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PRINCIPAL INVESTIGATOR: Xiao-kun Zhang, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

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Xiao-kun Zhang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Burnham Institute
La Jolla, California 92037

E-Mail: xzhang@burnham.org

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13. ABSTRACT *(Maximum 200 Words)*
TR3 is an orphan member of the steroid/thyroid/retinoid receptor superfamily and is known to induce apoptosis of cancer cells. We have investigated the mechanism of TR3-mediated apoptosis in breast cancer cells. Our results demonstrate that TR3 exerts its apoptotic effect in breast cancer by translocating from the nucleus to mitochondria in response to apoptotic retinoids. We have also identified a new apoptotic retinoid MM002 that effectively induces TR3 mitochondrial targeting and apoptosis of breast cancer cells. In studying the mechanism by which TR3 migrates from the nucleus to the cytoplasm, we found that the migration of TR3 from the nucleus to the cytoplasm requires RXR through their heterodimerization. We also observed that RXR contains a nuclear export sequence that is required for its cytoplasmic localization and is regulated by RXR ligands. Furthermore, we discovered that TR3 can physically interacts with Bcl-2 and that Bcl-2 acts as a mitochondrial receptor of TR3 and is required for apoptotic effect of TR3 in breast cancer cells. Our results not only enhance our understanding of the molecular mechanism by which TR3 exerts its apoptotic effect in breast cancer cells but also provide novel approaches to induce apoptosis of Bcl-2-expressing breast cancer cells.

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INTRODUCTION

Breast cancer is the most prevalent cancer in women and it has the second highest mortality rate for U.S. women. The only available drug for breast cancer chemoprevention for women at risk is the antiestrogen tamoxifen. Unfortunately, responsiveness to tamoxifen decreases as breast cancer cells lose their estrogen dependence for growth. The lack of therapies for estrogen-independent breast cancer has contributed to the increased mortality rates for the last 20 years. Novel therapies are therefore urgently needed.

Retinoids, analogs of vitamin A, are promising chemopreventive agents for breast cancer. Unfortunately, classical retinoids, such as all-*trans* retinoic acid (RA), did not show therapeutic benefit in preventing the occurrence of breast cancer, especially in patients with hormone refractory breast cancer (1,2). Recently, a new class of synthetic retinoids related to 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437) were found to potently induce apoptosis of both hormone-dependent and –independent breast cancers (3,4), indicating that it may be representative of a novel class of compounds suitable for treatment of estrogen-independent breast cancers. We subsequently showed that TR3, an orphan member of the steroid/thyroid/retinoid receptor superfamily, is required for induction of apoptosis of lung and prostate cancer cells by AHPN/CD437 and other apoptotic stimuli (5,6). Moreover, we discovered that TR3, in response to apoptosis stimuli, is translocated from the nucleus to the cytoplasm, where it targets mitochondria to induce cytochrome c release and apoptosis in LNCaP prostate cancer cells (6).

The objective of this application is to develop evidence that TR3 is a suitable molecular target for developing drugs against estrogen-independent breast cancer. In the proposed studies, we plan to establish whether TR3 is required for proliferation and apoptosis of breast cancer cells in response to various stimuli. We will also determine whether the novel nuclear-to-mitochondrial pathway for apoptosis occurs in breast cancer cells. In addition, we will study mechanism by which translocation of TR3 from the nucleus to cytoplasm is regulated by studying effect of phosphorylation. Furthermore, we will study physical interactions between TR3 and Bcl-2 and its effect on Bcl-2-mediated mitochondrial and post-mitochondrial activities. Results from these studies will provide evidence whether TR3 is a suitable molecular target for developing drugs for breast cancer prevention and treatment.

KEY RESEARCH ACCOMPLISHMENTS

In the past funding year, we have conducted various experiments to address the specific aims proposed in the grant application and have made significant progress as described below.

1. The novel nuclear-to-mitochondrial pathway for apoptosis in breast cancer cells

Expression of TR3 and its regulation in breast cancer cells. Our previous results indicate that TR3 expression is required for AHPN-induced apoptosis of human lung cancer and prostate cancer cells (5,6). We also showed that TR3 was expressed in both estrogen-dependent ZR-75-1 and -independent MDA-MB-231 cells (7). Taking into account that AHPN/CD437 effectively induces apoptosis of both types of breast cancer cell lines, we investigated the possible role of TR3 in this process. The AHPN/CD437 analog SR11453 (MM11453) that effectively induces apoptosis of estrogen-dependent and -independent breast cancer cells (data not shown) was used to treat MDA-MB-231 breast cancer cells. TR3 expression in the cells was analyzed by Northern blotting. Figure 1 shows that upon SR11453 treatment, TR3 expression was significantly induced. TR3 expression was also induced by another

apoptosis-inducing retinoid (SR11384), TPA, and the mitogenic agent epidermal growth factor (EGF).

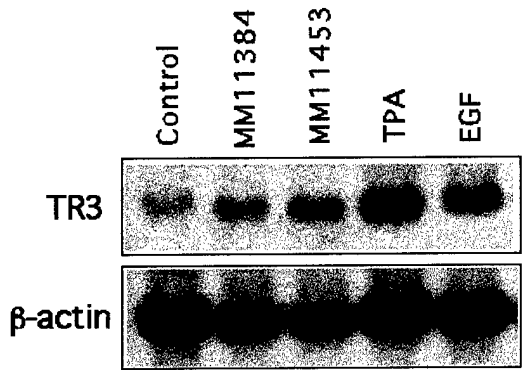

Figure 1. Induction of TR3 expression by SR11453 and other apoptotic and mitogenic stimuli. Total RNAs were prepared from MDA-MB-231 cells treated with MM1153 (10^{-6} M), SR11384 (MM11384)(10^{-6} M), TPA (100 ng/ml) or EGF (200 ng/ml) for 3 h and analyzed by Northern blotting.

AHPN/CD437 analog SR11453 causes disruption of mitochondrial membrane potential ($\Delta\psi m$). We also studied $\Delta\psi m$ in breast cancer cells treated with or without SR11453 using Rh123. As shown in Figure 2, SR11453 induced significant increases in the percentage of cells experiencing $\Delta\psi m$ loss. Disruption of $\Delta\psi m$ was observed in both estrogen-dependent MCF-7 and -independent MDA-MB-231 cells. Thus, induction of apoptosis by AHPN/CD437 analog SR11453 involves mitochondrial-mediated apoptotic pathway.

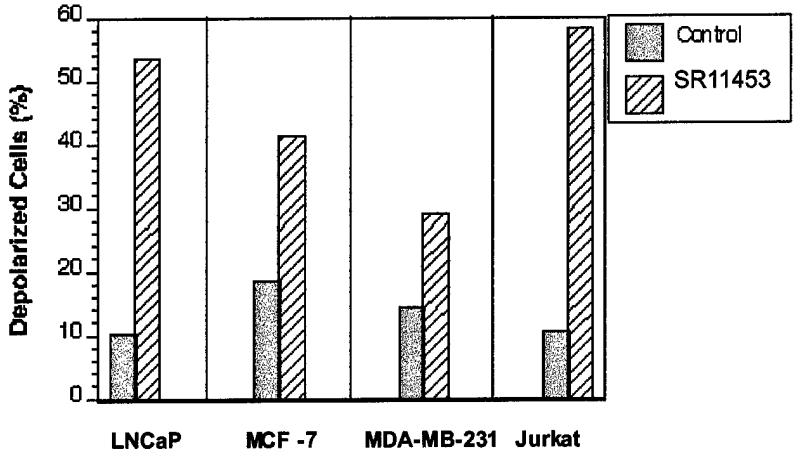

Figure 2. SR11453 disrupts $\Delta\psi m$. The indicated cells were treated with or without 10^{-6} M SR11453 for 18 h. Cells were then incubated with Rh123 for 30 min and analyzed by a FACScalibur cytometery. The percent of cells fluorescencing within the range of Rh123 were considered as depolarized.

