cells that were preincubated with increasing concentrations of Z-FA-fmk showed reduced levels of DEVDase activity and DNA fragmentation when treated with the RARγ-selective RRM MX2870-1 or the antagonist MX781 (Fig. 1A). The induction of DEVDase activity and DNA fragmentation by these two RRMs were also well prevented in the presence of low concentrations (5–20 μM) of Z-VAD-fmk or Z-DEVD-fmk (data not shown; see Ref. 10). Similarly, preincubation with Z-VD-fmk or with high concentrations of Z-FA-fmk significantly inhibited the externalization of phosphatidylserine induced by either MX2870-1 or MX781 (Fig. 1B). To explore whether this effect of Z-FA-fmk was specific to RRM-induced apoptosis, we also investigated the generation of DEVDase activity by different apoptotic stimuli in the absence or in the presence of Z-FA-fmk. This dipeptide efficiently inhibited the induction of DEVDase activity not only by the RRMs but also by other apoptotic insults, including etoposide-, ceramide-, and CD95/Fas receptor-mediated pathways (Fig. 1C).

**Z-FA-fmk Inhibits Caspase Activity in Vitro.** To determine whether the effect of Z-FA-fmk on RRM-induced apoptosis was a direct effect on caspase activity, we examined whether Z-FA-fmk could inhibit caspase activity in crude extracts prepared from apoptotic cells. Cell lysates obtained from MX2870-1-treated Jurkat cells showed a significant amount of DEVDase activity, which was eliminated on incubation with Z-FA-fmk (Fig. 2A). Z-VAD-fmk, or Z-DEVD-fmk (data not shown). Using a cell-free assay to measure activation of caspases, we repressed the induction of DEVDase activity by preincubation with Z-FA-fmk in a concentration-dependent manner (Fig. 2B). Similarly, incubation with Z-FA-fmk after stimulation with cytochrome c and dATP also completely suppressed DEVDase activity (not shown), suggesting that the peptide could effectively inhibit the activity of mature caspases.

We, therefore, investigated the effect of increasing concentrations of Z-FA-fmk on the activity of all of the purified recombinant caspases that are involved in apoptosis. We found that Z-FA-fmk was an efficient inhibitor of all of the effector caspases, being most effective against caspase 2 (Fig. 2C). Z-FA-fmk also inhibited caspases 7 and 3, and blocked caspase 6 to a lesser extent. In contrast, it showed no effect on death receptor-associated initiator caspases 8 and 10 (Fig. 2C). The other initiator caspase, caspase 9, was only partially inhibited in the presence of high concentrations of the peptide inhibitor. These initiator caspases, however, were efficiently inhibited by their cognate inhibitors Z-IE TD-fmk and Z-LEHD-fmk, respectively, as well as by the pan-caspase inhibitor Z-VAD-fmk (Table 1).

**Z-FA-fmk Binds Covalently to the Large Subunit of Active Caspases.** To provide evidence of a direct interaction between Z-FA-fmk and effector caspases, we carried out an in vitro affinity-labeling approach with biotinylated...
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Z-FA-fmk. We observed that biotin-Z-FA-fmk bound to the large subunit of purified caspases 2, -3 and -7 (Fig. 3A). In agreement with the inhibition studies, binding to caspase 6 was significantly weaker. However, we did not detect binding of biotin-Z-FA-fmk to initiator caspases 8, -9, or -10. As expected, biotin-Z-FA-fmk bound very strongly to all of the caspases, which correlated with a potent inhibition of their activity. Competition with an excess of unlabeled Z-VAD-fmk, but not with a granzyme B inhibitor, demonstrated that the binding of biotin-Z-FA-fmk to caspase 7 was specific (Fig. 3B).

