

# The Retinoid Antagonist MX781 Induces Clusterin Expression in Prostate Cancer Cells via Heat Shock Factor-1 and Activator Protein-1 Transcription Factors

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## ABSTRACT

Retinoids mediate numerous biological responses through the transcriptional activation of nuclear retinoid receptors. Due to their antiproliferative activity, retinoids have shown promise as anticancer agents. Synthetic analogs have been described that selectively activate one subset of the retinoid receptors or inhibit their transcriptional activity. Some of these compounds exhibit strong anticancer activity, which is associated with their ability to induce apoptosis. Here we describe that the retinoid antagonist MX781 causes a substantial increase of clusterin mRNA and protein levels in prostate carcinoma cells. In contrast, retinoic acid and other synthetic agonists and antagonists show no effect on clusterin mRNA/protein levels. Induction of clusterin mRNA is associated with transcriptional activation of the clusterin promoter, which requires the proximal –218-bp region containing binding sites for heat shock factor (HSF)-1, activator protein (AP)-2, and AP-1 transcription factors. MX781 slightly induces AP-1 DNA binding activity, and mutation of the AP-1 site differentially affects the activation of the clusterin promoter in a cell type-specific manner. In contrast, a robust increase of HSF-1 DNA binding activity is observed in all cancer cell lines examined, and mutation of the heat shock element site in the clusterin promoter completely abolishes MX781-induced transcriptional activation in PC3 and DU145 cells. Other agonist retinoid-related molecules also induce AP-1 activity, but not HSF-1, and elicit no effect on clusterin expression levels. These data point to HSF-1 as an important factor regulating clusterin expression in response to MX781, although AP-1 activity may also participate in a cell type-specific manner.

## INTRODUCTION

Retinoids are natural and synthetic derivatives of vitamin A that regulate gene expression by binding to and modulating the transcriptional activity of the nuclear retinoid receptors, retinoic acid receptors (RARs), and retinoid X receptors (reviewed in Ref. 1). Retinoids have been of particular interest in cancer prevention and treatment, but their broad activities mediated by transactivation of their receptors are often associated with secondary effects that have limited clinical applications. Selective analogs that target individual receptors or antagonize receptor activity are expected to display reduced toxicity. Synthetic retinoid-related molecules (RRMs), including the RAR $\gamma$ -selective agonists (CD437, CD2325, and MX2870-1) and one RAR antagonist (MX781), have shown exceptional anticancer activity through the induction of apoptosis (see Refs. 2 and 3 and the references therein), which seems independent of the nuclear receptors (3, 4). Other RRM of the heteroarotinoid class also induce apoptosis independently of RARs (5), whereas *N*-(4-hydroxyphenyl)retinamide can initiate cell death in a receptor-dependent or -independent manner (6). CD437 and MX2870-1 induce apoptosis in the absence of transcription/translation in leukemia cells (2, 7). In contrast, up-regulation

of several transcription factors [including activator protein (AP)-1, c-myc, and p53] appears to be required for CD437-induced cell death in cell lines derived from solid tumors (8–10).

Clusterin is an enigmatic glycoprotein with a nearly ubiquitous tissue distribution and is apparently involved in a wide range of biological processes (11). Clusterin expression has been associated with tumor formation and prostate cancer progression to androgen independence (12, 13). Elevated levels of clusterin have been observed in normal and malignant tissues undergoing apoptosis, including prostate tumor xenografts treated with an inhibitor of ribonucleotide reductase or with the phthalocyanine photosensitizer Pc4 (14, 15). In contrast, an antiapoptotic role has also been suggested (12). Overexpression of clusterin in prostate xenografts models conferred chemoresistance, whereas antisense oligonucleotides or small interfering RNA induced spontaneous apoptosis and increased the anticancer activity of chemotherapeutic agents (16–18). Clusterin is present in several different forms, existing predominantly as a ~70–80-kDa dimer that is secreted to extracellular fluids (19). Intracellular clusterin has been documented to exhibit various degrees of glycosylation or maturation (20), and one nuclear form of ~55 kDa (21) has been shown to induce apoptosis (22, 23). Clusterin may function as a molecular chaperone (24, 25), protecting cells against environmental stresses (26, 27).

Whereas a wealth of information has been documented on the induction of apoptosis by CD437 and its analogs, very little is known about the mechanism of MX781 action. We found that this RAR antagonist increased clusterin mRNA and protein expression levels in prostate carcinoma cells. This activity relied on an intact heat shock element (HSE) and AP-1 binding sites in the clusterin promoter and correlated with a strong and sustained activation of heat shock factor (HSF)-1.

## MATERIALS AND METHODS

**Cells and Reagents.** Prostate carcinoma cells (PC3, DU145, and LNCaP) were obtained from American Type Culture Collection and cultured in RPMI 1640. CV-1 cells were maintained in Dulbecco's modified Eagle's medium. All media contained 10% fetal bovine serum, 2 mM glutamine, and antibiotics. Synthetic RRM were obtained from Maxia Pharmaceuticals and Galderma R&D, and 10 mM stock solutions were prepared in DMSO. RRM treatments were performed in medium containing 0.5% serum. Polyclonal antibodies against clusterin (H-330 and M-18) were acquired from Santa Cruz Biotechnology. Antibodies against HSF-1, Hsp-60, Hsp-70, Hsc-70, and Hsp-90 were from Stressgen. Anti-poly(ADP-ribose) polymerase (PARP) antibody (C2-10) was from BD Biosciences.

Cell proliferation of prostate cancer cells was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. Cells (3000 cells/well seeded in 96-well plates) were treated (in triplicate points) with increasing concentrations of the RRM, and the percentage of live cells was measured at different times with respect to control cells grown in the presence of solvent.

**Total RNA Extraction and Expression Analysis.** Total RNA was isolated using an RNeasy kit (Qiagen). RNase protection assay was performed with the template hAPO-5 (BD Biosciences), as recommended by the manufacturer. Protected RNAs were analyzed by gel electrophoresis using 6% Tris-borate EDTA-urea polyacrylamide gels and autoradiography. Total RNA (2.5  $\mu$ g) was used to perform reverse transcription using Superscript (Invitrogen). Quantitative real-time PCR assays were performed in triplicate using an ABI

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PRIM 7700 (Applied Biosystems), and quantifications were normalized to an endogenous control [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. The primers used to measure clusterin expression were TGTCCTGGCTG-GCAA (forward primer) and AGTGACACCGGAAGAACGT (reverse primer). A melting curve was used to identify a temperature at which only the amplified fragment, and not primer dimers, accounted for SYBR Green-bound fluorescence.

