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Reduced concentrations of serum enhance the antiproliferative activity of retinoid-related molecules and accelerate the onset of apoptosis

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Abstract

Retinoid-related molecules (RRMs) that are selective agonists for the retinoic acid receptor-γ and one retinoid antagonist are potent inducers of apoptosis in various cancer cell lines. This cell-killing activity makes them promising candidates for their use as anticancer drugs. We have observed that reducing the amount of serum in the cell culture medium significantly increased the antiproliferative activity of these RRMs in a serum concentration dependent manner. The induction of caspase activity, DNA fragmentation, and externalization of phosphatidylserine by the RRMs was markedly reduced when cells were treated in medium containing 10% serum, as compared to cells treated in low serum. High concentrations of serum also inhibited the activation of stress kinases by RRMs and higher amounts of the retinoid derivatives were necessary to cause quantitatively similar effects as compared to treatments in medium containing low serum. We have demonstrated that high concentrations of serum in the culture medium prevented the intracellular accumulation of MX3350-1 (agonist). Moreover, pre-incubation of cells in low serum-containing medium accelerated the onset of apoptosis as evidenced by the rapid activation of caspases and formation of apoptotic bodies. The release of cytochrome *c* and Smac induced by RRMs occurred earlier in cells that had been pre-incubated in 0.5% serum, while the activation of JNK and p38 stress kinases was unaffected.

Keywords: Retinoid-related molecules; Serum; Apoptosis; Stress kinases

1. Introduction

Retinoids are natural and synthetic derivatives of Vitamin A that exert strong effects on critical biological processes including development, cell growth and differentiation, metabolism and homeostasis [1,2]. Retinoid actions are mediated through binding to and activation of the nuclear retinoid receptors, retinoic acid (RA) receptors (RARs) and retinoid X receptors [3]. In addition, retinoids can interfere with AP-1 activity in a receptor-dependent manner [4]. Retinoids have shown antiproliferative and cell differentiation activities in cancer cell lines *in vitro* and *in vivo*, and they therefore represent promising candidates for the chemoprevention and treatment of certain types of cancers [5]. However, the high levels of toxicity, probably derived from the broad biological responses mediated by the retinoid receptors, are still the main limitation for the clinical use of currently available retinoids [6]. Novel synthetic RRMs have been developed that show selective activities, which are expected to exhibit lower side effects. Using cell-based screenings, several RARγ-selective RRMs have been identified that show strong antiproliferative activity against several cancer cell lines. Of particular interest is MX3350-1, which is structurally related to CD437 [7] and has been found effective against solid tumors derived from lung cancer cells in nude mice with tolerable side effects [8]. The antiproliferative activity of CD437 and MX3350-1 has been correlated with the induction of apoptosis in several cancer cell lines [9–13]. A second class of selective RRMs that induces apoptosis is represented by the RAR antagonist MX781, which was found to eliminate tumors derived from estrogen-independent breast cancer cells with no overt signs of toxicity [14].

The mechanism of apoptosis induction remains unknown at the molecular level. CD437 and several analogs (including MX2870-1 and MX3350-1) induced apoptosis in the absence of protein synthesis and independently of the

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Abbreviations: AFC, 7-amino-trifluoromethyl coumarin; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; JNK, c-Jun N-terminal kinase; PI, propidium iodide; RAR, retinoic acid receptor; RRM, retinoid-related molecule.

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retinoid receptors [10,15–17]. Different lines of evidence suggested that RRMs induce apoptosis through the intrinsic pathway, via the mitochondria, which is also the mechanism believed to be followed by most anticancer drugs [18,19]. CD437 has been shown to directly target the mitochondria and to cause programmed cell death in the absence of a nucleus [20]. Caspase inhibitors, such as Z-VAD-fmk and Z-DEVD-fmk, did not impede the release of cytochrome *c* induced by RRMs, although they prevented the activation of caspases and the induction of DNA fragmentation [21,22]. We have recently demonstrated that the release of cytochrome *c* and subsequent activation of caspases by the RARγ-selective RRMs required the activation of stress kinases JNK and p38 [21]. JNK activity has been implicated in apoptosis [23], probably through the phosphorylation and inactivation of Bcl-2 and Bcl-XL [24,25]. In addition, JNK can also phosphorylate Bad, enhancing its pro-apoptotic activity [26].

We found that the antiproliferative activity of these selective RRMs, in particular the antagonist MX781, was significantly enhanced when cells were treated in the presence of low concentrations of serum in the culture medium [14]. In this study we have investigated the effect of serum on the induction of apoptosis by these RRMs and found that high concentrations of serum inhibited all apoptotic phenotypes induced by RRMs, but not by other stimuli. Pre-incubation of cells in the presence of low serum concentrations significantly sensitized Jurkat cells to the apoptotic activity of selective RRMs and expedited the activation of caspases and subsequent formation of apoptotic bodies, which was linked to an accelerated release of cytochrome *c* and Smac/Diablo. This suggested that the sensitization of Jurkat cells to RRM-induced apoptosis occurred at the mitochondrial level.

2. Material and methods

2.1. Reagents

RA was obtained from Sigma. Selective RRMs were obtained from MAXIA Pharmaceuticals and Galderma R&D. Stock solutions of 10 mM of the RRMs were made in DMSO, which were subsequently diluted in culture medium.