Mitochondrial localization of TR3 in breast cancer cells. To study whether the TR3 nuclear-to-mitochondrial targeting pathway occurs in breast cancer cells, we transfected a green fluorescent protein (GFP)–TR3 fusion construct into MDA-MB-231 cells. The GFP–TR3 fusion protein was predominately present in the nucleus in nonstimulated cells. However, upon treatment with SR11453 and SR11384, GFP–TR3

translocated to mitochondria (Figure 3). Furthermore, GFP–TR3/ΔDBD (a mutant that lacks the DNA-binding domain) was targeted to mitochondria in MDA-MB-231 and ZR-75-1 cells in the absence of any treatment to cause cytochrome c release (Figure 4).

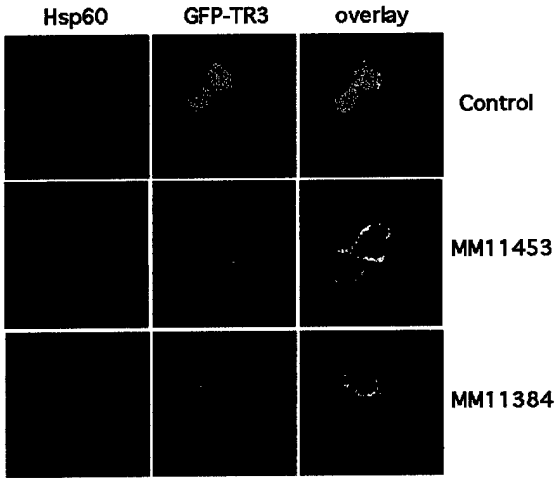

Figure 3. TR3 is targeted to mitochondria in MDA-MB-231 cells in response to an apoptosis inducer. GFP–TR3-transfected MDA-MB-231 cells were treated with or without SR11453 (10^{-6} M) or SR11384 (10^{-6} M) for 1 h, then immunostained with anti-Hsp60 antibody (Sigma), followed by Cy3-conjugated secondary antibody (Sigma) to detect mitochondria. GFP–TR3 and mitochondria (Hsp60) were visualized using confocal microscopy, and the two images were overlaid (see Overlay).

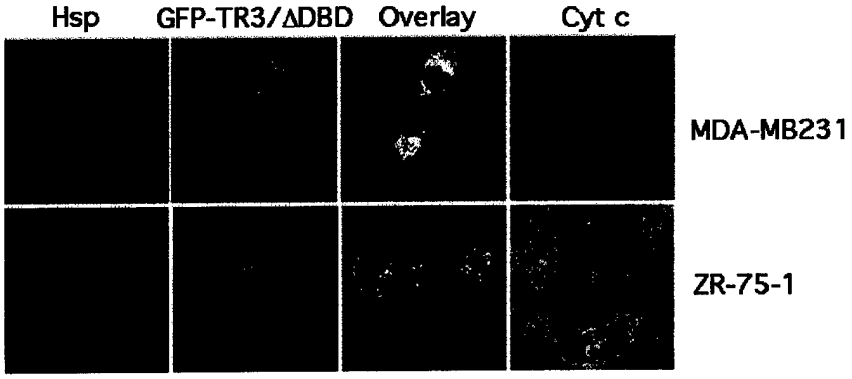

Figure 4. Mitochondrial targeting of TR3/ΔDBD is associated with cytochrome c release. GFP–TR3/ΔDBD was transiently transfected into ZR-75-1 or MDA-MB-231 cells, which were stained for mitochondria (Hsp60) and cytochrome c (Cyt c), and analyzed by confocal microscopy.

MM002 induces mitochondrial localization of endogenous TR3 in MDA-MB-231 cells. AHPN analog MM002 effectively induces apoptosis in breast cancer cells and retarded breast tumor growth in murine models (data not shown). To determine whether this new AHPN analog functioned through TR3, MDA-MB-231 cells were treated with MM002 and cellular localization of endogenous TR3 was analyzed by confocal microscopy. Figure 5 shows that the endogenous TR3 was found exclusively in the cytoplasm and colocalized with mitochondria in MM002-treated cells. Thus, the anti-breast cancer activities of MM002 may be mediated by TR3 mitochondrial localization.

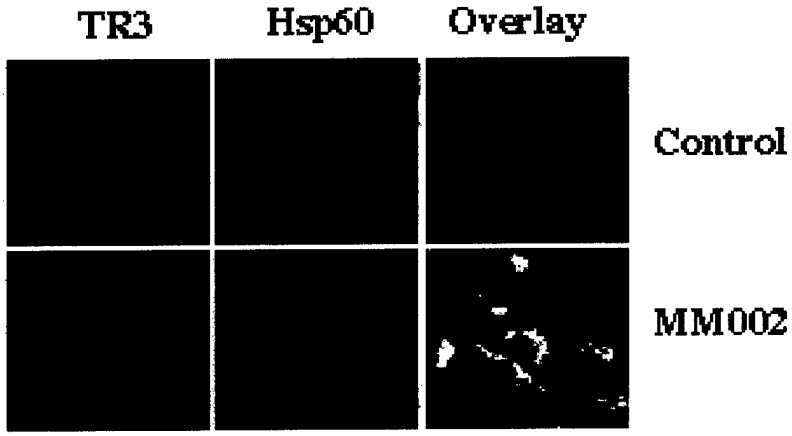

Figure 5. A new AHPN analog MM002 induces mitochondrial localization of endogenous TR3 in MDA-MB-231 cells. MDA-MB-231 cells were treated with or without MM002 (10^{-6} M) for 1 h, then immunostained with anti-TR3 antibody (Geneka) followed by Cy3-conjugated secondary antibody to detect endogenous TR3, or with anti-Hsp60 antibody followed by Cy5-conjugated secondary antibody (Sigma) to detect mitochondria. TR3 and mitochondria (Hsp60) were visualized using confocal microscopy, and the two images were overlaid (overlay).

Subcellular localization of TR3 correlates with apoptosis of mammary tumor cells. We studied cellular localization of TR3 expressed in mammary tumors derived from transgenic mice bearing polyomavirus middle T antigen (kindly provided by Dr. W. Muller). The tissue sample was also analyzed by the TdT assay for apoptosis. TR3 was localized in the cytoplasm in cells undergoing extensive apoptosis, while it was found in the nucleus in nonapoptotic cells (Figure 6). Thus, subcellular localization of TR3 may also play a role in regulating apoptosis in tumors.

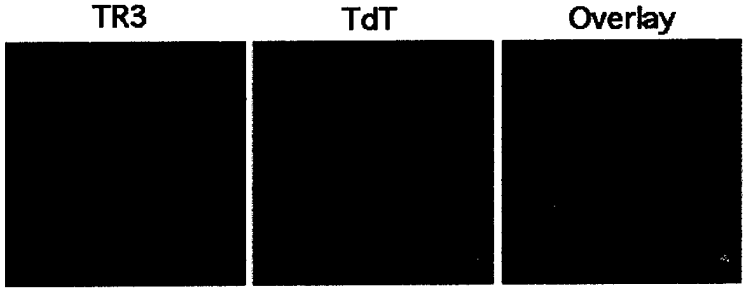

Figure 6. Correlation between TR3 subcellular localization and apoptosis in a mammary tumor. Mammary tumor tissue from transgenic mice bearing polyomavirus middle T antigen was stained with anti-TR3 antibody followed by Cy3-conjugated secondary antibody to detect TR3 expression and subcellular localization by confocal microscopy. The tissue was also analyzed by the fluorescein-conjugated TdT enzyme (Oncogene) to detect DNA fragmentation (TdT-labeled cells are indicated by green color). The two images were overlaid (overlay) to assess the correlation between apoptosis and TR3 subcellular localization.