**Transient Transfections and Luciferase Assay.** The pGL2 basic luciferase reporter vector containing the rat clusterin 5'-regulatory region from +57 to -1297 (pRAL) and different deletions has been described previously (28). Mutations were introduced in the pRAL-M/Nsi construct using the QuikChange site-directed mutagenesis kit (Stratagene), and all constructs were confirmed by sequencing. Cells ( $5 \times 10^4$ ) were seeded in 24-well plates the day before transfection. CV-1 cells were transfected with 100 ng of the luciferase reporter using a calcium phosphate precipitation procedure for 16 h. Cancer cells were transfected with 500 ng of the reporter using Superfect (LNCaP and PC3) or GenePorter (DU145) transfection reagents for 4–16 h. After transfection, cells were incubated in medium containing 0.5% serum for 8 h and then stimulated with drugs for 16 h before cell lysis. Cell extracts were analyzed for firefly luciferase and normalized with protein concentration.

**Western Blots.** Cells were lysed in 50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM MgCl<sub>2</sub>, 20 mM NaF, 1 mM sodium vanadate, and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) or in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors]. Protein extract (25–50 μg) was resolved by SDS-PAGE and transferred onto an Immobilon-P membrane (Millipore). The membranes were blocked with Blotto (5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20) and incubated at 4°C for 16–20 h with the primary antibodies. Incubation with secondary antibodies was subsequently carried out for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia).

**Electrophoretic Mobility Shift Assay.** Nuclear extracts were obtained from  $10^6$  cells following standard procedures. The sequences (in sense orientation) for the oligonucleotides (wild type) containing binding elements from the clusterin promoter were as follows: HSE, GCTTCCAGAAAGCTCCTA; AP-2, ATTCCCGGCATTCT; AP-1, CTGGCGTGAGTCACG; AP-2/AP-1, ATTCCCGGCATTCTCTGGCGTGAGTCACG; and CLU, GCTTCCAGAAAGCTCCTAGTGCATTCCCGGCATTCTCTGGCGTGAGTCACG. Electrophoretic mobility shift assays (EMSA) were performed basically as described previously (3). Where indicated, samples were incubated with specific antibodies (Active Motif) as recommended by the manufacturer or with an excess of unlabeled oligonucleotides for 30 min at 4°C before the addition of the labeled probe.

**Determination of Caspase Activity.** DEVDase activity was measured in cell extracts prepared in CE 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]. Ten μg of protein were diluted in 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 Acetyl-Asp-Glu-Val-Asp-(DEVD)-7-amino-trifluoromethyl coumarin (Enzyme System Products). Reactions were incubated at 37°C for 30 min, and the release of free AFC was measured every 2 min at 510 nm emission on excitation at 390 nm, using a Victor2 microplate reader (Perkin-Elmer). Slopes were calculated to reflect changes in fluorescence units per minute.

## RESULTS

**Retinoid-Related Molecules Inhibit Proliferation and Induce Apoptosis in Prostate Cancer Cells.** The antiproliferative activity of synthetic RRM against prostate carcinoma cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Treatment with 1 μM of the agonists MX2870-1 and CD2325 or the RAR antagonist MX781 significantly inhibited cell proliferation in a time-dependent manner (Fig. 1A). In contrast, little or no effect was observed with all-*trans*-retinoic acid (RA), which inhibited cell

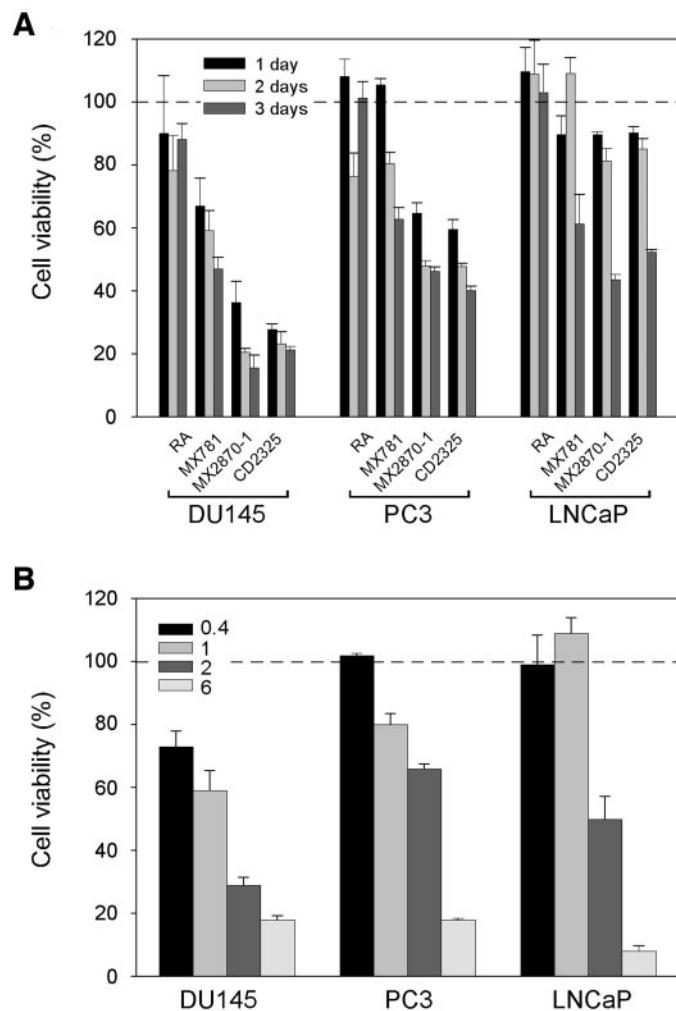


Fig. 1. Selective RRMs inhibit growth of prostate carcinoma cells. RRMs inhibit prostate cancer cell proliferation in a time (A) and concentration (B)-dependent manner. Cells were incubated with 1 μM RA or the indicated RRMs for 1, 2, or 3 days (A) or with the indicated concentrations of MX781 (in μM) for 48 h (B). The percentage of cell viability was compared with control cells grown in the absence of RRMs. Data show the average  $\pm$  SD of two independent experiments.

growth only after prolonged periods of treatment (data not shown). The growth-inhibitory activity of the synthetic RRMs was enhanced in a dose-dependent manner (Fig. 1B; data not shown). This activity correlated with the induction of apoptosis in PC3, DU145, and LNCaP cells, as demonstrated by the induction of caspase activity, DNA fragmentation, and changes in cellular and nuclear morphology (see Ref. 3; data not shown).

