Retinoid-related Molecules Induce Cytochrome c Release and Apoptosis through Activation of c-Jun NH2-Terminal Kinase/p38 Mitogen-activated Protein Kinases

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ABSTRACT

Retinoid-related molecules have been described that induce apoptosis in a variety of cancer cell lines. Of particular interest is the apoptotic activity of the all-trans-retinoic acid receptor γ-selective molecules MX2870-1 and MX3350-1. These compounds have been shown to be effective in vitro against lung cancer and could therefore serve as important leads for novel anticancer drugs. We analyzed the death signaling pathways activated by these molecules. We observed that apoptotic retinoid-related molecules (RRMs) cause the release of cytochrome c from the mitochondria and subsequent activation of caspases 9 and 3. This was preceded by a strong and sustained activation of c-Jun NH2-terminal kinase as well as p38 kinase, which was independent of caspase activity. Inhibition of p38 kinase activity by the specific inhibitor SB203580 did not affect the induction of apoptosis by MX2870-1. However, interference with the activation of c-Jun NH2-terminal kinase and p38 stress kinases by PD169316 completely blocked all signs of apoptosis, including caspase activity, DNA fragmentation, and phosphatidylserine externalization. PD169316 also prevented the cleavage of Bid and the release of cytochrome c induced by this class of RRMs. Furthermore, processing and activation of different caspases by MX2870-1 was completely inhibited by increasing concentrations of PD169316. Thus, the investigated RRMs induce a death pathway, which is independent of Fas ligand, that is also activated by UV radiation and other agents. Our findings open the possibility for the future use of this class of RRMs in combination therapies with other anticancer drugs.

INTRODUCTION

Retinoids are vitamin A derivatives that regulate important biological functions, including cell growth and differentiation, development, and carcinogenesis (1). Retinoids regulate the expression of a large number of target genes upon binding and activation of the nuclear retinoid receptors, RARs3 and retinoid X receptors (Ref. 2 and references therein). Natural retinoids, such as RA, can induce cell differentiation and inhibit growth of certain cancer cells in vitro and in vivo; therefore, retinoids have been considered as potential agents for the prevention and treatment of cancer. However, the broad range of biological responses mediated by retinoids causes numerous secondary effects that have limited their clinical use. A new class of synthetic RRMs was recently described that show retinoid receptor selectivity and that induce apoptosis in cancer cells (3, 4). Importantly, some of these novel RRMs were also found to have lower side effects at concentrations at which they showed effective activities against solid tumors in vivo. Of these RRMs, CD437 was first reported to selectively activate RARγ and to cause apoptosis in breast cancer cells (5, 6). However, CD437, as well as the closely related analogue MX2870-1, were also found to induce apoptosis in a rather indiscriminate manner in many cancer cell types (3, 6–9), which was associated with high levels of general toxicity. Another structurally related compound, MX3350-1, has been observed to induce apoptosis more selectively in certain cancer cell types and to be effective against tumor growth in vivo at concentrations at which it does not induce overt signs of toxicity (3). We have also described another selective molecule, the agonist MX781, that induces apoptosis in breast cancer cells in vitro and is effective against human breast tumors in nude mice (4). The mechanism of RRM-induced apoptosis is not well understood. MX2870-1-induced cell death occurs via the activation of caspases and, interestingly, does not require transcription/translation (7), suggesting a novel mechanism of action that might be independent of the retinoid receptors, as suggested previously (10). CD437 has been shown to activate AP-1 in melanoma cells (11), and activation of c-Jun can lead to the induction of apoptosis in lung cancer cells (12), indicating that CD437 and related compounds might induce apoptosis through the activation of JNK. This contrasts to the well-known inhibition of JNK activity and AP-1 induction by RA, as well as other retinoids and hormones, which occurs in a receptor-dependent manner (13–17).