Together, our studies demonstrate that retinoid-related AHPN/CD437 analogs can effectively induce apoptosis of breast cancer cells through a mitochondria-dependent pathway. Moreover, our data show that migration of orphan receptor TR3 from the nucleus to mitochondria is involved in mediating apoptotic effect of AHPN/CD437 analogs.

2. Regulation of TR3 mitochondrial targeting.

Regulation of TR3 nuclear export by RXR and its ligands. How TR3 translocates from the nucleus to mitochondria to induce cytochrome c release is unknown. Previous studies demonstrated that retinoid X receptor (RXR) is required for its ligand 9-*cis*-RA to inhibit activation-induced apoptosis of T-cells and thymocytes (8,9). TR3 is known to heterodimerize with RXR (10,11). To determine the effect of RXR and its ligands on TR3-dependent apoptosis, we studied the cellular localization of RXR in response to the apoptosis-inducing agent TPA in LNCaP prostate cancer cells. RXR was found exclusively in the nucleus in the absence of treatment. However, when cells were treated with TPA, RXR translocated to the cytoplasm, where it colocalized with mitochondria (Figure 7A). Pretreatment of cells with RXR ligands 9-*cis*-RA or SR11237 prevented RXR mitochondrial localization. Mitochondrial localization of RXR was also demonstrated by Western blotting, showing accumulation of RXR in cells treated with apoptotic stimuli TPA and MM11453 (Figure 7B). The mitochondrial localization of RXR depended on TR3 expression, as RXR failed to reside on mitochondria in cells expressing TR3-antisense RNA (Figure 7C). These results suggest that RXR may target mitochondria as a TR3/RXR heterodimer.

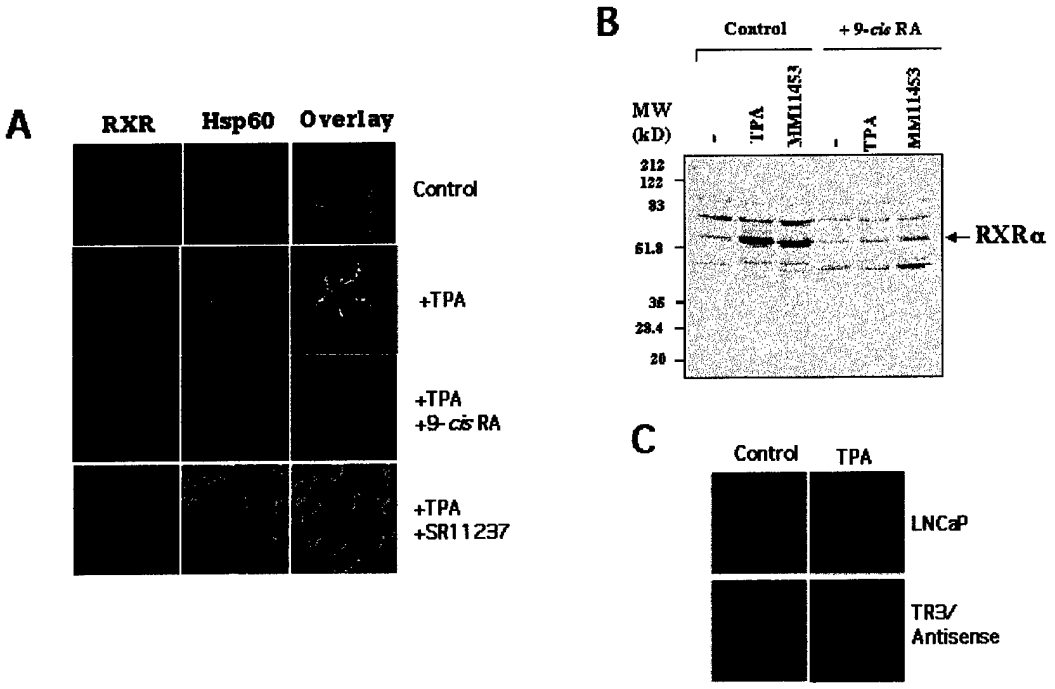

Figure 7. Localization of RXR in mitochondria in a TR3-dependent manner. **A**. Regulation of RXR mitochondrial localization by apoptosis inducer TPA and RXR ligands. LNCaP cells were pre-treated with or without RXR ligands 9-cis RA (10^{-7} M) or SR11237 (10^{-6} M) for 12 hr before TPA treatment (1 hr), then immuno-stained with anti-RXR antibody followed by Cy3-conjugated secondary antibody (Sigma) to detect RXR, or with anti-Hsp60 followed by Cy5-conjugated secondary antibody (Sigma) to detect mitochondria. RXR and mitochondria (Hsp60) were visualized using confocal microscopy and the two images were overlaid (overlay). **B**. Apoptotic stimuli induce accumulation of RXR in mitochondria. LNCaP cells were treated with TPA (100 ng/ml) or MM11453 (10^{-6} M) for 3 hr in the absence or presence of 9-*cis* RA (10^{-6} M), and the HM fraction was analyzed for expression of RXR by Western blotting. **C**. Mitochondrial localization of RXR is TR3 dependent. LNCaP cells or LNCaP cells stably expressing TR3

antisense RNA (TR3/Antisense) were treated with or without TPA for 1 hr, then immuno-stained with anti-RXR antibody followed by Cy3-conjugated secondary antibody (Sigma) to detect RXR.

Interaction of TR3 with RXR. To determine how RXR is involved in regulating TR3 nuclear export and mitochondrial targeting, we investigated interaction of RXR with TR3 and the regulation of TR3 nuclear export by RXR mutants. Figure 8 showed that TR3 could strongly interact with RXR, revealed by the GST-pull-down assay. Interestingly, deletion of the C-terminal end of RXR did not affect its interaction with TR3, while the same deletion largely abolished its interaction with RAR. This data suggests that TR3 interacts with RXR in a manner that is different from the interaction of RAR and RXR. The significance of this different heterodimerization in the regulation of nuclear receptor export is currently under investigation.

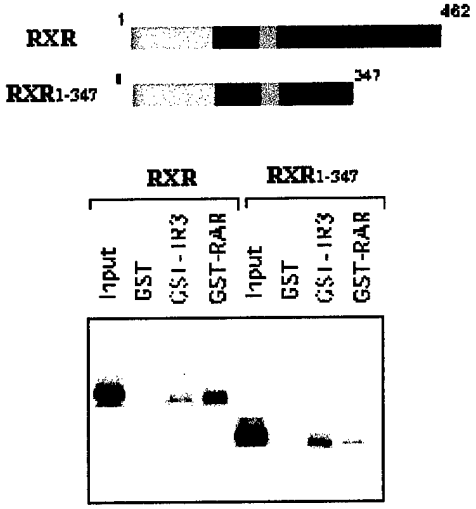

Figure 8. Heterodimerization between TR3 and RXR. RXR and its C-terminal deletion mutant, RXR1-347, were labeled by ^{35}S-methinion using in vitro transcription/translation. Labeled RXR proteins were subjected to analysis for their interaction with TR3 or RAR using the GST-pull-down assay. For control, the GST protein was used.

Identification of RXR nuclear export sequence. To provide more direct evidence that RXR plays a critical role in the regulation of TR3 nuclear export, a TR3 mutant deleted with the C-terminal end, TR31-467, was analyzed for its subcellular localization when it was co-transfected into 293T cells with RXR mutants that are capable of interacting with TR3 (Figure 8). When TR31-467 was co-expressed with RXR1-416, it was found exclusively in the cytoplasm. However, when it was co-expressed with RXR1-347, it was only in the nucleus (Figure 9). These data, therefore, demonstrate that subcellular localization of TR3 is highly regulated by RXR.