**MX781 Stimulates the Expression of Clusterin in Prostate Cancer Cells.** Using an RNase protection assay, we found that DU145 and LNCaP cells exhibited a robust increase of clusterin mRNA levels on short times of exposure to MX781, which was confirmed by quantitative real-time PCR (Fig. 2A). Induction of clusterin expression was very weak in PC3 cells treated for 4 h, but it increased markedly after prolonged exposure times (Fig. 2B). In contrast, cells treated with RA or other synthetic RAR $\gamma$ -selective agonists exhibited no elevated clusterin mRNA levels. The induction of clusterin mRNA levels by MX781 was dependent on the time of exposure and the concentration of RRM (Fig. 2B). Furthermore, we performed immunoblot analysis to demonstrate a significant increase in the protein levels of both full-length (Fig. 2C) and nuclear (data not shown) forms of clusterin in all three prostate carcinoma cell lines.

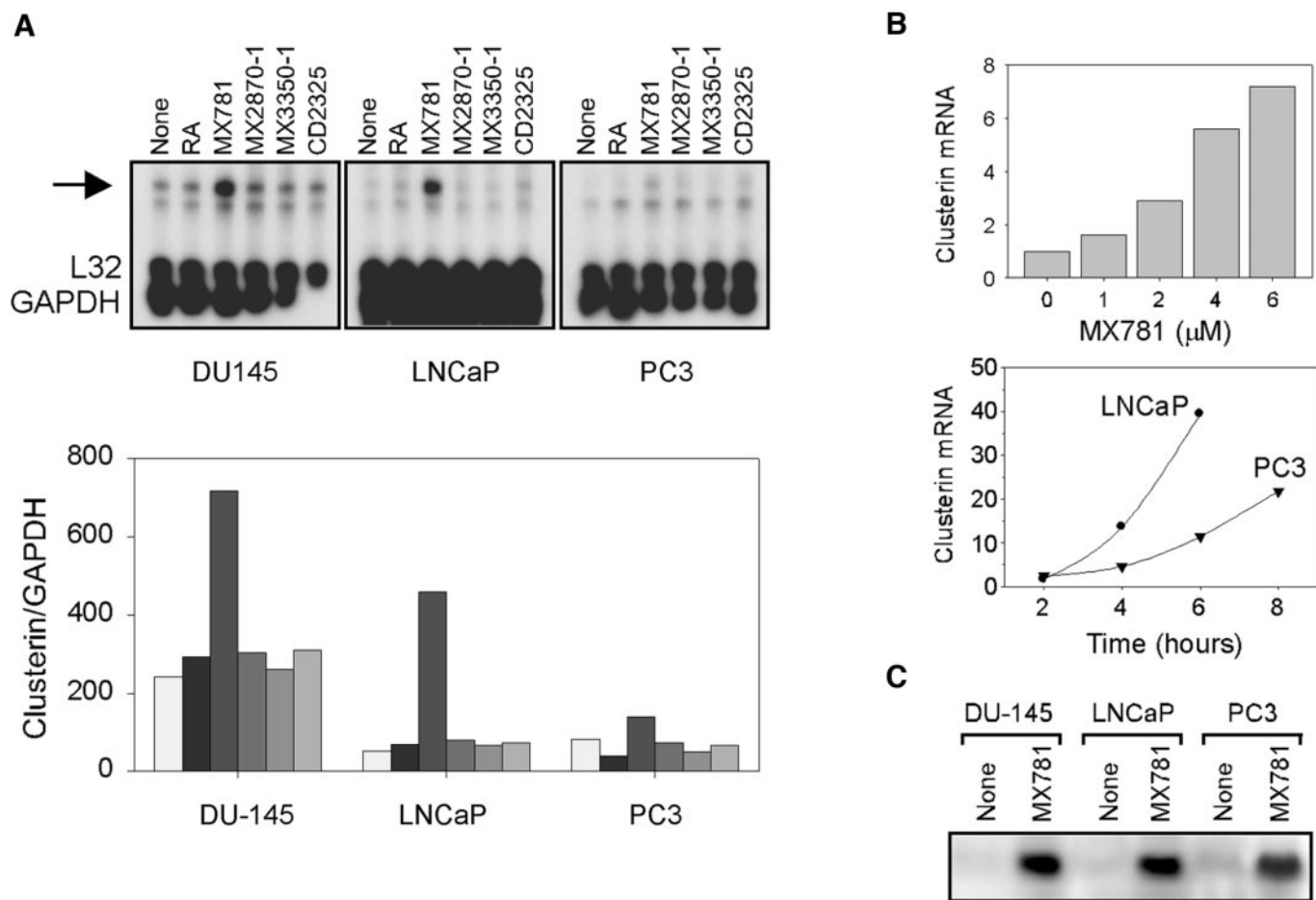


Fig. 2. Induction of clusterin in response to the RRM antagonist MX781. *A*, induction of clusterin mRNA levels by RRM antagonists. Cells were treated with  $6 \mu\text{M}$  of the indicated RRM antagonists, RA, or vehicle (*None*). Total RNA was isolated after 4 h of treatment. *Top panels*, the results of an RNase protection assay are shown, and clusterin mRNA is indicated with an arrow. L32 and GAPDH served as endogenous controls. The experiment was repeated at least three times with identical results. *Bottom panel*, quantification of clusterin and GAPDH mRNA levels by quantitative real-time PCR. The RNAs prepared as described above were used in the PCR. The ratio between the amounts of clusterin and GAPDH mRNA (arbitrary units) is shown. *B*, induction of clusterin mRNA levels is dependent on MX781 concentration and the time of exposure. *Top panel*, LNCaP cells were treated with the indicated concentrations of MX781 for 4 h. *Bottom panel*, RNA was isolated from LNCaP and PC3 cells incubated with  $6 \mu\text{M}$  MX781 for the indicated periods of time. Clusterin mRNA was quantitated by quantitative real-time PCR and normalized with GAPDH levels. These experiments were repeated twice with identical results, and the fold induction measured in one representative experiment is shown. *C*, induction of clusterin protein levels by MX781. Cells were treated with  $6 \mu\text{M}$  MX781 or vehicle (*None*) for 8 h. Whole cell extracts were prepared and analyzed by immunoblot with a clusterin (M-18) antibody. The full-length ( $\sim 60$  kDa) form is shown.