Z-FA-fmk Specifically Inhibits Effector Caspases and Does Not Prevent Cytochrome c Release in Intact Cells. We next examined whether this differential effect toward effector caspases that was elicited in vitro could also be illustrated in a cellular system, and whether it could be exploited to decipher the pathway of caspase activation initiated by selective RRMs. We found that Z-FA-fmk completely blocked the activation of caspase 8 induced by RRMs but not that induced by anti-Fas. However, it did not inhibit the autoproteolytic cleavage of caspase 9 at Asp-315 (p35 fragment) was not affected, Z-FA-fmk inhibited the full maturation of caspase 3 to its active form p17, and only reduced amounts of the intermediate fragment p19 were detected in RRM-treated cells. Both Z-FA-fmk and Z-VAD-fmk effectively inhibited the induction of DEVDase activity by all of the stimuli. Finally, we examined the effect of the peptide inhibitors on cytochrome c release. Z-VAD-fmk, but not Z-FA-fmk, prevented the release of cytochrome c in cells stimulated with anti-Fas antibody, which is initiated by caspase 8-mediated cleavage of Bid and which, furthermore, indicated that Z-FA-fmk was inactive against caspase 8 in intact cells. However, both peptides failed to block the release of cytochrome c induced by the RRMs. Taken together, these findings demonstrate a major role for the mitochondrial pathway in RRM-induced apoptosis.

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Z-FA-fmk Inhibits the Processing and Activation of Most Caspases in RRM-treated Cells. We further examined the processing of other effector caspases induced by MX2870-1 in the absence or in the presence of increasing concentrations of Z-FA-fmk using immunoblots. Caspases 2, -3, -6, -7, -8, and -9 were all activated in Jurkat cells treated with MX2870-1 for 3 h, as demonstrated by the appearance of mature or intermediate proteolytic fragments of the caspases, as well as the disappearance of the procaspase (Fig. 5 and data not shown). Preincubation with increasing amounts of Z-FA-fmk completely abolished the activation of caspase 8. Although the autoproteolytic cleavage of caspase 9 at Asp-315 (p35 fragment) was not affected, Z-FA-fmk substantially inhibited the cleavage at Asp-330 (fragment p37), which is mediated by caspase 3 in vivo (16). The cleavage of effector caspases 2, -3, and -7 was significantly inhibited by Z-FA-fmk (Fig. 5). This further confirmed the selectivity of Z-FA-fmk toward effector caspases in cellular assays. MX2870-1-mediated cleavage of Bid, a substrate of caspase 8, was totally inhibited by increasing amounts of Z-FA-fmk, correlating with the inhibition of caspase 8 activation and proving that caspase 8 activation is mediated by Z-FA-fmk sensitive (effector) caspases (Fig. 5). It is interesting to note that at least 10 μM Z-FA-fmk was necessary to inhibit the proteolytic processing of caspases as detected by immunoblot, whereas lower concentrations of the inhibitor (3 μM) were sufficient to significantly reduce DEVDase activity by 25%. This suggested that low concentrations of Z-FA-fmk could partially inhibit the activity of mature effector caspases in intact cells, whereas higher concentrations were required to prevent the cleavage and activation of effector caspases initiated by caspase 9 correlating with partial inhibition of mature caspase 9.

Cathepsins Are Not Involved in RRM-induced Apoptosis. Cathepsins B and D have been shown to be involved in apoptosis induced by several stimuli, including the RRM analogue CD437 (50). Although we have presented evidence...
Table 1  Inhibition of caspases by fmk derivatives

Recombinant purified caspases were preincubated with increasing amounts of the different fmk derivatives for 30 min at 37°C before the caspase activity was measured using appropriate AFC substrates, as indicated in “Materials and Methods.” Triplicate points were measured and the experiment was repeated at least twice with each caspase. The values represent the concentration of the fmk derivative (in μM) that inhibits 50% of the total caspase activity measured in the absence of the inhibitor.