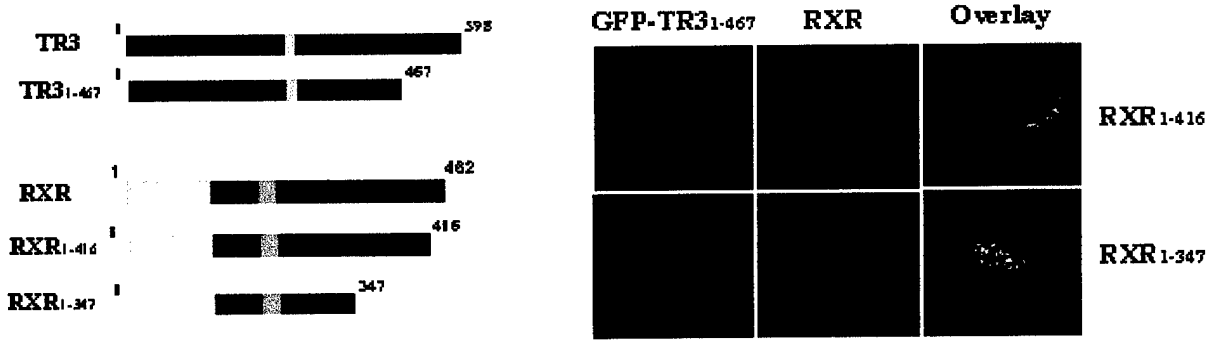

Figure 9. Effect of RXR mutations on TR3 subcellular localization. The TR3 mutant, TR31-467, was co-transfected into 293T cells together with the indicated RXR mutants and

the subcellular localization of TR3 and RXR mutants was determined by confocal microscopy analysis.

The above data suggest that RXR is actively involved in nuclear export of TR3 through their heterodimerization. To further study the nuclear export function of RXR, a number of RXR mutants were constructed and analyzed for their subcellular location in 293T cells (Figure 10). Our confocal microscopy analysis of these mutants showed that a small region in the ligand-binding domain of RXR was localized exclusively in the cytoplasm.

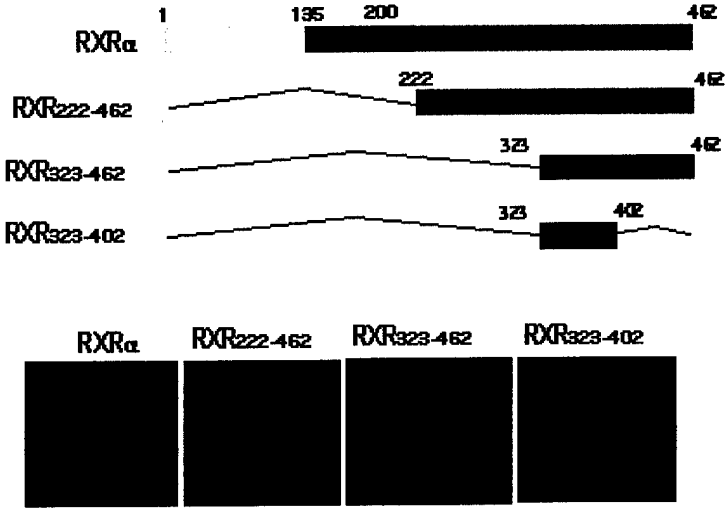

Figure 10. Mutational analysis of RXR mutants for their subcellular localization. RXR mutants fused with GFP were transfected into 293T cells and their subcellular localization was determined by confocal microscopy analysis.

The observation that GFP-RXR323-402 was constitutively localized in the cytoplasm indicated that sequences required for RXR nuclear export are located within this mutant. To determine whether this region contains a classical leucine-rich nuclear export sequence, GFP-RXR348-419 was transfected into 293T cells in the absence or presence of leptomycin B (LMB), which specifically inhibits formation of a complex consisting of CRM1, RanGTP and NES-containing proteins. Figure 11 showed that treatment of cells with LMB completely blocked cytoplasmic localization of RXR349-419, suggesting that nuclear export of RXR are regulated in a CRM1 dependent mechanism. We then cloned a Leucine-rich sequence in the region into GFP-containing vector, and the resulting GFP-fusion (RXR/NES) exclusively resided in the cytoplasm, while a similar fusion with a Leu replaced with Ala was diffusely distributed in cells. Together, these studies demonstrate that RXR contains a Leu-rich nuclear export sequence and that the sequence may be required for nuclear export of TR3 through TR3/RXR heterodimerization.

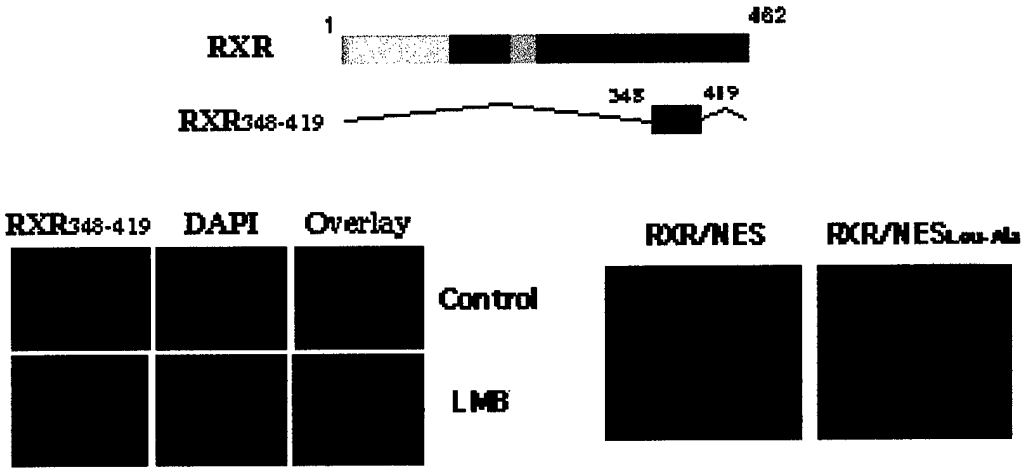

Figure 11. Identification of a nuclear export sequence in RXR. A fragment of RXR from a 348 to 419 or a leucine-rich sequence in the region was cloned in a GFP-containing vector and the resulting fusions, RXR348-419 and RXR/NES, respectively, were transfected into 293T cells and analyzed for their subcellular distribution by confocal microscopy analysis. For comparison a Leu in the leucine-rich sequence was replaced with Ala and the resulting mutant RXR/NES/Leu-Ala was also analyzed.

Effect of retinoids on RXR subcellular localization. To determine the effect of RXR ligands on nuclear export of RXR, we evaluated the cellular localization of RXR222-462 (Figure 10) in response to various RXR ligands (Figure 12). RXR222-462 in 293T cells was exclusively localized in the cytoplasm (Figure 12). However, when cells were treated with RXR ligands 9-cis RA, SR11237, and SR11345, RXR222-462 was localized in the nucleus. Treatment with RAR ligands all-trans-RA or AM80 did not affect the cytoplasmic localization of RXR222-462. Thus, RXR subcellular localization is regulated by its ligands.

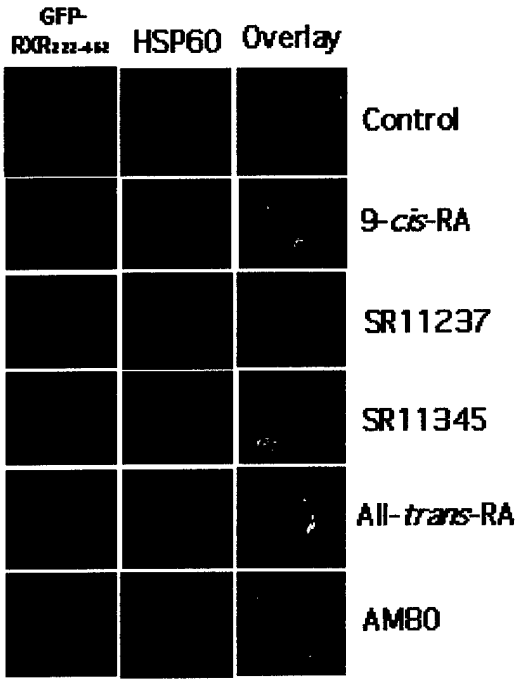

Figure 12. Effect of RXR ligands on RXR cytoplasmic localization. GFP-RXR222-462 was transfected into 293T cells and the transfected cells were then treated with the indicated retinoids. The cellular localization of RXR222-462 was analyzed by confocal microscopy.