**MX781 Induces Transcriptional Activity of the Clusterin Promoter via Heat Shock Element and Activator Protein-1 Sites.** To investigate whether MX781-mediated induction of clusterin was a consequence of transcriptional activation of the clusterin promoter, we performed transient transfection experiments in CV-1 cells. We analyzed the 1.3-kb 5'-promoter region and several deletions of the rat clusterin gene (28). MX781 induced a strong activation (5-fold) of the clusterin promoter, slightly lower than that induced by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). Co-stimulation with RA significantly increased the activation by MX781, whereas RA alone elicited no effect (Fig. 3A). This suggested that transcriptional activation of RARs alone is not sufficient to induce transactivation of the clusterin promoter. Deletion of the proximal region containing HSF-1, AP-2, and AP-1 binding sites completely abrogated MX781-induced activity, whereas TPA was still able to induce luciferase activity (Fig. 3A), probably via distal AP-1 sites (29). We next investigated the effect of other selective RRM antagonists on the clusterin promoter. As observed with RA, various antagonists (CD2366, CD2665, and CD2701) and the RAR $\gamma$ -selective agonists MX2870-1 and MX3350-1 were inactive, whereas CD2325 evoked a minimal but reproducible activation (almost 2-fold) of the promoter (Fig. 3B).

Deletion studies identified the region between  $-30$  and  $-218$ ,

which contains the sites for HSF-1, AP-2, and AP-1, as responsible for MX781-mediated activity. We therefore explored the relative contribution of these sites to the full activation of the clusterin promoter by introducing mutations that abrogate protein binding within the pRAL-M/Nsi luciferase reporter (Fig. 3C). Mutation of the HSE site completely abolished promoter activity in DU145 and PC3 cells but partially prevented activation in LNCaP cells (Fig. 3C). On the other hand, eliminating AP-1 binding partially inhibited clusterin promoter transcriptional activation in LNCaP and DU145 cells, whereas it completely blocked MX781-mediated transactivation in PC3 cells. Mutation of the AP-2 site had no effect. Simultaneous mutation of AP-1 and HSE sites was required to completely abrogate MX781 induction of luciferase activity, indicating that binding of both AP-1 and HSF-1 is needed for the optimal induction of the clusterin promoter. In CV-1 cells, mutating the HSE site completely abrogated the induction of luciferase activity by MX781, whereas mutation of AP-2 or AP-1 sites had no effect (Fig. 3D).

**Apoptotic Retinoid-Related Molecules Induce Activator Protein-1 DNA Binding Activity in Prostate Cancer Cells.** We then analyzed the effect of MX781 on AP-1 DNA binding activity by EMSA. A substantial DNA binding activity was found using an AP-2/AP-1 oligonucleotide in untreated DU145 cells, which was