2.2. Cell culture and retinoid treatments

All leukemia cells (Jurkat T, CCRF-CEM, CEM-T4, Hut-78, and K-562) were grown in RPMI 1640 medium containing 10% heat-inactivated FBS, 200 mM glutamine, penicillin and streptomycin sulfate. CEM-T4 cells were a gift of Dr. W. Raschke (Sidney Kimmel Cancer Center) and all other cell lines were obtained from ATCC. Unless indicated otherwise, cells treated with the retinoids in medium containing 0.5% FBS were pre-incubated for 16–20 hr in the presence of 0.5% FBS. Controls containing the same concentrations of solvent (DMSO) were included in all the experiments.

2.3. Cell proliferation assay

Cell survival was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, 25,000 cells well^{-1} were seeded in 96-well plates in 100 μL of medium containing the appropriate amount of serum. Cells were treated with RRMs or appropriately diluted solvent (DMSO) for 1 day, when 10 μL well^{-1} of 5 mg mL^{-1} MTT was added. After 4 hr of incubation at 37°, tetrazolium crystals were solubilized overnight at 37° with 100 μL of 10% SDS, 10 mM HCl and absorbance at 595 nm was measured.

2.4. Determination of caspase activity

Cytosolic extracts were obtained by incubating the cells for 20 min in ice with CE buffer (25 mM PIPES pH 7, 25 mM KCl, 5 mM EGTA, 1 mM DTT, 10 μM cytochalasin B, 0.5% NP-40, and a mixture of protease inhibitors consisting of 1 mM PMSF, 1 μg mL^{-1} leupeptine, and 1 μg mL^{-1} aprotinin), and cleared by centrifugation at 20,000 *g* for 30 min at 4°. Protein concentration was determined using Protein Assay (Bio-Rad). Ten to twenty micrograms of protein extract 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% CHAPS, and 10% sucrose) containing 100 μM Ac-DEVD-AFC (Enzyme Systems Products) [27]. Reactions were incubated at 37° and the release of AFC was measured as emission at 510 nm upon excitation at 390 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 in pmol of cleaved AFC.

2.5. Measurement of DNA fragmentation

The degree of DNA fragmentation was determined using a Cell Death Detection ELISA (Roche). The appropriate volume of cytosolic extract containing 2 μg of protein was used for the ELISA, which was performed as instructed by the manufacturer. The OD at 405 nm was measured and the fold induction of apoptosis was calculated using untreated cells as control.

2.6. Labeling of apoptotic cells with Annexin-V

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).

2.7. Kinase activity

JNK and p38 kinase activities were measured with an immune complex kinase assay (KA) using anti-JNK1 (G151-333, PharminGen) or anti-p38 (C-20, Santa Cruz Biotechnology) antibodies as previously described [21]. Alternatively, JNK activity was examined using a solid phase KA. Activation of JNK and p38 kinase was also analyzed by western blot. Twenty to fifty micrograms of whole cell extracts were separated on a 12.5% SDS–polyacrylamide gel (PAGE) and transferred to Immobilon-P (Millipore) membranes following standard procedures. Phospho-proteins were first examined using anti-phospho-p38 and anti-phospho-JNK antibodies (Cell Signaling Technology) following the manufacturer's recommendations. Subsequently, the blots were stripped and the levels of total JNK and p38 proteins were analyzed using anti-JNK1/JNK2 (G151-666, PharMingen) or anti-p38 (C-20, Santa Cruz Biotechnology) antibodies, respectively.

2.8. Measurement of intracellular concentrations of RRM

The RRM MX3350-1 contains a naphtalene moiety that confers fluorogenic properties. Cells treated with MX3350-1 were washed twice with cold PBS and cell extracts were prepared in CE buffer. Ten to twenty micrograms protein of cell extract was used to measure the fluorescence as emission at 400 nm upon excitation at 330 nm using a Hitachi F2000 fluorometer. A standard fluorescence curve was performed with known concentrations of the RRM diluted in CE buffer.

2.9. Immunoblot analysis of cytochrome c and Smac release from the mitochondria

Cytosol extracts were prepared from 2.5×10^6 Jurkat cells in 50 μL of ICM buffer (120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 20 mM HEPES–Tris pH 7.1, 2 mM