JNK protein kinases are a group of MAPKs, activated by exposure to cytokines and environmental stress, that regulate inflammatory responses, cell growth, proliferation, survival, and apoptosis (18, 19). Activation of p38 kinase, a second group of MAPKs, is also achieved by cellular stress, and a role in apoptosis has been noted (20, 21). JNK and p38 kinase were initially proposed to mediate apoptosis in neuronal cells (20), and phosphorylation of c-Jun is necessary for neuronal cell death (22). The use of kinase inhibitors and overexpression of dominant-negative mutant forms of MAPKs have demonstrated a role of JNK and/or p38 kinase in apoptosis induced in nonneuronal cells by various stimuli, including estrogen, chelerythrine, UV-B radiation, thapsigargin, and singlet oxygen (23–27). JNK also participates in anoikis, a process of cell death that occurs upon loss of extracellular matrix contacts (28), although this observation has been disputed (29). In contrast, activation of JNK is not involved in the induction of apoptosis by Fas or TNF receptor pathways (30, 31). Although JNK-mediated Fas ligand induction has been observed in T cells treated with DNA-damaging agents or under environmental stress (32, 33), Fas ligand expression might not be essential for the proapoptotic activity of JNK. Under certain circumstances, the activation of JNK and p38 MAPK has been observed to be dependent on caspase activity, and therefore, kinase activation can be a consequence of apoptosis. Thus, MEK1, a MAPK kinase that activates both JNK and p38 MAPK through MKK4, is cleaved and activated by caspase 3 (28, 34). Clarification on the role of JNK in apoptosis has come from recent knock-out experiments. The induction of apoptosis by various stimuli has been analyzed in mouse embryo fibroblasts obtained from Jnk1−/− Jnk2−/− embryos lacking JNK protein and activity (35). These fibroblasts are resistant to the induction of apo-
ptosis by UV radiation, anisomycin, and methyl methanesulfonate, but not by anti-Fas antibody. Interestingly, UV-mediated apoptosis does not require gene expression, indicating that JNK can regulate apoptosis independently of transcription (35).

To gain insight into the mechanism of apoptosis induced by RRM s that are selective for RARs, we analyzed the activation of different caspases and the induction of JNK and p38 MAP kinases. The inhibition of DEVDEFase activity, as a measurement of caspase activity, in a time- and RRM-concentration dependent manner. This was accompanied with the release of cytochrome c from the mitochondria and the processing of several caspases, including the upstream caspases 8 and 9. We observed a strong and sustained activation of both JNK and p38 MAPK in Jurkat cells upon exposure to RRM that correlated with the induction of caspase activity and apoptosis by various RAR-selective RRM s but was not observed with nonapoptotic retinoids. Furthermore, MAPK activation was independent of caspase activity. The induction of apoptosis by MX2870-1, as determined by DNA fragmentation, caspase activity, and externalization of phosphatidylserine, was completely prevented when cells were incubated with PD169316 (a potent inhibitor of RRM-mediated activation of JNK and p38 MAPK), but not with the selective p38 kinase inhibitor SB203580. The release of cytochrome c and subsequent activation of caspases were also inhibited by PD169316, but not by SB203580. Thus, our data indicate that activation of JNK (either alone or in combination with p38 MAPK) represents a necessary and early step for the induction of apoptosis by this class of RRM s in Jurkat T cells.

MATERIALS AND METHODS

Reagents. RA was obtained from Sigma Chemical Co. (St. Louis, MO). Selective RRM s were obtained from MAXIA Pharmaceuticals and Galdema Research Inc. Stock solutions containing 10 mM of the RRMs were made in DMSO. Anti-Fas antibody (CH-11 clone) was purchased from Kamiya Biomedical Company (Seattle, WA). Caspase inhibitors were obtained from Enzyme Systems Products (Livermore, CA). Kinase inhibitors were purchased form Calbiochem (San Diego, CA) or Alexis Biochemicals (San Diego, CA).

Antibodies against the different caspases were obtained from Cell Signaling Technology (Beverly, MA; caspases 3, 6, and 9), Pharmingen (San Diego, CA; caspases 6 and 7), and R&D Systems (Minneapolis, MN; caspase 8).

Cell Culture and RRM Treatment. Jurkat T cells were grown in RPMI 1640 containing 10% heat-inactivated FBS, 200 mM gluconate, nonessential amino acids, penicillin, and streptomycin sulfate. Before RRM treatment, cells were grown overnight (16–20 h) in medium containing 0.5% heat-inactivated FBS and subsequently stimulated in the presence of the same medium with low concentrations of serum. Synthetic RRMs were diluted in culture medium containing 0.5% FBS, keeping the DMSO concentration below 0.5%. Appropriate controls containing the same amount of solvent were included in each experiment.

Determination of Caspase Activity. Protein cell extracts were prepared from Jurkat cells treated or not with RRMs in extraction buffer (25 mM IPES (pH 7), 25 mM KCl, 5 mM EGTA, 1 mM DTT, 10 mM cysteine, 0.5% NP40, and a mixture of protease inhibitors consisting of 1% phenethyl- sulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml aprotinin) and then centrifuged at 20,000 × g for 30 min at 4°C. Protein (10–20 µg) were diluted in a total volume of 500 µl of caspase buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, 50 mM DTT, 0.1% CHAPS, and 10% sucrose] containing 100 µM Ac-DEVD-AFC (Enzyme Systems Products; Ref. 36). Reactions were incubated at 37°C, and the release of AFC was measured as emission at 505 nm with excitation at 400 nm. Fluorescence measurements were taken every 5 min for 30–60 min. Caspase activity was calculated from the initial slope. A standard curve with known concentrations of free AFC (Enzyme Systems Products) was performed to calculate the amount of cleaved AFC.