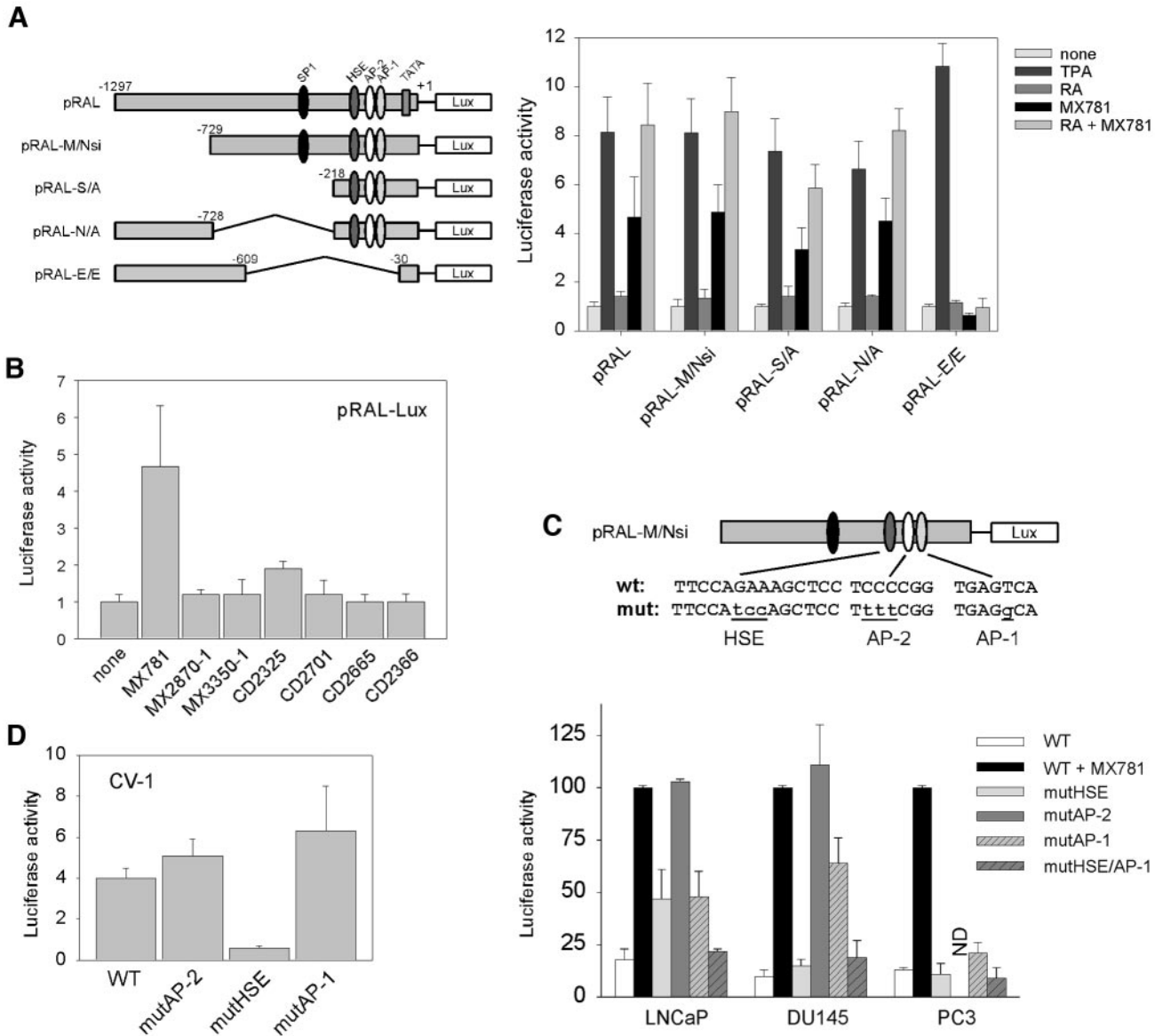


Fig. 3. Deletion and mutational analysis of the rat clusterin promoter region. *A, left panel*, schematic representation of the rat clusterin promoter and deletion mutants. *A, right panel*, CV-1 cells were transfected with the indicated reporters and stimulated with TPA (200 ng/ml), MX781 (2  $\mu$ M), RA (2  $\mu$ M), or MX781 + RA (2  $\mu$ M each) for 18 h. The fold induction relative to nonstimulated cells (average  $\pm$  SD) of three to seven experiments performed in duplicate is shown. *B*, CV-1 cells were transiently transfected with pRAL-Lux reporter and stimulated with 2  $\mu$ M of the indicated RRM for 18 h. The data represent the average  $\pm$  SD of two independent experiments performed in triplicate. *C, top panel*, schematic representation of the pRAL-M/Nsi reporter constructs containing the indicated mutations at the HSE, AP-2, or AP-1 binding sites. *C, bottom panel*, cancer cells were transfected with the indicated reporters, and luciferase activity was measured after 18 h of treatment with 2  $\mu$ M MX781. The percentage of luciferase activity with respect to cells transfected with wild-type pRAL-M/Nsi is indicated. *White columns* show the activity in nonstimulated cells transfected with wild-type pRAL-M/Nsi. *ND*, not determined. *D*, CV-1 cells were transfected with the indicated reporters and stimulated with 2  $\mu$ M MX781 for 18 h. Luciferase activity is shown as fold induction with respect to nonstimulated cells transfected with the different constructs. These experiments (*C* and *D*) show the average  $\pm$  SD from three to six experiments performed in duplicate.

further increased by MX781 exposure (Fig. 4A). The complex was specifically competed with an excess of unlabeled DNA and with an AP-1 oligonucleotide, but not with a DNA containing the AP-2 site alone. Similar binding was observed when the nuclear extracts were incubated with a DNA fragment containing the AP-1 site, whereas no binding to the AP-2 site was observed. Moreover, mutations at the individual binding sites further confirmed the presence of an AP-1 complex, but not AP-2 (Fig. 4A), correlating with the results of the transactivation experiments.

Antibody supershift experiments demonstrated the presence of cJun, JunD, and JunB in the AP-1 complex of resting as well as MX781-treated DU145 cells, whereas antibodies against FosB and CREB-1 did not recognize any protein-DNA complex (Fig. 4B); identical results were observed in LNCaP and PC3 cells and when

cells were stimulated with the agonist CD2325 (data not shown). However, cFos was detected only in MX781-treated DU145 cells but not in nonstimulated cells. This same cFos antibody did not supershift the AP-1 complex in extracts prepared from MX781-treated PC3 or LNCaP cells (data not shown). A more substantial increase of AP-1 DNA binding activity was seen in LNCaP cells, but not in PC3 and CV-1 cells; the latter exhibited higher levels of basal AP-1 DNA binding activity as compared with prostate carcinoma cells (Fig. 4C). Together, these data demonstrate a variable induction of AP-1 DNA binding activity by the apoptotic RRM, despite a strong activation of c-Jun NH<sub>2</sub>-terminal kinase [JNK (data not shown)].

**MX781 but not Agonist Retinoid-Related Molecules Induces Heat Shock Factor-1 DNA Binding Activity.** We next investigated protein binding to the HSE located 5' next to the AP-2/AP-1 site (30).