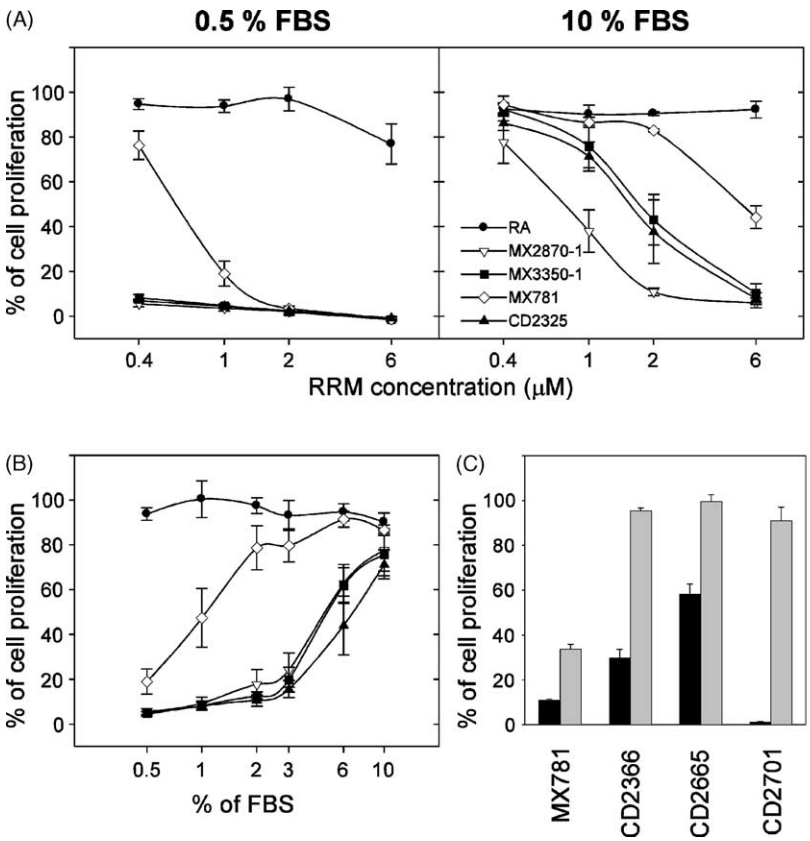

Fig. 1. Antiproliferative activity of selective RRMs. (A) Jurkat cells were treated for 24 hr with increasing concentrations of the indicated RRMs in medium containing 0.5% (left) or 10% FBS (right). Cells treated with RA were included for comparison and the percentage of proliferation was calculated relative to control cells grown in the presence of solvent. Cell proliferation was measured by MTT staining. The experiment was performed three times with triplicates and the average ± SD is indicated. (B) Effect of increasing concentrations of serum on the antiproliferative activity of RRMs. Jurkat cells were treated with 1 μM of RA, MX3350-1, CD2325, or MX781, or 0.4 μM of MX2870-1 in the presence of the indicated concentrations of serum. After 24 hr, cell proliferation was measured by MTT. The experiment was repeated four times with triplicate data points and the mean ± SD is shown. (C) Serum inhibits the antiproliferative activity of retinoid antagonists. Jurkat cells were treated with 2 μM of the indicated compounds for 1 day in 0.5% FBS (black columns) or in 10% FBS for 3 days (gray columns), when cell viability was measured by MTT. The percentage of cell proliferation with respect to untreated cells as control (100%) is indicated. The experiment was performed two times with triplicates and averaged data ± SD are shown.

succinate, 100 μg mL^{-1} digitonin and protease inhibitors). Twenty to fifty micrograms of protein extracts were analyzed in a 17.5% SDS–PAGE and transferred to Immobilon-P membrane. Cytosolic cytochrome *c* and Smac were detected using antibodies obtained from Dr. X. Wang (University of Texas Medical Center). Blotting with the primary antibodies was performed in TBS (10 mM Tris–HCl pH 7.6, 150 mM NaCl) containing 5% nonfat dry milk and 0.05% Tween-20, overnight at 4°. Incubation with secondary antibodies, horseradish peroxidase-coupled donkey anti-rabbit or sheep anti-mouse, was subsequently carried out for 1 hr at room temperature. Proteins were detected by chemiluminiscence using ECL (Amersham Biosciences).

3. Results

3.1. Effect of the serum concentration on the antiproliferative/cell death inducing activity of selective RRMs against human leukemia cells

To investigate the effect of serum on the antiproliferative/cell-killing activity of various selective RRMs against leukemia cells, Jurkat cells were grown with increasing amounts of RRMs for 24 hr in the presence of 0.5 or 10% serum. A very potent cell-killing activity was found with concentrations below 1 μM of the RARγ-selective agonists MX3350-1, MX2870-1, and CD2325 when tested in the presence of 0.5% FBS (Fig. 1A). The RAR antagonist MX781, but not RA, also inhibited cell viability by 80% when used at concentrations as low as 1 μM. In contrast, cells treated with the RRMs in the presence of 10% FBS were less susceptible to their antiproliferative/cell-killing activity, and higher concentrations of the RRMs were required to significantly induce cell-killing. The effect of serum concentration was more pronounced with the antagonist MX781, which was ineffective even at a 2 μM concentration in the presence of 10% FBS while completely eliminating over 90% of cells when treated in the presence of 0.5% FBS. The antiproliferative/cell-killing activity of the selective RRMs was inhibited by serum in a concentration-dependent manner (Fig. 1B). Interestingly, the three RARγ-selective molecules MX2870-1, MX3350-1, and CD2325, which are structurally related [21], showed a very similar dependency on the amount of serum, and a significant increase in cell-killing activity was seen when