Measurement of DNA Fragmentation. The degree of DNA fragmentation was determined using a Cell Death Detection ELISA (Roche, Indianapolis, IN), which measures the amount of histone-associated DNA fragments present in the cytosol of apoptotic cells. Jurkat cells (50,000) were stimulated for the indicated periods of time. Cells were harvested by centrifugation and lysed in 0.5 ml of the lysis buffer provided with the kit. Two µl of the extract were used, and the ELISA was performed as instructed by the manufacturer. Alternatively, cytosol extracts prepared for measurement of caspase activity were also used. The reaction of conjugated peroxidase with ABTS was quantitated by measuring the absorbance at 405 nm, and the fold induction of apoptosis was calculated using untreated cells as control.

Labeling of Apoptotic Cells with Annexin-V. Jurkat cells (500,000) were treated with RRMs as indicated. Cells were washed with PBS and stained with Annexin-V-FITC (PharMingen) and PI in binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM CaCl 2 ] for 15 min at room temperature in the dark. Cells were subsequently analyzed by flow cytometry (FACS Calibur) for apoptosis (FITC) and viability (PI).

Kinase Activity. JNK and p38 kinase activities were measured with an immune complex kinase assay using anti-JNK1 (G151-333; PharMingen) or anti-p38 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies basically as described (37). Briefly, the protein kinase was immunoprecipitated from whole-cell extracts (100 µg for JNK or 200 µg for p38 kinase) prepared in lysis buffer and purified with protein A-Sepharose beads for 3 h at 4°C. A kinase assay was subsequently performed for 30 min at 30°C with purified GST-c-Jun (1–79) or GST-ATF2 (1–254) as substrates for JNK and p38, respectively. Alternatively, JNK activity was also measured using a solid-phase kinase assay with GST-c-Jun (1–79) bound to the glutathione-Sepharose beads (Amersham Pharmacia, Piscataway, NJ) or an in-gel kinase assay essentially as described (38). Gels were quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Activation of JNK and p38 kinase was analyzed by Western blots with antiphosphospecific antibodies. Whole-cell extracts (20–50 µg) were separated on a 12.5% SDS-polyacrylamide gel and transferred to Immobilon-P membranes (Millipore, Bedford, MA) according to standard procedures. Phosphoproteins were first examined with anti-phospho-p38 and anti-phospho-JNK antibodies (Cell Signaling Tech) according to the manufacturer’s recommendations. Subsequently, the blots were stripped, and the levels of total JNK and p38 proteins were analyzed with anti-JNK1/JNK2 (G151-666; PharMingen) or anti-p38 (C-20; Santa Cruz Biotechnology) antibodies, respectively. Films were analyzed with a Kodak digital camera to quantitate band intensity.

Analysis of Cytochrome c Release and Caspase Activation by Immunoblot. Cytosol extracts were prepared from Jurkat cells essentially as described (39). Cells (6 × 10 7 ) were stimulated, harvested by centrifugation at 200 × g for 5 min, and washed twice with cold PBS. Cells were resuspended in 0.3 ml of extraction buffer [20 mM HEPES-KCl (pH 7.4), 10 mM KCl, 250 mM sucrose, 1.5 mM EDTA, 1.5 mM EGTA, 1 mM MgCl 2 , 1 mM DTT, and mixture of protease inhibitors] and incubated for 20 min on ice. Homogenization was performed with a glass Dounce homogenizer with a B pestle, and extracts were cleared by centrifugation at 20,000 × g for 30 min at 4°C. Cytosol extracts were concentrated using Microcon YM-3 spin columns (Milli- pore) and stored at −70°C until needed. Protein extracts (20–50 µg) were analyzed in a 17.5% SDS-polyacrylamide gel and transferred to Immobilon-P membranes. Immunoblotting was performed with an anticytochrome c monoclonal antibody (7H8.2C12; Pharmingen) as described (39). The membrane was stripped and subsequently probed with antibodies against cleaved caspases 3 and 9 and with antibodies recognizing the proenzyme and at least one proteolytic fragment of caspases 6, 7, and 8. Protein levels were normalized by probing the same membrane with an antiaxin monoclonal antibody (AC-15, Sigma). Incubation with various antibodies was performed in Tris-buffered saline [10 mM Tris-HCl (pH 7.6), 150 mM NaCl] containing 5% dried nonfat milk and 0.05% Tween 20, or as suggested by the manufacturers, overnight at 4°C. Incubation with secondary antibodies, horseradish peroxidase-coupled donkey antirabbit or sheep antimouse antibody, was subsequently carried out for 1 h at room temperature. Proteins were detected with chemiluminescence using ECL (Amersham Pharmacia).