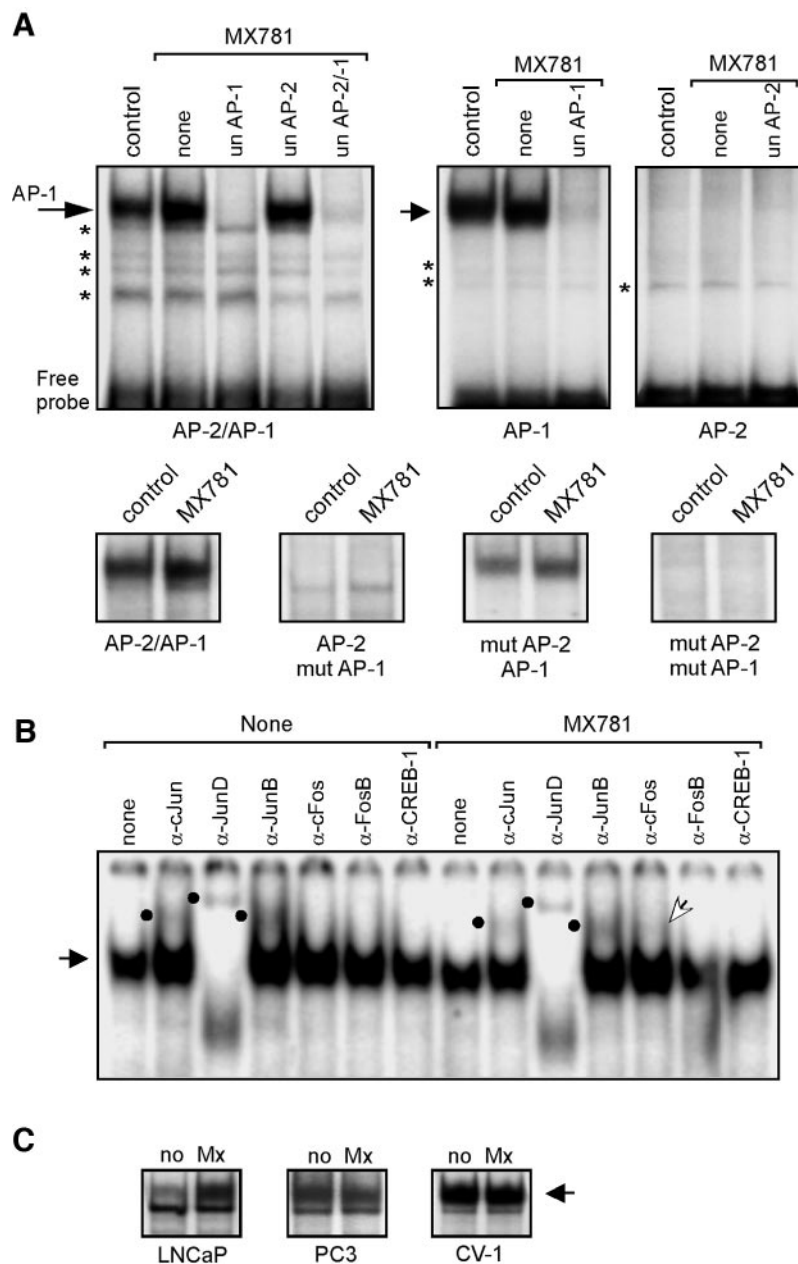


Fig. 4. MX781 induces AP-1 DNA binding activity in prostate cancer cells. **A**, induction of AP-1 DNA binding activity by MX781. DU145 cells were treated with 6  $\mu$ M MX781 or vehicle (*control*) for 4 h, and then nuclear extracts were prepared and assayed by EMSA using the indicated wild-type or mutated (*mut*) oligonucleotides. Where indicated, a 50-fold molar excess of unlabeled oligonucleotide (*un*) was included in the binding reaction. The AP-1 complex is indicated with an *arrow*. The *asterisks* indicate nonspecific bands. **B**, antibody supershift of the AP-1 complex. Nuclear extracts were prepared from DU145 cells treated with MX781 or not treated as described above. Binding reactions to a labeled AP-1 oligonucleotide were carried out in the absence (*None*) or presence of the indicated antibodies before electrophoresis. The specific AP-1 DNA complex is shown (*black arrow*). The antibody supershifted complexes are indicated by *black circles*. The *open arrow* shows the supershift detected exclusively in MX781-stimulated cells with the anti-cFos antibody. **C**, the indicated cells were left untreated (*no*) or stimulated with 6  $\mu$ M MX781 (*Mx*) for 4 h. Nuclear extracts were assayed for DNA binding activity to the AP-2/AP-1 probe. The specific complex is indicated by an *arrow*. All of the experiments shown in this figure were carried out two to four times, producing similar outcomes, and one representative result is shown.

A rapid and strong induction of HSF-1 DNA binding activity was observed in DU145 cells treated with MX781, which was dependent on the time of incubation and the RRM concentration (Fig. 5A). Mutation of the core binding site completely abrogated binding (Fig. 5B). The protein-DNA complex was totally displaced on incubation with unlabeled wild-type oligonucleotide, but not with mutant DNA. Furthermore, binding was totally abrogated when protein extracts were incubated with anti-HSF-1 antibody, but not with an unrelated antibody (Fig. 5B), proving the presence of HSF-1 in the bound complex. Induction of HSF-1 binding was not observed in cells treated with MX2870-1 or MX3350-1, whereas CD2325 produced a very weak induction of HSF-1 DNA binding (Fig. 5C). Similar results were seen in all prostate cancer cells (data not shown) and correlated with a marginal stimulation of clusterin promoter activity in CV-1 cells (see Fig. 3B). Strong induction of HSF-1 DNA binding by MX781 was also observed in nuclear extracts prepared from LNCaP, PC3, and CV-1 cells. Enhanced HSF-1 activity was not due to

increased levels of HSF-1 protein (see below), but it was associated with posttranslational modifications and nuclear translocation of HSF-1 protein, as demonstrated by immunoblot (Fig. 5D).

**MX781 Induces a Heat Shock Response in Prostate Carcinoma Cells.** Having established that MX781 induces HSF-1 DNA binding and transcriptional activity, we sought to examine the effect of MX781 treatment on certain heat shock proteins (HSPs) at the protein level. As shown in Fig. 6A, all prostate cancer cells treated with MX781, but not with RA, exhibited elevated levels of Hsp-70 and Hsp-90 after 8–24 h of stimulation, concurrent with increased levels of clusterin expression. In contrast, the protein levels of Hsp-60 and Hsc-70 remained unchanged. The agonist CD2325 also enhanced Hsp-70 and Hsp-90 levels after 24 h of treatment in PC3 and DU145 cells, although to a lesser extent as compared with MX781 (Fig. 6A). This correlated with a weaker induction of HSF-1 DNA binding activity shown earlier. Increased levels of inducible HSPs were associated with posttranslational changes of HSF-1 but not enhanced amounts of HSF-1. Reduced mobility of HSF-1 was

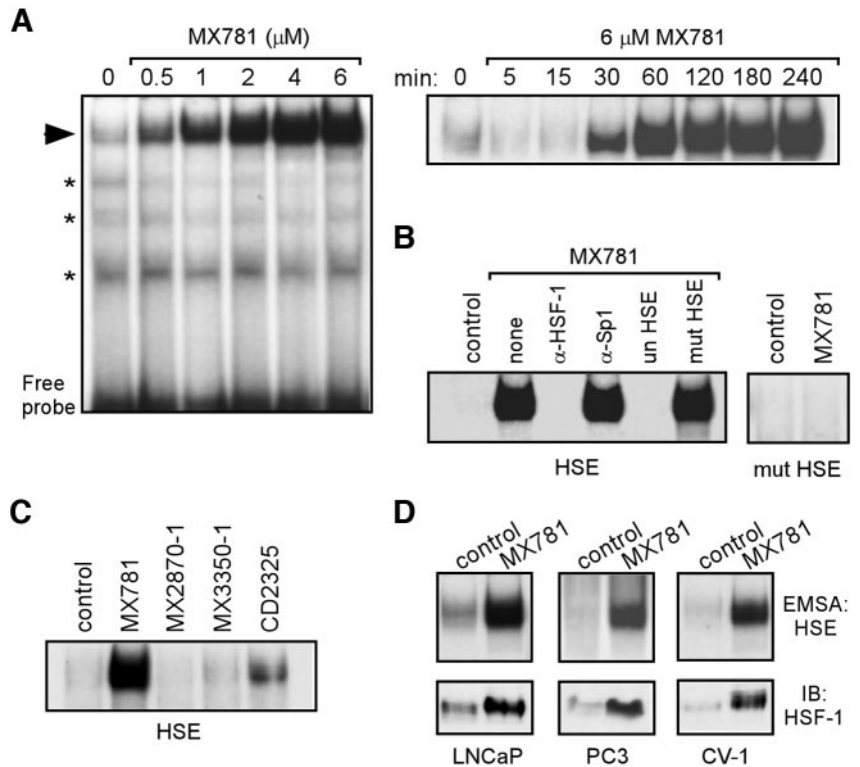


Fig. 5. Induction of HSF-1 DNA binding activity by MX781. **A**, MX781 induces binding to a HSE in a time- and concentration-dependent manner. DU145 cells were treated with the indicated concentrations of MX781 for 4 h. On the *right panel*, DU145 cells were incubated with 6  $\mu$ M MX781 for the indicated periods of time. The binding activity of nuclear extracts to the clusterin HSE was analyzed by EMSA. The specific protein-DNA complex is indicated with an *arrowhead*, and the *asterisks* show nonspecific bands. **B**, binding to the HSE involves HSF-1. Nuclear extracts prepared from DU145 cells stimulated with 6  $\mu$ M MX781 for 4 h were incubated with labeled wild-type or mutated HSE binding site in the absence (*none*) or presence of the indicated antibodies, or with a 50 $\times$  molar excess of unlabeled wild-type (*un*) or mutated (*mut*) oligonucleotides, as indicated. **C**, effect of selective RRM on HSE binding. DU145 cells were incubated with 6  $\mu$ M of the indicated RRM. Nuclear extracts obtained after 4 h of treatment were assayed for HSE DNA binding activity. **D**, the indicated cell lines were incubated with solvent (*control*) or 6  $\mu$ M MX781 for 4 h, and then nuclear extracts were prepared and analyzed by EMSA and immunoblot. All of the experiments presented in this figure were performed two to three times with identical outcomes, and one representative result is shown.