Together, our studies demonstrate that RXR is required for TR3 nuclear export and that RXR and its ligands may play a role in regulating AHPN/CD437-induced TR3 mitochondrial targeting and apoptosis in breast cancer cells.

2. Mitochondrial receptor of TR3

Members of the Bcl-2 family are important regulators of cell death and survival (12,13). Many of which, such as Bcl-2, are located predominantly in the outer mitochondrial membrane

(12,13). We investigated the possibility that TR3 targeted mitochondria by interacting with Bcl-2 that is known to reside on the outer membrane of mitochondria. TR3/ΔDBD, which constitutively resides on mitochondria (6), was analyzed in a co-immunoprecipitation (Co-IP) assay for its interaction with Bcl-2 by transfecting it into human embryonic kidney cell line 293T alone or with Bcl-2 expression vector. Co-IP assay showed that a significant amount of TR3/ΔDBD was co-precipitated with Bcl-2 by anti-Bcl-2 antibody (Figure 13a). In a reporter gene assay in CV-1 cells, transactivation of TR3 on its responsive element (NurRE-tk-CAT) was potently inhibited by cotransfection of Bcl-2, but not by Bax (Figure 13b). The TR3/ΔDBD and Bcl-2 interaction was also demonstrated by the GST-pull down assay, showing that ^{35}S-labeled Bcl-2 was pulled down by GST-TR3 but not by GST (Figure 13c). Transfected Bcl-2 and TR3/ΔDBD colocalized in LNCaP cells as revealed by confocal microscopy analysis (Figure 13d). Thus, TR3 interacts specifically with Bcl-2 and Bcl-2 may function as a receptor for TR3 to target mitochondria.

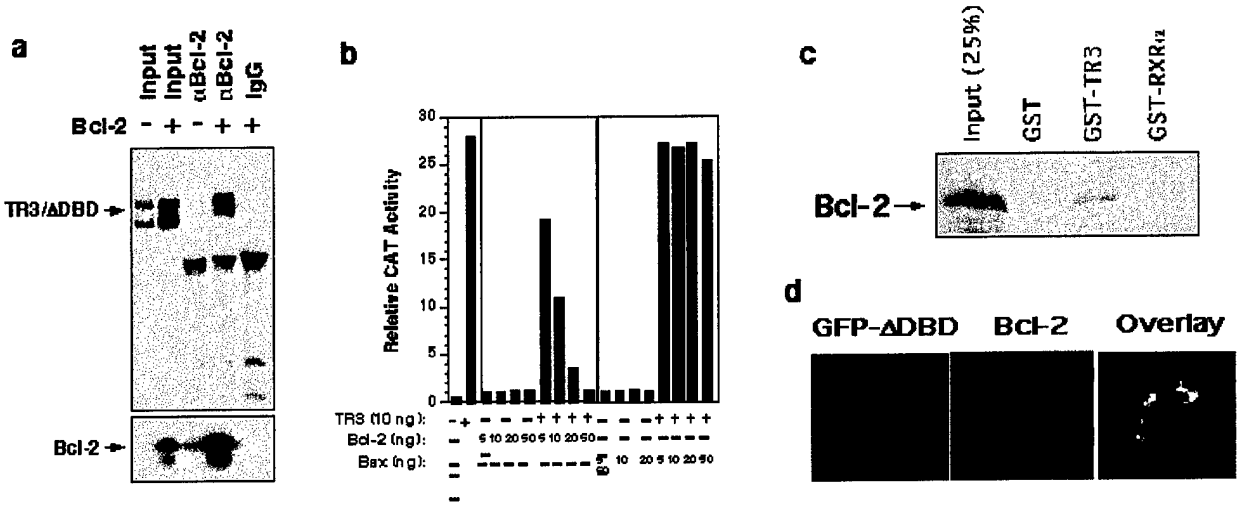

Figure 13. Interaction of TR3 with Bcl-2. a. *In vivo* Co-IP assay. GFP-TR3/ΔDBD was cotransfected alone or with Bcl-2 expression vector into 293T cells. The expressed GFP-TR3/ΔDBD mutant protein was then precipitated by using either anti-Bcl-2 antibody or control IgG and detected by western blotting using anti-GFP antibody. The same membranes were also blotted with anti-Bcl-2 antibody to determine precipitation specificity and efficiency. Input represents 10% of total cell extract used in the precipitation assays. **b.** Inhibition of TR3-dependent transactivation by Bcl-2. CV-1 cells were transfected with the NurRE-*tk*-CAT (6) with or without TR3 expression vector (25 ng) together with or without the indicated amount of Bcl-2 or Bax expression vector. CAT activity was then determined. **c.** GST-pull down of Bcl-2 by TR3. GST-TR3, GST or GST-RXR immobilized on 20 μl of glutathione-Sepharose was incubated with 10 μl of *in vitro* synthesized ^{35}S-labeled Bcl-2. Bound proteins were analyzed by SDS-PAGE autoradiography. **d.** Confocal microscopy analysis. Expression vectors for GFP–TR3/ΔDBD and Bcl-2 were cotransfected into LNCaP cells. After 20 h, cells were immunostained with anti-Bcl-2 antibody then Cy3-conjugated secondary antibodies (Sigma). GFP–fusion and Bcl-2 were visualized using confocal microscopy.

Bcl-2 is required for apoptotic effect of TR3 in breast cancer cells. The above data suggest that Bcl-2 is required for mitochondrial targeting of TR3 through their physical interaction. To determine whether Bcl-2 was required for apoptotic effect of TR3 in breast cancer cells. TR3/ΔDBD was cotransfected with or without Bcl-2 expression vector into MCF-7 breast cancer cell. Cells were then stained by DAPI for their nuclear morphology. DAPI staining indicated that TR3/ΔDBD only caused nuclear fragmentation or condensation of MCF-7 cells when

Bcl-2 was cotransfected (Figure 14). Thus, the TR3/ΔDBD-Bcl-2 interaction is required to induce apoptosis of breast cancer cells.

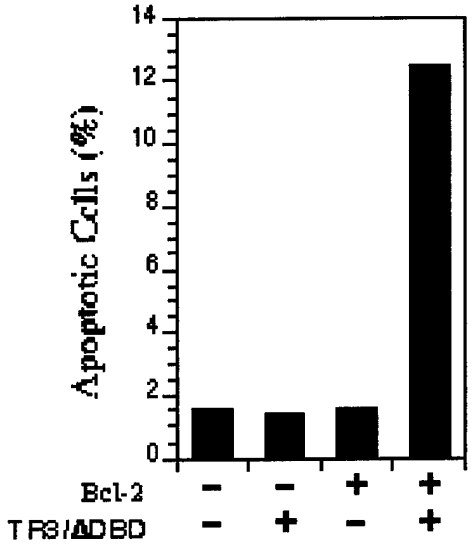

Figure 14. Bcl-2 is required for apoptotic effect of TR3/ΔDBD in MCF-7 cells. MCF-7 cells transfected with GFP-TR3/ΔDBD together with or without Bcl-2 expression vector. Transfected cells were stained by DAPI. Apoptotic cells were then scored.

REPORTABLE OUTCOMES

Dawson, M.I., Hobbs, P., Peterson, V., Leid, M., Lange, C., Feng, K., Chen, G., Gu, J., Li, H., Kolluri, S., Zhang, X-k., Zhang, Y., and Fontana, J. Induction of apoptosis in cancer cells by a novel analog of 6-[3- (1-Adamantyl) –4- hydroxyphenyl} –2- naphthalenecarboxylic acid (AHPN) lacking retinoid receptor transcriptional activation activity. Cancer Research. 61:4723-4730, 2001.