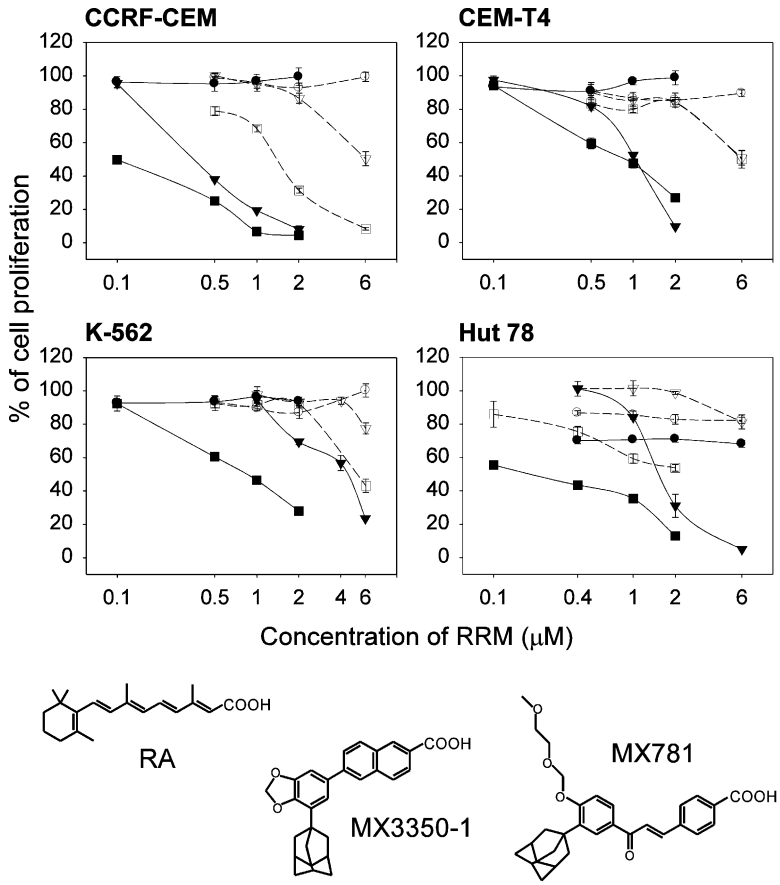

Fig. 2. Effect of serum on the antiproliferative activity of RRMs against leukemia cell lines. Twenty-five thousand cells per well were seeded in 96-well plates in medium containing 0.5% FBS (closed symbols) or 10% FBS (open symbols). Cells were treated with increasing concentrations of RA (circles), MX3350-1 (squares), or MX781 (inverted triangles) for 24 hr, when cell viability was measured using a MTT assay. Control cells were treated with an equivalent amount of solvent (DMSO, <0.1% v/v). The experiment was repeated twice with triplicates and the average ± SD is indicated. The chemical structure of these compounds is shown below for comparison.

the FBS concentration was reduced only to 6%. In contrast, the effect of serum on the antiproliferative activity of the antagonist MX781 was quite different and a reduction below 2% FBS was necessary to significantly increase the cell-killing activity of this RRM. RA exhibited no antiproliferative activity against Jurkat cells under these experimental conditions, even at the highest concentration. The effect of serum on the antiproliferative activity of other retinoid antagonists was also analyzed. CD2701, and to a lesser extent CD2366 and CD2665, showed antiproliferative activity against Jurkat cells after only 24 hr of exposure in medium containing low concentrations of serum. However, the antagonists were completely ineffective when tested in high serum, even after 3 days of treatment (Fig. 1C).

We next examined the response to RRMs of other leukemia cells in the presence of low or high concentrations of serum. The T leukemia cell lines CCRF-CEM, CEM-T4 and Hut 78, as well as K-562 chronic myelogenous leukemia cells, were incubated with the different RRMs for increasing periods of time in the presence of 0.5 or 10% FBS and cell viability was analyzed by MTT. Both MX781 and MX3350-1, as well as other RARγ-selective agonists, but not RA, elicited a strong antiproliferative/cell-killing activity in all the cell lines analyzed when incubated in the presence of 0.5% FBS (Fig. 2 and data not shown). As observed in Jurkat cells, about a 10-fold higher concentration of RRM was necessary in the presence of 10% FBS to induce similar effects as in medium containing 0.5% FBS.