detected in cells treated with MX781 and CD2325 as well (Fig. 6B), probably due to hyperphosphorylation of HSF-1 (31). These changes were seen even after 24 h of treatment in LNCaP and DU145, indicating a sustained activation of HSF-1.

In PC3 cells, however, treatment with MX781 for extended periods of time (24 h) caused a complete disappearance of HSF-1 protein (Fig. 6B). This occurred in parallel with caspase activation, as evidenced by degradation of PARP, a known substrate of caspases. MX781-induced degradation of PARP and HSF-1 was weaker in LNCaP and DU145 cells, suggesting a delay in the induction of apoptosis. To demonstrate that HSF-1 degradation was indeed a consequence of caspase activity, we incubated cells with the pan-caspase inhibitor *z*-Val-Ala-Asp-fluoromethyl ketone (*z*-VAD-fmk) before MX781 stimulation. This inhibitor efficiently blocked the induction of DEVDase activity (Fig. 6C) and prevented the degradation of HSF-1 and PARP (Fig. 6D) induced by MX781. In contrast, *z*-VAD-fmk elicited no effect on Hsp70 protein levels, indicating that heat shock response was independent of caspase activity.

## DISCUSSION

Complex signaling pathways are activated in response to cellular stress, which result in the activation of key transcription factors and the rapid synthesis of proteins that function to induce apoptosis or to repair and protect cells from dying. Certain pathways, such as JNK/AP-1 and I $\kappa$ B kinase (IKK)/nuclear factor  $\kappa$ B, can signal survival or apoptosis decisions in a cell type, context, and stimulus-dependent manner (32). Synthetic RRM with strong anticancer activity have been shown to activate (JNK) or inhibit (IKK) these pathways, which correlates with their ability to induce apoptosis (3, 33). Another signaling pathway that responds to cellular stress is the heat shock response, which results in the rapid synthesis of HSPs that function as molecular chaperones or proteases to repair damaged proteins or degrade them (34). Enhanced heat shock gene expression is regulated by the four-member HSF family of transcription factors (35), with

HSF-1 playing a major role in the cellular response to thermal stress (36). A large body of evidence supports a protective role of HSPs against cellular stress (37). Hsp-70 inhibits apoptosis downstream of cytochrome *c* release, perhaps by preventing recruitment of procaspase 9 to the apoptosome and interfering with apoptosis-inducing factor (38–40). However, strong and prolonged activation of heat shock response can also be detrimental and cause cell death (41), and a proapoptotic activity has been suggested for Hsp-60 and Hsp-90 (42).

In addition to elevated temperatures, heat shock response is activated by several cytotoxic chemicals, including hydrogen peroxide, antiproliferative prostaglandins, and sodium arsenite (43–45). These compounds can induce apoptosis, although a direct connection between the activation of heat shock and the induction of apoptosis needs to be established. We now report on a novel RAR antagonist, MX781, which induces a strong activation of HSF-1 and causes apoptosis *in vitro* and *in vivo* (3, 46). MX781 and the RAR $\gamma$  agonists MX2870-1 and CD2325 share structural similarities (including an adamantyl group and a carboxylic moiety) and induce cell death in a caspase-dependent manner. However, agonist RRM are more potent inducers of apoptosis than the antagonist MX781, which usually requires higher concentrations and reduced amounts of serum *in vitro* (47). In addition to exhibiting opposite RAR-dependent activities, the mechanism of apoptosis induction of agonist RRM and antagonist MX781 also seems quite distinct (48). Besides activating JNK and p38 stress kinases and inhibiting IKK (3, 47), MX781 also enhances HSF-1 transcriptional activity. The lack of heat shock stress in response to RA and other agonist RRM suggests that this novel activity of MX781 is probably independent of RAR-mediated transcriptional activity. This is further suggested by the fact that co-stimulation with RA enhances rather than inhibits MX781-mediated transactivation of the clusterin promoter.

The induction of HSF-1 DNA binding and transcriptional activity by MX781 is very strong and lasts for extended periods of time.