RESULTS

Apoptotic RRMs Induce Cytochrome c Release and Activation of Caspases 3, 6, 7, 8, and 9. We have previously shown that caspases 2 (Nedd2) and 3 (CPP32) are activated in T-leukemia cells.
exposed to MX2870-1 (7). To further characterize the activation of caspases by selective RRMs, we first analyzed the increase in caspase activity after treatment with MX2870-1. Fig. 1A shows that DEVDase activity increases in a time- and RRM concentration-dependent manner and that 0.4 μM MX2870-1 produced maximum activation. DEVDase activity was also increased after exposure to MX3350-1, CD2325, and CD437, but not RA (see below).

To determine the signaling cascade of RRM-mediated caspase activation, we investigated the release of cytochrome c from the mitochondria, which is a necessary step for the Apaf1-dependent activation of caspases during apoptosis (40, 41). Cytochrome c was released from the mitochondria in a time-dependent manner in cells treated with MX2870-1 or MX3350-1, but not with RA (Fig. 1B and data not shown). As expected, treatment with anti-Fas antibody also caused cytochrome c release. We then used immunoblotting to analyze the activation of various caspases. Treatment of Jurkat cells with the RRMs caused the activation of caspase 9 in a time- and concentration-dependent manner (Fig. 1B and data not shown), as indicated by the appearance of activated caspase 9 (p37). Activation of caspase 8 by the RRMs was evidenced by the detection of proteolytic fragments p43/p41 as well as the p18 subunit (Fig. 1B and data not shown). Activated caspase 3 was also detected after RRM treatment as a double band representing a p19 proteolytic fragment and the active subunit p17. Furthermore, caspases 6 and 7 were also activated by RRM exposure, but not by RA. Full-length caspases 6 (p34) and 7 (p35) completely disappeared in apoptotic cells, and processing of caspase 7 was further demonstrated by the appearance of a p31 proteolytic fragment (Fig. 1B). Treatment of Jurkat cells with anti-Fas antibody also produced activation of the different caspases analyzed.

Caspase 8 has been reported to cleave Bid, and truncated Bid triggers the release of cytochrome c (42, 43). To investigate whether caspase 8 activation mediated by RRMs preceded the release of cytochrome c, we examined the effect of specific caspase inhibitors on RRM-induced cytochrome c release. Fig. 1C shows that the caspase inhibitors did not prevent the accumulation of cytochrome c in the cytosol of MX2870-1-treated cells, supporting a caspase-independent mechanism for cytochrome c release. However, these caspase inhibitors, but not Z-FA-fmk, were able to prevent the activation of caspases 3, 7, 8, and 9 (Fig. 1C). Although caspase 3 p19 was accumulated in cells preincubated with Z-VAD-fmk or Z-DEVD-fmk, further proteolysis to yield a p17 fragment is required for the caspase to be active, and this processing was completely prevented by the caspase inhibitors. The induction of DEVDase activity by MX2870-1 was also totally inhibited by Z-VAD-fmk and Z-DEVD-fmk, but not by Z-FA-fmk (Fig. 1D).