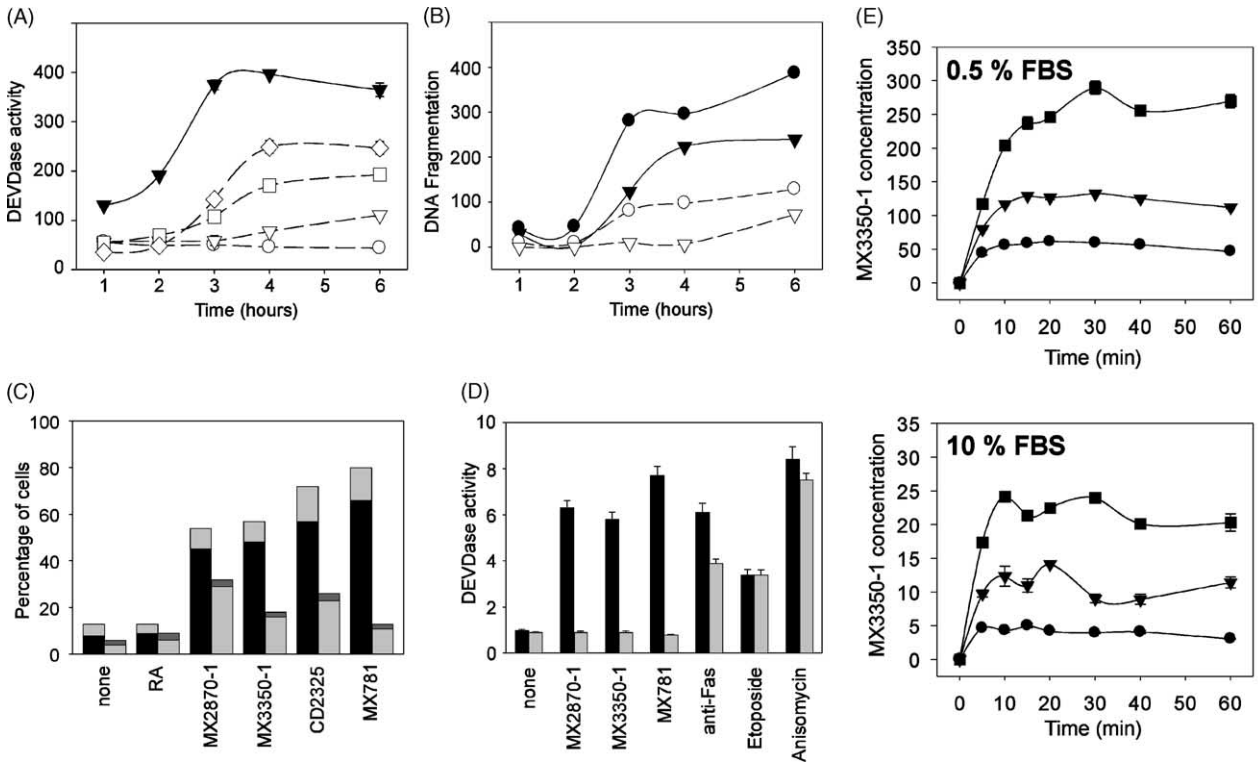

Fig. 3. Inhibition of RRM-induced apoptosis by serum. (A) Jurkat cells were incubated for the indicated periods of time with 0.4 μM (▽), 1 μM (□), or 2 μM (◇) of MX2870-1 in the presence of 10% FBS or with 0.4 μM MX2870-1 in 0.5% FBS (▼). As control, cells were incubated with solvent (○). Cytosolic extracts were prepared and analyzed for DEVDase activity (nmol AFC g^{-1} min^{-1}). A representative experiment performed in duplicate is shown. (B) Jurkat cells were treated with 2 μM MX2870-1 (circles) or 6 μM MX781 (inverted triangles) in the presence of 0.5% FBS (closed symbols) or 10% FBS (open symbols). Cytosol extracts were prepared at the indicated times and DNA fragmentation was measured by ELISA (as absorbance at 405 nm). The experiment was performed twice with duplicates and data from one representative experiment are shown. (C) Jurkat cells were incubated with 2 μM (MX2870-1, MX3350-1, CD2325) or 6 μM (RA, MX781) for 3 hr in medium containing 0.5% FBS (black columns) or 10% FBS (light gray columns). Control cells (none) were incubated with equivalent amounts of solvent. Cells were stained for externalization of phosphatidylserine and cell viability and analyzed by flow cytometry. The percentage of Annexin-V-positive, PI-negative cells is indicated. The stacked columns on top indicate the percentage of cells double-stained with PI and Annexin-V. The experiment was performed at least three times and the results from one representative experiment are shown. (D). Jurkat cells were incubated for 3 hr with 0.4 μM MX2870-1, 1 μM MX3350-1, 4 μM MX781, 125 ng mL^{-1} anti-Fas antibody (CH-11), 100 μM etoposide, or 0.1 μg mL^{-1} anisomycin, in the presence of 0.5% FBS (black columns) or 10% FBS (gray columns). Cell extracts were prepared and analyzed for DEVDase activity. The fold induction with respect to control cells (none) is indicated. The average ± SD of three independent experiments performed in duplicate is shown. (E) Intracellular concentration of MX3350-1 is reduced in the presence of high serum. Jurkat cells were pre-incubated in 0.5% or kept in 10% FBS and then treated with 0.4 (●), 1 (▼), or 2 (■) μM of MX3350-1 in the presence of low or high serum, as indicated. Cytosol protein extracts were prepared at increasing periods of time and the intracellular concentration of MX3350-1 (fluorescence units mg^{-1} protein) was measured. The mean ± SD of three independent experiments is shown.

3.2. Inhibition of RRM-induced apoptosis by high concentrations of serum

We next investigated the effect of serum concentration on the induction of apoptosis by the selective RRMs. We first analyzed the stimulation of DEVDase activity by increasing amounts of MX2870-1 in the presence of 10% FBS as a function of the time of incubation and compared to the induction of caspase activity by 0.4 μM MX2870-1 in 0.5% serum, which produced maximum DEVDase activity under these experimental conditions [21]. At least 1 μM MX2870-1 and 4 hr of incubation were necessary to significantly increase caspase activity when cells were incubated in the presence of 10% FBS, while 3 hr of exposure to 0.4 μM RRM in 0.5% FBS were sufficient to induce maximum DEVDase activity (Fig. 3A). Similarly, treatment with the RARγ-selective RRM MX2870-1, as well as the antagonist MX781, in medium containing 0.5% FBS induced significantly higher degree of DNA fragmentation in shorter periods of time (Fig. 3B). We then analyzed the effect of serum on the externalization of phosphatidylserine by the different apoptotic RRMs. As expected, the percentage of Annexin-V-positive/PI-negative apoptotic cells induced by all selective RRMs was notably higher when cells were incubated in the presence of 0.5% FBS (Fig. 3C). In contrast, the amount of serum in the culture medium did not significantly affect the induction of caspase activity by other stimuli, such as etoposide or anisomycin, although high concentrations of FBS partially inhibited the induction of DEVDase activity by anti-Fas antibody (Fig. 3D).

We next measured the intracellular concentration of MX3350-1 in cells incubated in medium containing different concentrations of serum. The time course of RRM uptake was very similar independently of the serum concentration, and maximum accumulation of the compound was observed after only 10–20 min of incubation depending on the amount of RRM added to the culture medium (Fig. 3E). The amount of MX3350-1 that entered the cells was dose-dependent, and almost 10-fold higher when cells were incubated in medium containing 0.5% FBS (Fig. 3E). This correlated with the higher antiproliferative activity of the RRMs observed in low serum (see Figs. 1 and 2).

3.3. Effect of serum on the induction of JNK and p38 MAP kinases

The activation of JNK and p38 stress kinases is necessary for the release of cytochrome *c* and subsequent activation of caspases by the RARγ-selective RRMs in Jurkat cells [21]. We therefore examined how the amount of serum affected the activation of these stress kinases by the RRM MX2870-1. As observed in Fig. 4, significantly higher concentrations of MX2870-1 were required to induce JNK and p38 kinase in cells treated in the presence of 10% serum than in cells grown in low serum. The highest activation of the kinases was seen with 6 μM MX2870-1 in 10% FBS, while 1 μM of the RRM was sufficient to cause maximum activity of both JNK and p38 kinase in the presence of 0.5% FBS (see Fig. 4 and Ref. [21]). Similar results were observed with the antagonist MX781 and other agonist RRMs (data not shown). This reduced JNK and p38 MAP kinase activation in the presence of 10% FBS correlated with diminished apoptosis induction and reduced antiproliferative/cell-killing activity.