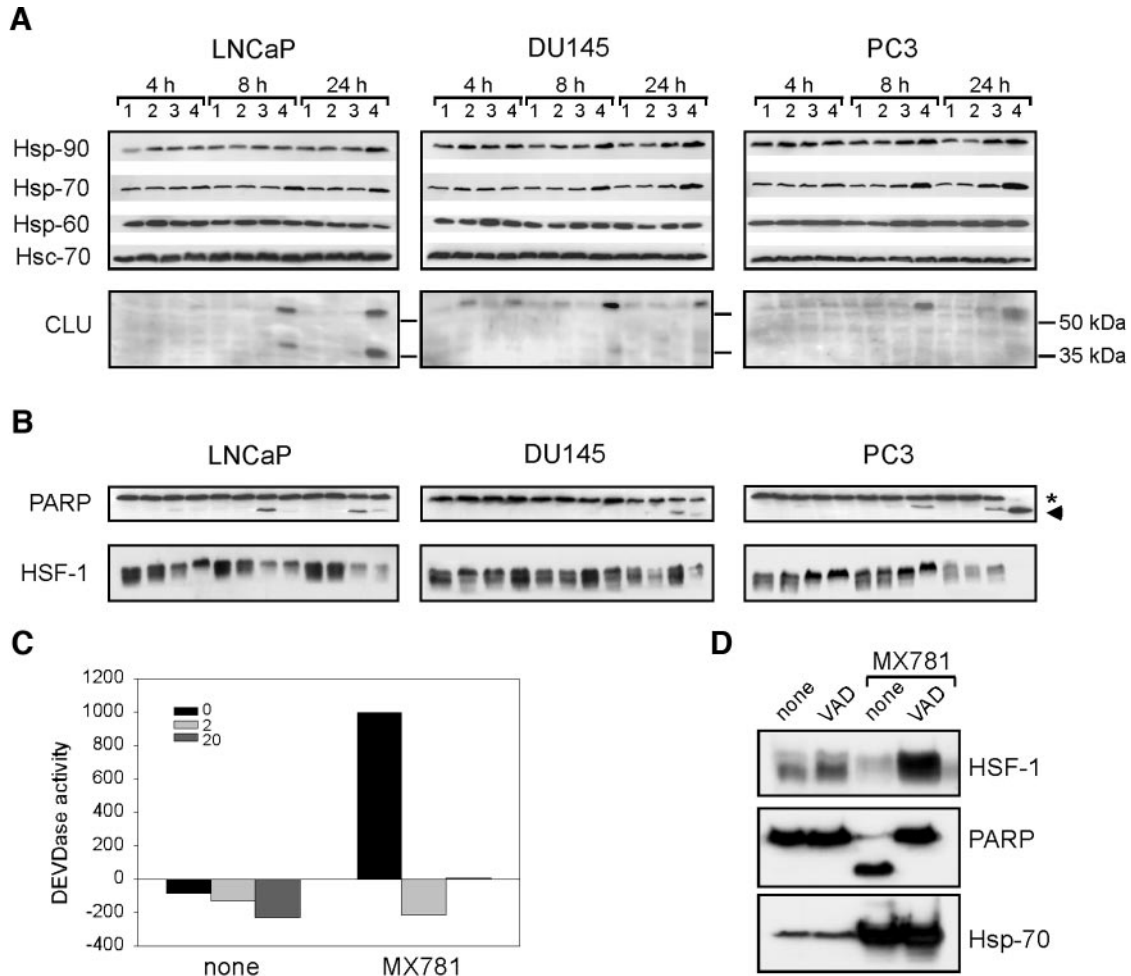


Fig. 6. Induction of HSP expression and apoptosis by MX781 in prostate cancer cells. *A*, cells were incubated with vehicle (Lanes 1) or 6  $\mu$ M RA (Lanes 2), CD2325 (Lanes 3), or MX781 (Lanes 4) for increasing periods of time. Whole cell extracts were prepared and analyzed by immunoblot with the indicated antibodies. Both full-length (~60-kDa) and mature (~40-kDa) clusterin (CLU) forms are shown. *B*, RRM-induced sustained activation of HSF-1 and cleavage of PARP. The cleavage product of PARP (~84 kDa) is indicated by an arrowhead, and the asterisk indicates full-length PARP. *C*, inhibition of DEVDase activity by z-VAD-fmk. PC3 cells were preincubated in the absence (black columns) or presence (gray columns) of the indicated concentrations of z-VAD-fmk (in  $\mu$ M) and subsequently stimulated with 4  $\mu$ M MX781 for 20 h. Cell extracts were prepared in radioimmunoprecipitation assay buffer and assayed for DEVDase activity (in arbitrary units). *D*, degradation of HSF-1 is prevented by z-VAD-fmk. Extracts obtained in the experiment shown before were resolved by SDS-PAGE and analyzed by immunoblot with the indicated antibodies.

Whether or not the activation of HSF-1 by MX781 affects the induction of apoptosis by the RRM is currently under investigation, but preliminary studies have shown that the presence of HSF-1 does indeed enhance MX781-induced apoptosis.<sup>3</sup> This correlates with other studies linking the overexpression of hyperactivated HSF-1 with cell death (41). An additional link between the activation of a heat shock response and apoptosis is the increase in clusterin mRNA and protein levels observed in MX781-stimulated cells. Maximum increase of clusterin levels relies on the presence of an intact HSE, although AP-1 transcription factors may also play a role in prostate carcinoma cells in a cell type-specific manner. Interestingly, the highest induction of clusterin mRNA expression observed in LNCaP cells coincided with the highest induction of AP-1 DNA binding activity. On the contrary, the induction of mRNA clusterin expression was considerably delayed in PC3 cells, which exhibit high levels of basal AP-1 DNA binding activity that are minimally affected by MX781 stimulation. Independently of the actual role of AP-1 activity or associated cofactors on MX781 function, activation of the JNK/AP-1 pathway alone is not sufficient for the induction of clusterin expression because the agonist

RRMs CD2325 and MX2870-1 had minimal effects on clusterin expression, despite a strong activation of JNK. Therefore, optimal induction of clusterin promoter activity relies on a complex interplay between HSF-1 and AP-1 transcription factors and probably additional cell type-specific cofactors as well.

Although clusterin was originally thought to mediate apoptosis (49) and has been used as an apoptotic marker, many studies have suggested an antiapoptotic function (26, 27), which is more in agreement with its hypothetical chaperone activity. Nevertheless, different functions can be attributed to the various cellular forms of clusterin. Overexpression of a nuclear truncated form of clusterin has been shown to cause apoptosis (22, 23). On ionizing radiation or transforming growth factor  $\beta$  stimulation, levels of truncated clusterin increase, and the protein undergoes posttranslational modifications (perhaps glycosylation) and translocation into the nucleus (21), where it can bind Ku70 protein and induce apoptosis independently of caspases (22). Thus, although the induction of full-length clusterin seems to have a cytoprotective effect, concurrent generation of the truncated nuclear form may lead to apoptosis in cell type- and stimulus-dependent manners. High levels of stress by continuous exposure to MX781 could turn the cytoprotective activity of clusterin

<sup>3</sup> F. Javier Piedrafita, Y. Bayon, and M. A. Ortiz. Involvement of HSF-1 in MX781-induced apoptosis, manuscript in preparation.



and certain HSPs (such as Hsp-70) into the proapoptotic activity of nuclear clusterin and/or other HSPs. Whether clusterin actively participates in MX781-induced apoptosis is being explored.

In conclusion, we have reported on a novel function of the RRM antagonist MX781 in prostate cancer cells, which is the activation of HSF-1 pathway and subsequent induction of clusterin mRNA and protein levels. Binding of HSF-1 is essential for the optimal activation of the clusterin promoter and cooperates with AP-1 for the full induction of clusterin expression by MX781. The involvement of HSF-1/AP-1/clusterin in MX781-induced apoptosis would argue in favor of a transcription/translation-dependent mechanism of action, as observed with CD437 and other agonist RRMs in cell lines derived from solid tumors.

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