<table>
<thead>
<tr>
<th>Caspase</th>
<th>FA</th>
<th>DEVD</th>
<th>IETD</th>
<th>VDVAD</th>
<th>LEHD</th>
<th>VAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casp-2</td>
<td>6.147</td>
<td>1.732</td>
<td>0.727</td>
<td>1.252</td>
<td>N.d.</td>
<td>1.954</td>
</tr>
<tr>
<td>Casp-3</td>
<td>15.410</td>
<td>0.027</td>
<td>0.269</td>
<td>0.067</td>
<td>N.d.</td>
<td>0.183</td>
</tr>
<tr>
<td>Casp-6</td>
<td>32.450</td>
<td>1.577</td>
<td>0.300</td>
<td>4.004</td>
<td>0.710</td>
<td>N.d.</td>
</tr>
<tr>
<td>Casp-7</td>
<td>9.077</td>
<td>0.062</td>
<td>0.663</td>
<td>0.326</td>
<td>N.d.</td>
<td>0.100</td>
</tr>
<tr>
<td>Casp-8</td>
<td>0.566</td>
<td>0.217</td>
<td>0.506</td>
<td>N.d.</td>
<td>0.070</td>
<td></td>
</tr>
<tr>
<td>Casp-9</td>
<td>110.700</td>
<td>N.d.</td>
<td>0.074</td>
<td>0.035</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casp-10</td>
<td>5.222</td>
<td>5.081</td>
<td>17.970</td>
<td>N.d.</td>
<td>6.639</td>
<td></td>
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</tbody>
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*FA, Z-FA-fmk; DEVD, Z-DEVD-fmk; IETD, Z-IETD-fmk; VDVAD, Z-VDVAD-fmk; LEHD, Z-LEHD-fmk; VAD, Z-VAD-fmk; Casp-, caspase; N.d., not determined.

that Z-FA-fmk can efficiently inhibit caspase activity both in vitro and in intact cells, we cannot rule out that cathepsins B/L may participate in RRM-induced apoptosis. We, therefore, examined the presence of cathepsin B and L activities in the cytosol of RRM-treated cells and further analyzed the effect of other protease inhibitors in RRM-induced apoptosis. Of all of the apoptotic stimuli tested, including UV radiation, TNFα, anisomycin, anti-Fas antibody and various selective RRMs, only ceramide provoked a substantial increase in the amount of cathepsin-B and L activities in the cytosol, although extensive augment of DEVDase activity was observed with all those stimuli (Fig. 6A and data not shown). CD437, but not MX2870-1, induced a small although reproducible increase of cytosolic cathepsin B/L activities. Cathepsin activity was not detected in the presence of low concentrations of Z-FA-fmk (1 μM) or CA-074-Me (a specific inhibitor of cathepsin B/L). However, CA-074-Me and low concentrations of Z-FA-fmk did not affect the induction of DEVDase activity by ceramide or the RRMs. Only high concentrations of Z-FA-fmk (100 μM) were effective in preventing the induction of caspase activity (Fig. 6A). Other protease inhibitors, including pepstatin A (inhibitor of cathepsin D) and E-64D (inhibitor of cysteine proteases) showed no effect on the induction of DEVDase activity and DNA fragmentation by any from among the selective RRMs MX2870-1, CD437, or MX781 (Fig. 6B). Taken together, these results suggest that lysosomal cysteine proteases are not involved in the induction of apoptosis by selective RRMs in Jurkat cells and that the effects of Z-FA-fmk are directly related to the inhibition of effector caspases.

Discussion

The cytotoxic activity of chemotherapeutic drugs usually correlates with the induction of apoptosis. Natural (anhydroretin) and synthetic retinoids (4-HPR and CD437) have
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been shown to be potent inducers of apoptosis in several cancer cell types (3, 4, 51–53). The mechanism of retinoid-induced apoptosis is largely unknown, but different lines of evidence support a nuclear retinoid receptor-independent mechanism of action that does not require transcription/translation (4). Alternatively, a novel retinoid action in the cytosol is more probably responsible for the induction of apoptosis. Similar to other chemotherapeutic agents (23, 43, 54), mitochondrial damage is most likely a major arbitrator of retinoid-induced apoptosis, as recently suggested for CD437 (55). Thus, generation of reactive oxygen species by anhydroretinol and 4-HPR has been reported (1, 2). In addition, the elevation of ceramide levels by 4-HPR, and CD437–anhydroretinol with nanomolar affinity (60, 61). It will be of interest to investigate whether c-Raf and protein kinase C are also targets for the selective RRMs described here.