RRMs Induce JNK and p38 Kinase Activities in Jurkat Cells. JNK is required for the release of cytochrome c induced by certain apoptotic stimuli, such as UV radiation and methyl methanesulfonate, but not when apoptosis is induced by death receptors (35). Because CD437 increases AP-1 activity in melanoma and lung carcinoma cells (11, 12), we investigated whether these related RRMs can induce JNK activity. Activation of JNK was already observed after 60 min of exposure to MX2870-1, and JNK activity reached a maximum after 90–120 min, similar to that observed after stimulation with 0.5 m sorbitol (Fig. 2A). p38 kinase activity was also stimulated by MX2870-1 and followed a similar time course. In contrast, p42/p44 extracellular signal-regulated kinase activity was not affected by RRM treatment (data not shown). Significant amounts of JNK and p38 kinase activities were still detected after 4–6 h of RRM exposure. Activation of JNK and p38 was further analyzed by Western blots with antiphosphospecific antibodies. With this approach, the pattern of JNK activation was identical to that observed in the kinase assay. p38 kinase activation was detected earlier in time (30 min) with the antiphosphospecific antibodies, and the activity of p38 that remained after 4–6 h of incubation was much higher, probably reflecting a higher sensitivity of this particular assay. The blots were subsequently...
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Fig. 2. MX2870-1 activates JNK and p38 kinase in a time- and concentration-dependent manner. A, time course activation of JNK and p38 kinase by RRM. Jurkat cells were stimulated with 2 μM MX2870-1 for the indicated periods of time (in hours) or with 0.5 M sorbitol for 30 min. JNK (top) and p38 (bottom) kinase activities (KA) were measured with an immune complex kinase assay with GST-c-Jun or GST-ATF2 as substrates, respectively. The fold activation with respect to untreated cells is shown below the KA panel. The relative amounts of phospho-JNK (PP-JNK) and phospho-p38 (PP-p38) were examined by Western blotting to confirm the KA data. The blots were further normalized for JNK and p38 total protein levels (JNK and p38). The activation of JNK and p38 depends on the concentration of MX2870-1. Cells were incubated with increasing concentrations of MX2870-1, and total cell extracts were analyzed for JNK (top) and p38 kinase (bottom) activities as above. Western blots were used to normalize for total JNK and p38 kinase proteins. These experiments were performed at least twice with identical results, and a representative experiment is shown.

We next investigated the activation of JNK and p38 kinase by increasing concentrations of MX2870-1 (Fig. 2B). Maximum JNK activation was observed with MX2870-1 concentrations as low as 0.4 μM. It is worth pointing out the close correlation observed between the activation of JNK/p38 kinase and the induction of DEVDase activity as a function of MX2870-1 concentration in Jurkat cells (compare Figs. 2B and 1A). This suggested that the activation of JNK/p38 by MX2870-1 might be linked to the induction of apoptosis. To support this, we studied the activation of JNK and p38 kinase by various RARα-selective RRM that induce apoptosis in Jurkat cells. Fig. 3A shows that the apoptotic RRM induce a strong activation of both JNK and p38 MAPK. In contrast, RA, as well as several RAR or retinoid X receptor-selective retinoids that did not induce apoptosis, had no effect on the stress kinases (Fig. 3A and data not shown). In-gel kinase experiments indicated that JNK1 and JNK2 were the major c-Jun kinases activated by the apoptotic RRM, similar to the activation observed after exposure to 12-O-tetradecanoylphorbol-13-acetate and A21387 (Fig. 3B). The RARα-selective molecules share a unique chemical structure with a characteristic adamantyl group (Fig. 3C), which is important for the induction of apoptosis.

**Activation of JNK and p38 Kinase Is Independent of Caspase Activity.** It has been shown that the induction of apoptosis by certain stimuli causes activation of JNK and p38 kinase activities in a caspase-dependent manner (44). Therefore, to determine whether the activation of the stress kinases preceded RRM-mediated apoptosis or whether it was a consequence of caspase activity, we examined the effects of caspase inhibitors on the activation of JNK and p38 by MX2870-1. As shown in Fig. 4A, the rapid induction of JNK activity by the RRM was not prevented by Z-VAD-fmk, Z-DEVD-fmk, or Z-FA-fmk. Furthermore, the caspase inhibitors had no effect on JNK activity observed after 4 h of retinoid exposure, indicating that sustained activation of JNK was not mediated by caspase activity. In contrast, both Z-VAD-fmk and Z-DEVD-fmk, but not the inactive compound Z-FA-fmk, completely inhibited DNA fragmentation (Fig. 4A). In addition, inhibition of caspases by Z-VAD-fmk did not prevent MX2870-1-mediated activation of p38 kinase (Fig. 4B). Other caspase inhibitors, such as Z-VDVAD-fmk (inhibitor of caspase 2) and the granzyme B inhibitor Z-AAD-fmk, did not prevent the activation of JNK and p38 kinase or the induction of DNA fragmentation by MX2870-1 (Fig. 4B). Taken together, these data demonstrate that the induction of JNK and p38 kinase activities by MX2870-1 is independent of the activation of caspases and therefore may be upstream of the apoptotic cascade.