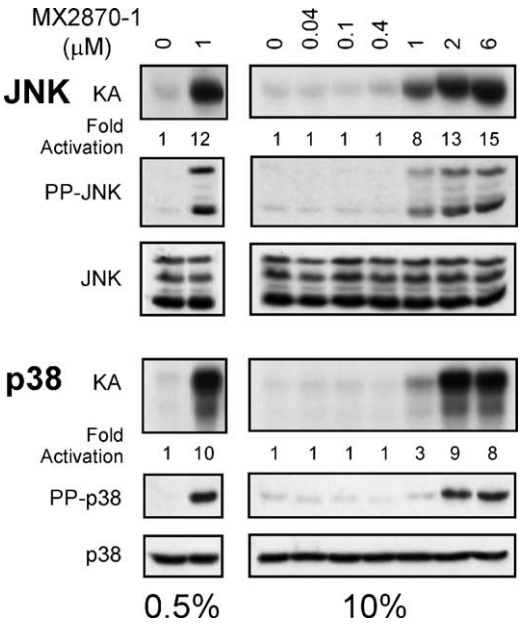

Fig. 4. Activation of JNK and p38 kinase by MX2870-1. Jurkat cells were incubated with the indicated concentrations of MX2870-1 in 0.5% (left) or 10% FBS (right) and total cell extracts were analyzed for JNK (top) and p38 kinase (bottom) activities using an immune complex KA. The fold induction relative to untreated cells is indicated under the KA panel. Western blots were further used to analyze active kinases (PP-JNK, PP-p38) and 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.

3.4. Pre-conditioning of Jurkat cells to RRM-induced apoptosis by low serum concentrations

We investigated next the effect that pre-incubation in low serum exerted on RRM-induced apoptosis. Jurkat cells were incubated in medium containing 0.5 or 10% FBS for 16–20 hr. Cells were subsequently treated in fresh medium containing 0.5% FBS with low concentrations of selective RRMs and the induction of apoptosis was examined after 2 hr of RRM exposure. The amount of DEVDase activity was significantly increased only in cells that had been pre-incubated in 0.5% FBS, but not in cells pre-incubated in high concentrations of serum (Fig. 5A). Similarly, mature apoptotic bodies were observed only

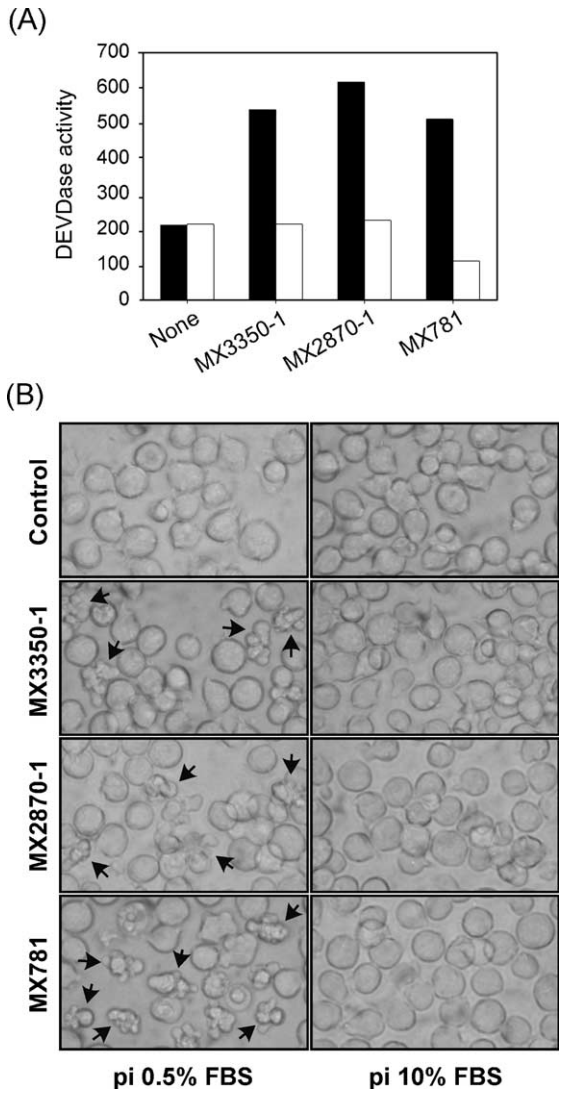

Fig. 5. Pre-conditioning of cells to RRM-induced apoptosis. (A) Jurkat cells (0.5 million mL^{-1}) were grown for 16 hr in medium containing 0.5% FBS (black columns) or 10% FBS (white columns). Then, cells were sedimented and resuspended in fresh medium containing 0.5% FBS with solvent (none), 1 μM MX3350-1, 1 μM MX2870-1, or 4 μM MX781. After 2 hr of treatment, cell extracts were prepared and assayed for DEVDase activity (arbitrary units). (B) RRM-induced formation of apoptotic bodies is enhanced in cells pre-incubated in low amounts of serum. Cells from the experiment described in (A) were examined before harvesting by light microscopy to visualize mature apoptotic bodies (some are indicated with arrows). The experiment was repeated at least three times with identical outcomes and data from one representative experiment are shown.