CD437 induces a rapid loss of inner mitochondrial transmembrane potential that precedes the generation of reactive oxygen species (55). In agreement, antioxidants have been shown to inhibit CD437-induced apoptosis (50). Using caspase inhibitors, we have established that MX2870-1 and MX781 induce the release of cytochrome c independently of caspase activity (see Fig. 4 and Ref. 10). In this study, we show that the inhibitor of cathepsins B and L, Z-FA-fmk, is also an efficient inhibitor of effector caspases and, interestingly, this selectivity is observed in intact cells as well. Thus, the activation of caspase 8 and the cleavage of caspase 9 at Asp330, induced by the RRMs, are effectively inhibited by Z-FA-fmk in Jurkat cells, arguing an effective inhibition of effector caspases 3 and 7. In contrast, Z-FA-fmk failed to inhibit the activation of caspase 8 induced by anti-Fas antibody, which initiates apoptosis through the extrinsic path-
way (62), whereas the pan-caspase inhibitor Z-VAD-fmk prevented Fas-mediated activation of caspase 8 and cytochrome c release. The activation of caspase 8 by effector caspases in RRM-treated cells further supports the conclusion that RRM s trigger the intrinsic pathway. An important role has now emerged for caspase 2 as an initiator caspase in stress- and chemotherapy-induced apoptosis (28). We have shown that Z-FA-fmk is an effective inhibitor of caspase 2 in vitro, and proteolytic processing of caspase 2 in Jurkat cells is partially reverted by preincubation with increasing amounts of Z-FA-fmk. Hence, our data suggest that caspase 2 activity is dispensable for the effective release of cytochrome c induced by RRM s because this is not blocked by Z-FA-fmk. However, we cannot rule out that Z-FA-fmk does not inhibit caspase 2 in vivo as efficiently as in vitro.

Although caspase-independent pathways have been described as participating in cell death and cathepsin D is apparently involved in CD437-induced apoptosis in HL-60 cells (50), we found no evidence that treatment with the apoptotic RRM s increases the amount of cytosolic cathepsin B or L activities in Jurkat cells. Similarly, cathepsin D activity was not detected in RRM-treated cells. Furthermore, several protease inhibitors as well as low concentrations of Z-FA-fmk that efficiently inhibited cathepsin B/L activity failed to prevent RRM-induced apoptosis. This suggested that the compounds analyzed here function independently of cathepsins B and L or of other lysosomal cysteine proteases, and caspases are the main executioners in RRM-mediated cell death in Jurkat cells.

Finally, our observations argue that certain protease inhibitors are not as specific as generally accepted. Z-VAD-fmk, for example, has been shown to bind to purified cathepsins B and H (45) and to inhibit the activity of calpains (44). Z-FA-fmk is often used as a negative control in experiments using synthetic caspase inhibitors. As demonstrated here, it is also an effective inhibitor of effector caspases at concentrations often used in many studies. Thus, an inhibition of apoptosis by Z-FA-fmk could be wrongly attributed to a potential role of cathepsins in apoptosis. Other studies have reported the inhibition of recombinant caspases by Z-FA-fmk in vitro. But none of these studies examined the effect on all of the apoptotic caspases simultaneously. Our in vitro data are in contrast to one study showing that Z-FA-fmk inhibited caspases 6, -7, and -9 with similar efficacy, although it was a poor inhibitor of caspase 3 (47). In general, we observed a very effective inhibition of all of the effector caspases using concentrations of Z-FA-fmk much lower than those previously reported. Because of the covalent nature of the fmk-mediated inhibition of caspases, this discrepancy is most likely caused by differences in the experimental conditions, including the time and temperature of incubation (63). In this regard, we found that incubation of the caspases with Z-FA-fmk for shorter periods of time or at lower temperatures dramatically reduced the inhibition of caspase activity. Another reason for the discrepancies among these studies could be differences in the purity and activity of the recombinant caspase preparations used. Importantly, in contrast to other fmk derivatives that inhibit various caspases (see Table 1), Z-FA-fmk is selective toward effector caspases and does not interfere with the activity of initiator caspases 8 and 10. Similarly, Z-FA-fmk does not prevent the autocleavage of caspase 9 at Asp-315 in intact cells. This selectivity of the peptide could be useful in deciphering caspase pathways that are activated by a particular stimulus, as we have demonstrated here.

Acknowledgments

We are grateful to Renata Hasim for technical assistance and Dr. Guy Salvesen for providing recombinant caspases. We thank Dr. Magnus Pflaai, Maxia Pharmaceuticals (San Diego, CA) and Galderma R&D (Sophia Antipolis, France) for the retinoid analogues. We also thank Marion Sauter for secretarial assistance.

References


Molecular Cancer Therapeutics

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