**Interference with the JNK and p38 Kinase Pathways Prevents the Activation of Caspases and the Induction of Apoptosis by RRM.** To examine whether JNK and/or p38 kinase activities are required for the induction of apoptosis by RRM, we examined the effect of protein kinase inhibitors on the induction of apoptosis by MX2870-1. Preincubation with SB203580 (45) at concentrations that completely blocked p38 kinase activity but minimally affected JNK activity has been shown to indicate the striking differences between both types of molecules.
DNA fragmentation (arbitrary units) was measured after 2.5 h of RRM treatment. Concentrations of PD169316 (5 × 10⁻⁷ M) were necessary to significantly inhibit DNA fragmentation (Fig. 6B). The effect of PD169316 on the processing of various caspases by RRM was concentration dependent. PD169316 was similarly observed when other RAR-γ-selective RRMs and several other stimuli were used to induce cell death. The induction of DEVDase activity by the apoptotic RRMs was completely inhibited by PD169316 (Fig. 6C). Similarly, the inhibitor also interfered with the apoptotic effects induced by UV radiation and anisomycin, but not by anti-Fas. These data suggest that the inhibition of apoptosis by PD169316 might be attributable to the blockade of the JNK cascade, similar to prior observations made with Jnk1⁻/⁻ Jnk2⁻/⁻ mouse embryo fibroblasts (35).

**Activation of Stress Kinases Is Required for RRM-mediated Release of Cytochrome c.** The effect of PD169316 on the release of cytochrome c and the activation of caspases induced by RRMs was analyzed using Western blotting. PD169316, but not SB203580, effectively prevented RRM-mediated release of cytochrome c as well as Bid cleavage (Fig. 7A). Consistent with the above observations, the kinase inhibitor also completely blocked the activation of caspases 8 and 9 induced by MX2870-1. When cells were treated with anti-Fas antibody, the cleavage of Bid, the release of cytochrome c, and the activation of caspases 8 and 9 were not inhibited by PD169316, confirming that JNK activity is not required for Fas-mediated cell death (35). In contrast, Fas-mediated effects were enhanced by preincubation with the kinase inhibitor (Fig. 7A), correlating with a higher DEVDase activity found in the absence of JNK/p38 activities (see Fig. 6C). The effect of PD169316 on the processing of various caspases by RRMs was concentration dependent. Fig. 7B shows that activity (data not shown) had no significant effects on the induction of apoptosis by MX2870-1 (Fig. 5). In contrast, the induction of caspase activity, DNA fragmentation, and externalization of phosphatidylserine by MX2870-1 was completely prevented when cells were preincubated with PD169316. Although it was originally described as a specific p38 kinase inhibitor (46), we found that PD169316 could efficiently inhibit RRM-mediated activation of JNK as well (see below). The kinase inhibitors had no effect on Jurkat cells when treated in the absence of RRMs (Fig. 5).

The effect of increasing concentrations of PD169316 on the activation of stress kinases by RRMs was further investigated. Low concentrations of PD169316 (2.5 μM) inhibited the activation of p38 kinase by 40% but did not affect JNK activity (Fig. 6A). Higher concentrations of PD169316 (5 μM) were necessary to significantly inhibit the activation of JNK by MX2870-1, and almost complete inhibition of both kinases was seen in the presence of 10 μM PD169316 (Fig. 6A). In contrast to SB203580, which inhibits p38 kinase activity by binding to the ATP binding site (45 and data not shown), immunoblot analysis with antiphosphospecific antibodies showed that PD169316 also prevented the phosphorylation, and therefore the activation, of JNK and p38 kinase (Fig. 6A).

We examined the effect of increasing concentrations of PD169316 on the induction of caspase activity by MX2870-1. The inhibition of DEVDase activity was dependent on the concentration of the kinase inhibitor (Fig. 6B). Interestingly, low concentrations of PD169316 (2.5 μM), which partially blocked p38 kinase activity but not JNK, elicited a partial inhibition (~30%) of caspase activity, and DEVDase activity was further inhibited in the presence of increasing amounts of PD169316. Total inhibition of DEVDase activity was observed with 10 μM PD169316, which also completely prevented the activation of both JNK and p38 kinase. We next investigated whether this effect of
Fig. 6. PD169316 inhibits RRM-induced JNK, p38 MAPK, and caspase activities in a dose-dependent manner. Jurkat cells were preincubated with the indicated concentrations (in μM) of PD169316 (PD) for 90 min prior to stimulation with 2 μM MX2870-1 for an additional 90 min. Cell extracts were prepared and subsequently analyzed for JNK and p38 kinase activities (A) as well as DEVDase activity (B). A. Kinase activities were measured by immune complex kinase assay (KA) and immunoblots with antiphospho-specific antibodies. The percentages of RRM-induced JNK or p38 activities remaining in the presence of increasing concentrations of PD are indicated below the KA panel, with 100% being the full activation obtained after MX2870-1 stimulation in the absence of PD169316. Levels of JNK and p38 proteins were analyzed by Western blots (JNK and p38). B. Effect of PD169316 on MX2870-1-mediated DEVDase activity. The plot shows the percentage of DEVDase activity as a function of the concentration of PD169316, with 100% being the caspase activity induced by RRM in the absence of PD. The results shown are the mean ± SE (bars) of three independent experiments. C. Effect of PD169316 on apoptosis induced by different stimuli. Jurkat cells were preincubated with 15 μM PD169316 (D) or DMSO (E) for 90 min prior to stimulation with 2 μM RRMs (CD457, MX2870-1, MX3350-1, and CD2523), 2 μM RA (RA), 100 J/m2 UV radiation (UV), 200 ng/ml anti-Fas (a Fas), or 10 μg/ml anisomycin (Anisomycin). Caspase activity (nmol AFC/g/min) was measured after 3 h of treatment using cytosol extracts and Ac-DEVD- AFC as substrate. The experiment was performed at least two times, and a representative result is shown.