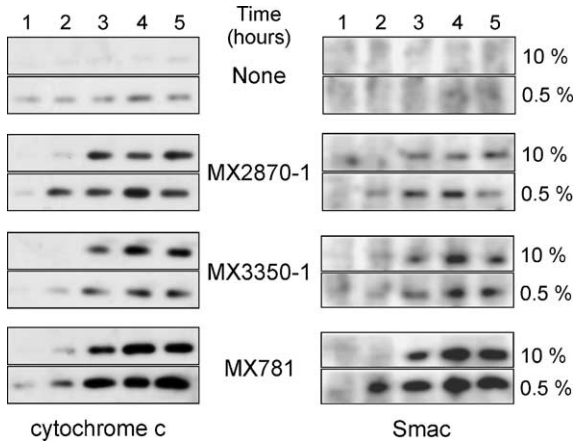

Fig. 6. Effect of serum on the release of cytochrome *c* and Smac/Diablo. Jurkat cells (2.5×10^6) that had been pre-incubated for 16 hr in medium containing 0.5 or 10% FBS were stimulated with 1 μM MX2870-1, 1 μM MX3350-1, or 4 μM MX781 in medium containing low (0.5%) serum. At the indicated times, cells were harvested and cytosol extracts were prepared in 50 μL of ICM buffer. Twenty-five micrograms of protein were analyzed by SDS–PAGE and immunoblot with anti-cytochrome *c* and Smac antibodies. This experiment was performed twice with identical results and data from one representative experiment is shown.

in RRM-treated cells that had been pre-incubated in low serum (Fig. 5B).

Translocation of cytochrome *c* into the cytosol precedes caspase activation in RRM-induced apoptosis [21] and is necessary for the formation of the apoptosome, which triggers activation of pro-caspase 9 [28]. Another protein that is released from the mitochondria during apoptosis, Smac, interacts with inhibitors of apoptosis, allowing for an efficient activation of caspases [29,30]. Therefore, we examined whether the enhanced sensitivity observed in cells pre-incubated in low serum correlated with an amplified or accelerated release of mitochondrial proteins, such as cytochrome *c* and Smac/Diablo. Immunoblot analysis of cytosol extracts obtained from RRM-treated Jurkat cells indicated that pre-incubation in 0.5% FBS-containing medium indeed accelerated the release of both cytochrome *c* and Smac (Fig. 6). Equal amounts of protein were loaded as demonstrated by the appearance of a nonspecific band at ~70 kDa detected with the anti-cytochrome *c* antibody (data not shown). Moreover, low amounts of cytosolic cytochrome *c*, but not Smac, were detected in control cells that had been pre-incubated in medium with 0.5% FBS (Fig. 6).

We examined next whether accelerated activation of JNK and p38 was responsible for the faster release of mitochondrial proteins. The antagonist MX781 efficiently activated JNK and p38 kinase (Fig. 7A and data not shown), although the time course was significantly delayed when compared to that of the agonist RRMs (Cf Fig. 7A and B). Pre-incubation in 0.5% FBS before RRM exposure had no significant effects on the time and fold induction of JNK (Fig. 7) and p38 kinase (not shown) activities, although slightly higher basal activity was detected in untreated control cells that had been pre-incubated in low serum. Measurements of the amount of DEVDase activity present in these extracts showed that pre-incubation in low serum significantly enhanced caspase activation by both MX781 and MX3350-1 (Fig. 7C).