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Zhang, X-k., Kolluri, S., Cao, X., Li, H., Lin, B., and Dawson, M.I. Subcellular Localization of Orphan Receptor TR3 Defines its Biological Activities. The FASEB Summer Conference on Retinoids. Tucson, Arizona, June 22-27, 2002.

CONCLUSIONS

We have investigated the mechanism of TR3-mediated apoptosis in breast cancer cells. Our results demonstrate that TR3 exerts its apoptotic effect in breast cancer by translocating from the nucleus to mitochondria in response to apoptotic retinoids. We have also identified a new apoptotic retinoid MM002 that effectively induces TR3 mitochondrial targeting and apoptosis of breast cancer cells. In studying the mechanism by which TR3 migrates from the nucleus to the cytoplasm, we found it requires RXR through their heterodimerization. We also observed that RXR contains a nuclear export sequence that is required for its cytoplasmic localization and is

regulated by RXR ligands. These data not only enhance our understanding of the molecular mechanism of TR3 nuclear export but also provide important information for developing RXR ligands for regulating apoptosis of breast cancer cells. Furthermore, we discovered that TR3 can physically interacts with Bcl-2 and that Bcl-2 acts as a mitochondrial receptor of TR3 and is required for apoptotic effect of TR3 in breast cancer cells. Our finding that Bcl-2 is apoptotic when it interacts with TR3 provides novel approach to induce apoptosis of Bcl-2-expressing breast cancer cells. The overall plan for the next year of research remains the same as proposed in the original grant application.

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APPENDICES

1. Dawson, M.I., Hobbs, P., Peterson, V., Leid, M., Lange, C., Feng, K., Chen, G., Gu, J., Li, H., Kolluri, S., Zhang, X-k., Zhang, Y., and Fontana, J. Apoptosis induction in cancer cells by a novel analogue of 6-[3- (1-Adamantyl) –4- hydroxyphenyl} –2- naphthalenecarboxylic acid (AHPN) lacking retinoid receptor transcriptional activation activity. Cancer Research. 61:4723-4730, 2001.

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Apoptosis Induction in Cancer Cells by a Novel Analogue of 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic Acid Lacking Retinoid Receptor Transcriptional Activation Activity[1]

Marcia I. Dawson,[2] Peter D. Hobbs, Valerie J. Peterson, Mark Leid, Christopher W. Lange, Kai-Chia Feng, Guo-quan Chen, Jian Gu, Hui Li, Siva Kumar Kolluri, Xiao-kun Zhang, Yuxiang Zhang, and Joseph A. Fontana

Department of Medicinal Chemistry, Molecular Medicine Research Institute, Mountain View, California 94043 [M. I. D., C. W. L., K-C. F., G-q. C.]; Retinoid Program, SRI, Menlo Park, California 94025 [P. D. H.]; Laboratory of Molecular Pharmacology, Oregon State University School of Pharmacy, Corvallis, Oregon 97331 [V. J. P., M. L.]; The Burnham Institute, La Jolla, California 92037 [J. G., H. L., S. K. K., X-k. Z.]; and John D. Dingell Veterans Affairs Medical Center and Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201 [Y. Z., J. A. F.]

ABSTRACT

The retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (AHPN) is reported to have anticancer activity *in vivo*. Induction of cell cycle arrest and apoptosis in cancer cell lines refractory to standard retinoids suggests a retinoid-independent mechanism of action for AHPN. Conformational studies suggested that binding of AHPN does not induce an unusual conformation in retinoic acid receptor (RAR) γ. The 3-chloro AHPN analogue MM11453 inhibited the growth of both retinoid-resistant (HL-60R leukemia, MDA-MB-231 breast, and H292 lung) and retinoid-sensitive (MCF-7 breast, LNCaP prostate, and H460 lung) cancer cell lines by inducing apoptosis at similar concentrations. Before apoptosis, MM11453 induced transcription factor TR3 expression and loss of mitochondrial membrane potential characteristic of apoptosis. MM11453 lacked the ability to significantly activate RARs and retinoid X receptor α to initiate $(TREpal)_2$-*tk*-CAT reporter transcription. These results, differential proteolysis-sensitivity assays, and glutathione *S*-transferase-pulldown experiments demonstrate that, unlike AHPN or the natural or standard synthetic retinoids, MM11453 does not behave as a RAR or retinoid X receptor α transcriptional agonist. These studies strongly suggest that AHPN exerts its cell cycle arrest and apoptotic activity by a signaling pathway independent of retinoid receptor activation.

INTRODUCTION

The natural RAs[3] and their synthetic analogues are being investigated as chemotherapeutic agents because they inhibit proliferation, induce apoptosis in cancer cells, and retard tumor xenograft growth (1). These standard retinoids exert their antiproliferative effects by influencing the transcriptional activity of RAR and RXR subtypes α, β, and γ (reviewed in Ref. 2). Retinoids complexed to a RXR/RAR can activate or repress gene transcription from RA response elements in the promoter of retinoid-sensitive genes. A retinoid bound to an RXR can modulate activation by other transcription factors with which it dimerizes (2). Retinoid receptor-ligand complexes also compete with other transcription factors for coactivator proteins (3, 4), whereas nonliganded dimers compete for corepressors (5).

The diversity from the six subtypes and variations in their expression patterns (2, 6–9), response element sequences, intermediary proteins, and other transcription factors (2) led to the identification of receptor-selective retinoids to enhance efficacy by reducing the systemic toxicity associated with retinoids activating all receptors (10). Receptor class and subtype-selective compounds (reviewed in Refs. 1 and 11) also provide a means for studying individual receptor-signaling pathways.

On evaluating RARγ-selective retinoids, we observed that AHPN (CD437 [1] in Fig. 1; Ref. 12) rapidly caused detachment of retinoid-sensitive MCF-7 breast and NIH:OVCAR-3 ovarian cancer cells (13, 14). This atypical retinoid activity extended to retinoid-resistant lines, including MDA-MB-231 breast cancer and HL-60R leukemia (13). AHPN induced cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ expression (13), G_0-G_1 cell cycle arrest (13), and apoptotic events, such as caspase activation, *gadd45* expression (15), poly(adenosyl diphosphate-ribose) polymerase cleavage, and DNA fragmentation (13). Interestingly, apoptosis occurred in the absence of functional tumor suppressor *p53* (13), the gene for which is mutated in many cancers (16). Apoptosis by AHPN and its derivatives and analogues was subsequently observed in other lines derived from tumors and their metastases (17–25).

The efficacy against retinoid-resistant cancer cells prompted studies on how AHPN induces apoptosis. To reduce complications, we conducted mechanistic studies in cells lacking functional retinoid receptors (26) and used apoptotic AHPN analogues lacking retinoid agonist transactivation activity, such as MM11453 [2]. MM11453 induced apoptosis by a cascade that included mitochondrial translocation of transcription factor TR3/nur77/NGFIB-II (TR3), cytochrome *c* release, caspase activation, and DNA fragmentation (27). Binding of MM11453 to RARs and RXRα did not cause the conformational changes of AHPN that led to corepressor loss and coactivator recruitment. We report here the characterization and anticancer activity of MM11453, the prototype for new nonretinoidal apoptotic agents with potential for cancer treatment.