PD169316 efficiently inhibited in a dose-dependent manner the appearance of activated caspases 3 and 9 induced by MX2870-1. The processing of caspases 6, 7, and 8 was also inhibited when cells were treated in the presence of increasing concentrations of PD169316. Together these data establish that MX2870-1 and other RARγ-selective RRMs activate the stress kinases JNK and p38, which results in cytochrome c release and subsequent activation of caspases that lead to cell death.

**DISCUSSION**

Apoptosis induction by certain RRMs represents a novel retinoid-associated activity that has not been well understood in terms of its signaling mechanism. In *in vitro* studies have shown that CD437 is a strong inducer of apoptosis in a wide variety of cancer cell lines as well as in normal mammary epithelial cells (47). The structurally related molecules MX2870-1 and MX3350-1 have also been reported to induce apoptosis in cancer cells. Interestingly, compounds such as MX3350-1 showed certain selectivity against some particular types of cancer (3), which could be attributable to minor differences in the chemical structures. The induction of apoptosis by CD437-like RRMs appears to be independent of transcription, as suggested by us (7) and others (48). Concurrent with this, we have reported on a novel retinoid antagonist, MX781 (which does not activate RAR-dependent transcription), that can induce apoptosis in breast cancer cells (4) as well as in Jurkat and other cancer cell lines. Thus that RAR-dependent transcriptional activation is not required for the induction of apoptosis is also implied by the usually high concentrations of retinoids that are needed to induce apoptosis in comparison with the optimal concentration necessary to activate transcription. It is also noticeable that another molecule that selectively activates RARγ, 4-hydroxyphenyl retinamide (or fenretinide; Ref. 49), and which is structurally not related to CD437, also induces apoptosis (7, 49–51) and activates JNK and p38 kinase in Jurkat T cells. Thus, certain retinoid-related structures, in particular those that bind the RARγ isofrom, can inherit additional activities that allow them to trigger apoptosis in cancer cells. However, any direct role of RARγ binding activity in the induction of apoptosis by these molecules remains to be demonstrated. For example, CD437 induced apoptosis in HL-60R cells (52), which do not express RARβ/γ receptors and harbor a truncated RARα gene (53), suggesting that retinoid receptors are dispensable for CD437-induced apoptosis within a HL-60 background. In agreement, we have observed that saturation of the receptors with an excess of RA or overexpression of RARγ has no effect on MX2870-1-induced apoptosis in Jurkat T cells. To elucidate the mechanism of apoptosis induction by these selective RRMs and to establish whether this is a unique mechanism characteristic of this class of molecules is of obvious interest.

Here we show that this type of apoptosis-inducing RRMs, represented by MX2870-1, causes a strong and sustained activation of JNK and p38 MAP kinases. JNK/p38 activation correlates with the induction of apoptosis, as determined by measurement of caspase activity, DNA fragmentation, and externalization of phosphatidylserine. All of the RARγ-selective RRMs that induced apoptosis induce a substantial increase of JNK and p38 kinase activity in Jurkat cells. Our data demonstrate that the activation of JNK and p38 kinase is an early step in the apoptosis-signaling cascade activated by these RRMs and that it is not dependent on caspase activation. Importantly, JNK activation alone is sufficient for the induction of apoptosis by RRMs because inhibition of p38 activity by SB203580 had no effect on RRM-induced apoptosis. Another inhibitor of p38 kinase, PD169316, blocked RRM-mediated apoptosis. However, this compound is not selective for p38 kinase and efficiently blocks the activation of both JNK and p38 MAPK by apoptotic RRMs in a dose-dependent manner. Although JNK activation is enough to induce apoptosis in the absence of p38 activity, we cannot completely exclude certain roles for p38 kinase in RRM-mediated apoptosis because partial inhibition of p38 kinase by low concentrations of PD169316 that had no effect on JNK activity significantly decreased caspase activity induced by MX2870-1. Experiments in cells lacking JNK protein and activity will be necessary to definitively prove a requirement for JNK activity in RRM-induced cell death and whether p38 kinase activation in the absence of JNK is sufficient to induce apoptosis.