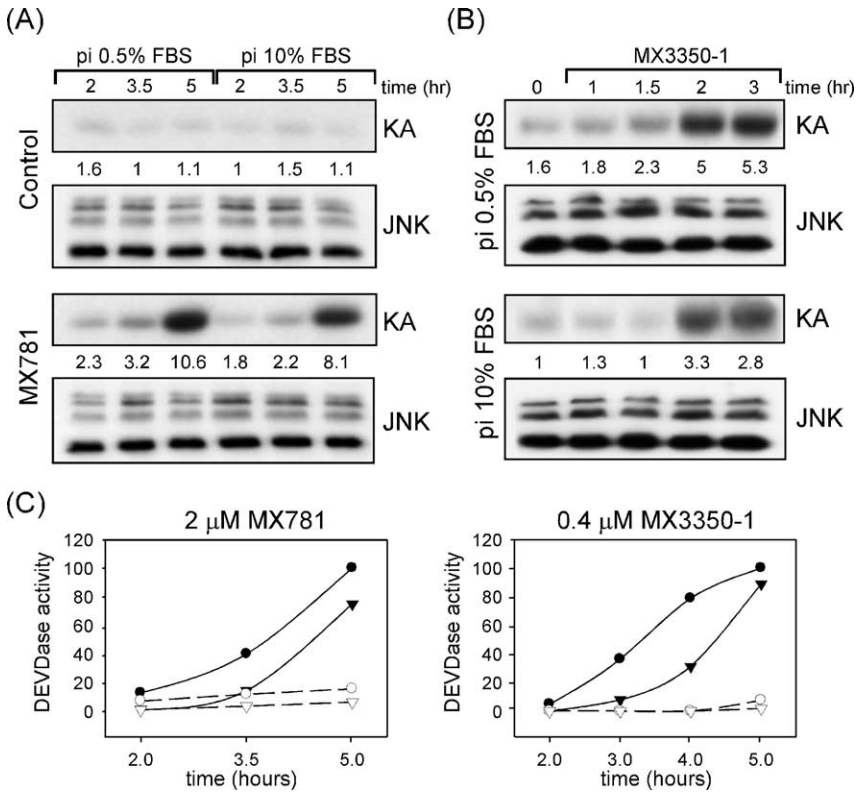

Fig. 7. Effect of low serum on RRM-mediated induction of JNK and caspase activities. (A) MX781 induces JNK activity in Jurkat cells. Cells that had been pre-incubated (pi) in 0.5 or in 10% FBS-containing medium were stimulated with 2 μM MX781 for the indicated periods of time (hr) in low serum. Whole cell extracts were prepared and analyzed for JNK activity (KA) using a solid phase KA with GST-c-Jun as substrate. The fold induction with respect to control cells pre-incubated in high serum is indicated under the KA panel and total JNK protein is shown below. The experiment was repeated three times with similar outcome and one representative experiment is shown. (B) Activation of JNK by MX3350-1. Jurkat cells pre-incubated in 0.5 or 10% FBS and subsequently stimulated with 0.4 μM MX3350-1 in the presence of low serum. Cell extracts were prepared at the indicated periods of time and JNK activity (KA) was examined using an immune complex KA. The fold induction was calculated as above. Below the KA panel, immunoblot analysis shows the amount of total JNK protein. Representative data from one experiment repeated twice is indicated. (C) DEVDase activity is enhanced in cells pre-incubated in low serum. Extracts obtained in the experiments described above (A,B) were used to measure caspase activity using Ac-DEVD-AFC as substrate. Jurkat cells pre-incubated in medium containing low (circles) or high (inverted triangles) amounts of serum for 16 hr were sedimented and resuspended in low serum-containing medium. Cells were then stimulated with the indicated RRMs (closed symbols, straight lines) or solvent as control (open symbols, dashed lines). The percentage of the maximum caspase activity observed in low serum-sensitized cells stimulated with RRM for 5 hr is represented.

4. Discussion

Certain RARγ-selective RRMs and one RAR antagonist are strong inducers of apoptosis in various cancer cell lines [9,10,12–14,21,22]. Here we show that the concentration of serum in the culture medium significantly affects the antiproliferative/cell-killing activity of the selective RRMs against leukemia cells. A similar effect has been observed with cells derived from solid tumors (see Ref. [14] and our unpublished observations). The inhibition of apoptosis by serum affected most dramatically the activity of the RAR antagonist MX781. Indeed, this compound, as well as other antagonists, efficiently inhibited cell proliferation of certain cancer cells only when treated in the presence of low concentrations of serum. The inhibition of the RRM-mediated antiproliferative activity correlated with reduced induction of apoptosis as well as with reduced activation of stress kinases, JNK and p38 kinase.

Our observations that MX781 is effective in estrogen-independent breast cancer xenografts [14] suggest that the results obtained *in vitro* do not always correlate with the outcome *in vivo*, and only those experimental conditions using low concentrations of serum yield results that are in agreement with the observations in animal studies. These remarks would have a clear impact in the design of the cell-based screenings of retinoid-like drugs before pre-clinical studies. In this regard, *in vitro* screens performed exclusively in regular culture conditions, normally in medium supplemented with 10% serum, would most probably ignore potential clinically relevant compounds like MX781, which has proven its anticancer activity in animal models [14]. Similarly, MX3350-1 is active *in vivo* against solid tumors derived from lung cancer cells [8], and this agonist is significantly more effective *in vitro* in the presence of reduced amounts of serum. The differences between *in vitro* and *in vivo* observations could be due

to unique molecular mechanisms of action of these RRMs in cultured cells or in the animal. Another explanation for this discrepancy can be the pharmacokinetics of these drugs and the potential activity of metabolites that are produced when administered to the animals.

One factor that limited the antiproliferative activity of the RRMs *in vitro* is the reduced intracellular concentrations in the presence of high serum. Interestingly, pre-incubation in 0.5% FBS significantly increased the sensitivity of Jurkat cells to the killing activity of RRMs. This effect was most notably observed when cells were exposed to suboptimal concentrations of the RRMs. This pre-conditioning seems to occur at the mitochondrial level, since a small but reproducible basal accumulation of cytochrome *c* in the cytosol and faster RRM-mediated release of both cytochrome *c* and Smac was detected in cells that had been pre-incubated in low serum. In contrast, incubation in medium containing low serum was not sufficient to release detectable amounts of Smac/Diablo in untreated cells, which has been shown to require caspase activity [31]. A massive release of cytochrome *c* precedes the activation of caspases in cells challenged with apoptotic stimuli [32]. The small amount detected in cells incubated in low serum could represent a small percentage of cytochrome *c* released in the whole cell population or a complete release in a small portion of cells, perhaps in a cell cycle dependent manner. In any case, these low levels of cytosolic cytochrome *c* are insufficient to induce caspase activity in the absence of Smac translocation. Moreover, a small but reproducible increase of basal JNK activity (about 2-fold) was consistently observed in cells that had been pre-incubated overnight in medium containing low levels of serum. This increased basal kinase activity could be responsible for the low amounts of cytochrome *c* detected in the cytosol of nonstimulated cells, as activated JNK has been recently demonstrated to translocate into the mitochondria, phosphorylate Bcl2, Bcl-X_L, and Bad, and cause the release of cytochrome *c* [24–26,33]. The correlation between JNK activation and DEVDase activity further supports our previous observations [21] suggesting a JNK-dependent induction of apoptosis. In addition, the sensitization at the mitochondrial level is in agreement with the RRMs inducing apoptosis through the intrinsic pathway, as suggested by us and others [20–22].

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