MATERIALS AND METHODS

Retinoids. AHPN [1] was prepared by modifying a reported procedure (28). AHPN (MM11453) [2] was synthesized as follows. The biaryl bond was introduced by palladium(0)-catalyzed coupling between 3-(1-adamantyl)-4-benzyloxybenzeneboronic acid and ethyl 6-bromo-3-chloro-2-naphthalenecarboxylate [palladium(triphenylphosphine)$_4$ (Aldrich, St. Louis, MO), aqNa_2CO_3, dimethoxyethane, reflux, 6 h], followed by chromatography (6% EtOAc/hexane on silica gel) to give the benzyl-protected ethyl ester of MM11453 (66%). Benzyl group cleavage [BBr_3, CH_2Cl_2, −78°C, 2 h] to the phenol (91%) and ester group hydrolysis (aq NaOH, ethanol, 90°C, 2 h; aq HCl) gave MM11453 (95%) as a white powder, melting point 294°C–296°C (decomp.). IR (KBr): 3200, 1706, 1277, 1244, 991, 815, and 680 cm^{-1}. ^1H nuclear magnetic resonance (300 MHz, Me_2SO-d_6, δ): 1.75, 2.06, 2.17 (s, 6, adamantyl CH_2; s, 3, adamantyl CH;

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[2] To whom requests for reprints should be addressed, at The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. Phone: (858) 646-3165; Fax: (858) 646-3195; E-mail: mdawson@burnham.org.

[3] The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; AHPN, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid; aq, aqueous; GST, glutathione *S*-transferase; *tk*, thymidine kinase; CAT, chloramphenicol acetyltransferase; DPSA, differential protease sensitivity assay; TRE, thyroid hormone receptor response element; TREpal, palindromic TRE; PF, protease-resistant fragment; Hsp, heat shock protein; LBD, ligand-binding domain; Met, methionine; NCoR, nuclear receptor corepressor; Rh123, rhodamine green; TTAB, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid.

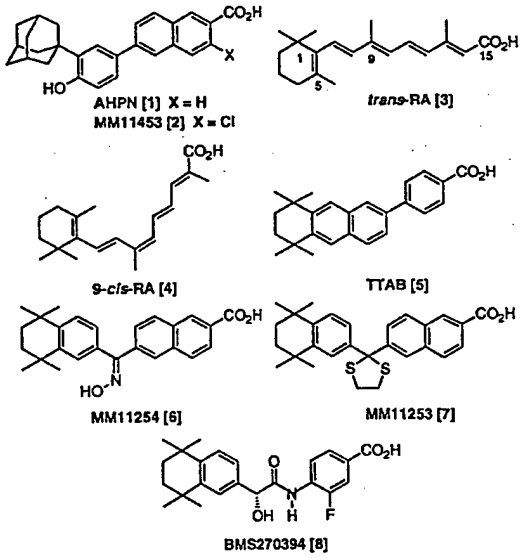

Fig. 1. AHPN [1], MM11453 [2], *trans*-RA [3], 9-*cis*-RA [4], TTAB [5], MM11254 [6], MM11253 [7], and BMS270394 [8].

s, 6, adamantyl CH_2), 6.97 (d, J = 9.0 Hz, 1, ArH-5), 7.51 (s, 1, ArH-2), 7.52 (d, J = 9.0 Hz, 1, ArH-6), 7.98 (d, J = 9.0 Hz, 1, NapH-8), 8.06 (s, 1, NapH-5), 8.25 (d, J = 8.6 Hz, 1, NapH-7), 8.27 (s, 1, NapH-4), 8.60 (s, 1, NapH-1), 9.68 (s, 1, ArOH). High-resolution mass spectrometry for $C_{27}H_{25}ClO_3$ (M^+): calculated, 432.1492; found, 432.1492. *trans*-RA [3] was purchased (Sigma Chemical Co.), as was [11,12-$^3H)_2$]9-*cis*-RA (specific activity, 43 Ci/mmol; DuPont NEN, Boston, MA). 9-*cis*-RA [4] was prepared as reported (29).

Computational Analysis. CAChe Software (Fujitsu, Beaverton, OR) was used to identify low-energy conformers within 2 kcal of the global energy minimum (MM3 force field, conjugate-gradient minimization, 30° search label variation, exclusion of ≥9 Å van der Waals interactions, and energy change <0.001 kcal/mol). Conformers were superimposed by using least-squares rigid fit of atoms corresponding to the 1, 5–9, and 15 carbon molecules of *trans*-RA.

Receptor Transcriptional Activation.[4] CV-1 cells (1,000 per well) were grown in DMEM (Irving Scientific, Santa Ana, CA) with 10% charcoal-treated FCS (Tissue Culture Biologicals, Tulare, CA) for 16–24 h before transfection, as described (30, 31). Briefly, 100 ng of $(TREpal)_2$-*tk*-CAT reporter, β-galactosidase expression vector pCH 110 (Pharmacia, Piscataway, NJ), and a RAR expression vector (or 20 ng of RXRα) were mixed with carrier DNA (pBluescript; Stratagene, La Jolla, CA) to give 1 μg of total DNA/well. CAT activity was normalized using β-galactosidase as the control. Activation after subtraction of constitutive activity is expressed relative to that of 1.0 μM *trans*-RA for RARs (100%) or 1.0 μM 9-*cis*-RA for RXRα (100%) and represents the average of three determinations.

Receptor Binding. Competitive radioligand binding on crude bacterial lysates at 0°C for 2 h used ~25 μmol of recombinant human RAR subtype or mouse RXRα-GST fusion proteins in 200 μl of binding buffer [10 mM HEPES (Sigma Chemical Co.; pH 7.8), 100 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, and 10% glycerol] with 1–2 nM [$^3H_2$]9-*cis*-RA (43 Ci/mmol). Bound [^{3}H]9-*cis*-RA was isolated (Sephadex G-50; Pharmacia) and counted. Nonspecific [^{3}H]9-*cis*-RA binding at 1 μM nonlabeled 9-*cis*-RA generally was <10% of total label bound.

DPSA. [^{35}S]Met-labeled RARα, RARβ, RARγ, and RXRα, prepared by *in vitro* transcription/translation (32), were used in DPSA as described (33). [^{35}S]Met-labeled receptors were incubated with 0.1% ethanol alone, 1.0 μM 9-*cis*-RA, or MM11453 for 30 min at 0°C. Limited proteolysis (trypsin-tosyl phenylalanyl chloromethyl ketone; Sigma Chemical Co.) for 15 min at 22°C, followed by termination by Laemmli sample buffer and boiling and separation (10% acrylamide gel under denaturing conditions), afforded PFs for visualization by autoradiography (33, 34).

GST-Pulldown. Experiments were performed as described using GST-p300 1–450 (35) and GST-NCoR 2110–2453 (36) fusion proteins and [^{35}S]Met-labeled human RARγ.

Cell Lines and Culture. RA-resistant HL-60R cells, having a mutant RARα that does not significantly bind *trans*-RA and lacking RARβ and RARγ (26), and MDA-MB-231 cells were grown as described (20). MCF-7, LNCaP prostate, H460 and retinoid-resistant H292 lung cancer cells and Jurkat lymphoma cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 (Irving Scientific) with 10% charcoal-treated FCS.

Cell Growth Inhibition. HL-60R and MDA-MB-231 cells (50,000 and 100,000 per well, respectively) and 0.1–1.0 μM MM11453, AHPN, or Me_2SO alone were incubated for 24 or 120 h (72-h medium change), respectively. Results are expressed relative to Me_2SO control as mean ± SE of triplicate experiments. SEs were <10%. MCF-7, LNCaP, H292, and H460 cells (3,000 per well in 96-well plates) were treated with 1.0 μM MM11453, AHPN, *trans*-RA, or ethanol alone for 48 h before viable cell numbers were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (7, 9, 27). Data shown are representative of three experiments.

Apoptosis Detection. DNA fragmentation and apoptotic bodies were assessed in at least 500 HL-60R or MDA-MB-231 cells after incubation with MM11453 for 24 or 120 h, respectively, as described above, and acridine orange staining (15). The percentage of apoptotic cells was expressed relative to the Me_2SO control as the mean ± SE of triplicate experiments. MCF-7, LNCaP, H292, H460, and Jurkat cells (3,000 per well) were treated with 1.0 μM MM11453, *trans*-RA, or ethanol alone for 48 h, trypsinized, washed (PBS), fixed (3.7% paraformaldehyde), and stained with 4′,6-diamidino-2-phenylindole (1 μg/ml) to visualize nuclei by fluorescent microscopy (21). Cells with apoptotic nuclear morphology were scored in each 400-cell sample using a fluorescence microscope. The data are representative of three experiments.