As observed with other drugs, such as anisomycin and methyl methanesulfonate (35), activation of JNK is necessary for the release of mitochondrial cytochrome c mediated by RRMs. Our data suggest that the apoptosis pathways activated by RRMs are similar to those induced by stress (including UV radiation and anisomycin) downstream of JNK activation and that they all are sensitive to inhibition by PD169316. Released cytochrome c interacts with Apaf1 and procaspase 9 in a dATP-dependent manner, leading to the activation of caspase 9 and other caspases downstream (41). Using Western blot analysis, we have indeed observed activation of caspase 9 after RRM...
ACTIVATION OF JNK AND p38 KINASES IN RRM-INDUCED APOPTOSIS

Fig. 7. PD169316 prevents cytochrome c release and processing of caspases by MX2870-1. A, Jurkat cells were preincubated with 30 μM SB203580 (SB), 15 μM PD169316 (PD), or solvent (none) for 90 min prior to stimulation with 2 μM MX2870-1 for 2.5 h as indicated (R). Cells treated (PD) or not (--) with PD169316 were also stimulated with 200 ng/ml anti-Fas antibody (clone CH-11; αFas). Appropriate controls with no RRM (--) were included. The presence of cytochrome c (Cytc) in the cytosol, cleavage of Bid, and the activation of caspases 8 and 9 (Casp 8 and 9) are shown. The arrowsheads indicate the position of full-length Bid (p22) and caspase 8. Arrows indicate the position of truncated Bid (p15) and processed caspase 8 (p43/p41). B, protein extracts obtained in the experiment described in the legend of Fig. 6 were used in Western blots to examine the activation of different caspases by MX2870-1 (R) in the presence of increasing amounts of PD169316. The activated caspase 3 (Casp 3; p19/p17) and caspase 9 (Casp 9; p37) are shown. Arrowheads indicate the position of full-length caspase 6 (Casp 6; p34), caspase 7 (Casp 7; p35), and caspase 8 (Casp 8; p55). The appearance of the small subunit of caspase 6 (p31) and caspase 8 (p41/p43) is indicated by arrows as evidence of the activation of caspases.

In summary, we report here on the activation of an apoptotic signaling pathway by a novel class of anticancer compounds. In contrast to natural retinoids and other synthetic RRMs that inhibit apoptosis, the RRMs analyzed here therefore show promise for use in combination with anticancer therapies that use the Fas/TNF receptor pathways to induce apoptosis. In addition, they can be effective against tumors that carry p53 mutations, where other therapies have failed. Elucidation of the target(s) upstream of JNK/p38 that lead to the induction of apoptosis will convey new insights for understanding the mechanism of RRM-induced cell death in cancer cells. This knowledge would have substantial input to the future development of novel anticancer molecules.

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of transcription, in agreement with our previous observations (7). Activation of JNK/p38 kinases in Jurkat cells might lead to the phosphorylation of certain pro- or antiapoptotic protein(s), causing a gain or loss of activity that contributes to the induction of apoptosis. In fact, several reports showed that JNK phosphorylates the antiapoptotic proteins Bcl-2 and Bcl-XL, although the consequences of this phosphorylation are not clear (63–65). However, it is possible that phosphorylation of these proteins impairs their antiapoptotic function, contributing to the induction of apoptosis (63, 66). Whether these phosphorylation events occur and participate in RRM-induced apoptosis in Jurkat cells remains to be examined.

In contrast to natural retinoids and other synthetic RRMs that inhibit AP-1 activation, MX2870-1 and related RARγ-selective RRMs use activation of the JNK/p38 kinase cascades to induce tumor cell death. This pathway does not involve death receptor (Fas or TNF) signaling and is independent of p53. The RRMs analyzed here therefore show promise for use in combination with anticancer therapies that use the Fas/TNF receptor pathways to induce apoptosis. In addition, they can be effective against tumors that carry p53 mutations, where other therapies have failed. Elucidation of the target(s) upstream of JNK/p38 that lead to the induction of apoptosis will convey new insights for understanding the mechanism of RRM-induced cell death in cancer cells. This knowledge would have substantial input to the future development of novel anticancer molecules.

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Retinoid-related Molecules Induce Cytochrome c Release and Apoptosis through Activation of c-Jun NH₂-Terminal Kinase/p38 Mitogen-activated Protein Kinases

Maria A. Ortiz, Francisco J. Lopez-Hernandez, Yolanda Bayon, et al.

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