Northern Analysis. Total RNAs were prepared (RNeasy Mini kit; Qiagen, Germany), and TR3 expression was determined on 30 μg of total RNA from each line treated with 1.0 μM MM11453, *trans*-RA, or ethanol alone. Blotting conditions were as described (27) with β-actin expression as the control.

TR3 Mitochondrial Targeting. The expression vector for TR3/ΔDBD-GFP, a TR3 mutant lacking the DNA-binding domain fused to the green fluorescent protein expression vector, was transiently transfected into H460 cells, as described for LNCaP cells (27). Cells were treated with 1.0 μM MM11453 or ethanol alone for 6 h and then immunostained with anti-Hsp60 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Cy3-conjugated secondary antibody (Sigma Chemical Co.) to indicate mitochondria to which Hsp60 is restricted. Confocal microscopy was used to detect TR3/ΔDBD-GFP (green fluorescence) and Hsp60 (red). Images were overlaid to show colocalization.

Mitochondrial Membrane Potential Assay. LNCaP, MCF-7, and MDA-MB-231 cells (10,000,000) were treated with 1.0 μM MM11453 for 18 h before incubation with 5 μg/ml Rh123 for 30 min at 37°C. Rh123-fluorescing cells were scored depolarized by flow cytometry (FACScalibur system; BD Biosciences, San Jose, CA; Ref. 37). The data shown are representative of three experiments. Wild-type Jurkat cells or Jurkat cells stably expressing either Bcl-2 or control vector (38) were treated similarly.

RESULTS

Close-Fitting of Energy-minimized AHPN and Retinoid Conformers. Energy-minimized conformers of AHPN [1], RAR-selective *trans*-RA [3], and RAR-selective TTAB [5] (39) were overlapped. The *trans*-RA conformer was that reported in the RARγ LBD (40, 41). Three orthogonal views of these overlaps are shown in Fig. 2*A*. The major structural difference was the 1-adamantyl group of AHPN, which extended 2.2 Å more than the *trans*-RA 18-methyl group. In Fig. 2*B*, the energy-minimized conformers of RARγ-selective agonists AHPN and MM11254 [6], a (*Z*)-oxime (14) of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl-carbonyl)-2-naphthalenecarboxylic acid (42), are shown overlapped with RARγ-selective agonist BMS270394 [8], as found in the ligand-binding pocket of crystallized holo-RARγ (40). The

[4] Assay conducted at The Burnham Institute, La Jolla, CA, under a license agreement with Ligand Pharmaceuticals, San Diego, CA.

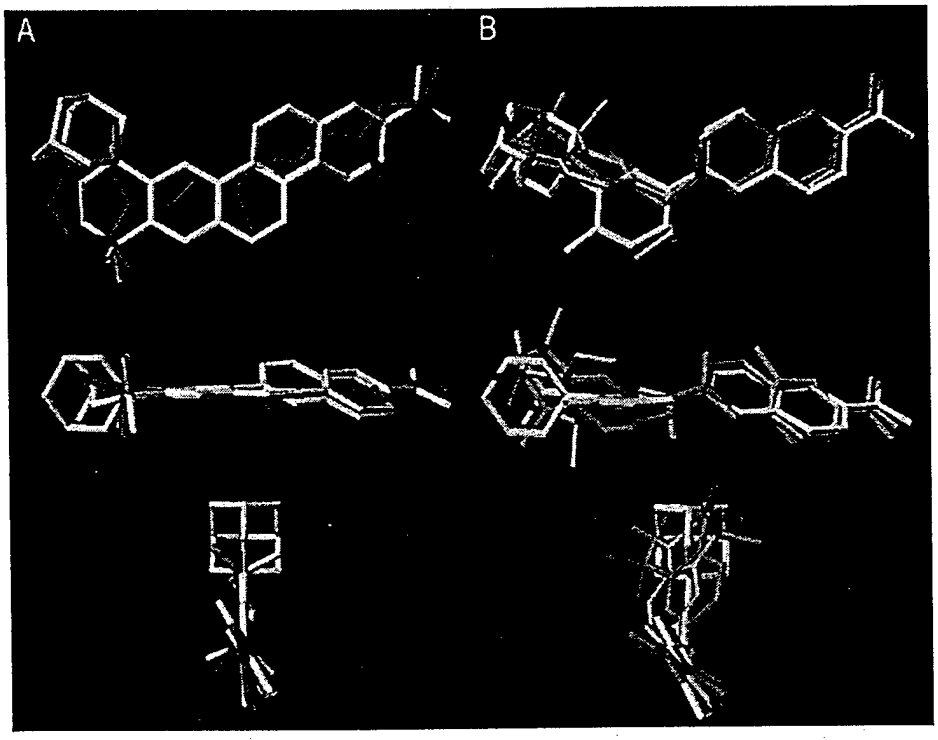

Fig. 2. Comparison of energy-minimized AHPN and retinoid conformers. Conformational analysis was performed as described in "Materials and Methods." *A*, orthogonal views of superimposed conformers of AHPN (*blue*), *trans*-RA (*red*), and TTAB (*yellow*). *B*, superimposed conformers of AHPN (*blue*), MM11254 (*green*), and BMS270394 (*magenta*).

AHPN 1-adamantyl group overlaps the saturated portion of the 5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalene rings of both RARγ-selective retinoids, and the AHPN phenolic oxygen is near the oxygen molecules in the oxime group of MM11254 (2.5 Å) and the bridge hydroxyl of BSM270394 (3.9 Å). Such hydroxyl groups are reported to confer RARγ selectivity by hydrogen bonding to the Met-272 sulfur molecule of RARγ (41). Placement of these overlapped conformers (Fig. 2*B*) in the RARγ ligand-binding site gives ligand O–Met-272–S distances of 4.09, 2.55, and 3.32 Å, respectively. These studies suggest that binding of AHPN to RARγ occurs in the same manner as that of standard retinoid agonists.

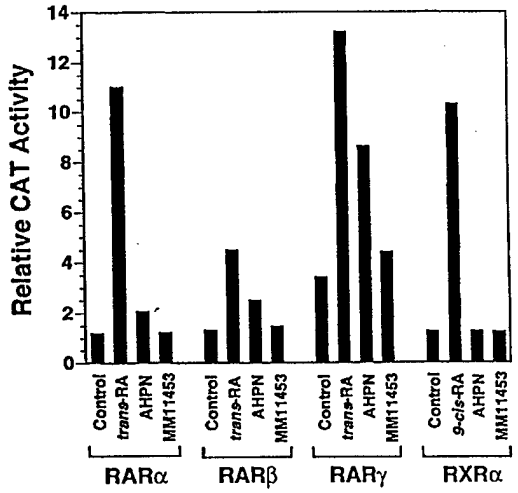

Fig. 3. Transcriptional activation of retinoid receptors by MM11453 on the $(TREpal)_2$-*tk*-CAT reporter. CV-1 cells were transiently transfected as described in "Materials and Methods," treated with 1.0 μM MM11453, AHPN, *trans*-RA, or 9-*cis*-RA, and assayed for CAT activity after 24 h. Reporter gene activation is expressed relative to 1.0 μM *trans*-RA on the RARs or 1.0 μM 9-*cis*-RA on RXRα.

MM11453 Lacked RAR Transcriptional Activation of AHPN. Although originally reported as RARγ selective on the $(TREpal)_2$-*tk*-CAT reporter in cotransfected HeLa cells (12), we observed on the $(TREpal)_2$-*tk*-CAT in CV-1 cells (4) that high RARγ selectivity occurred at 0.1 μM and below (14). At 0.5–1.0 μM, at which the natural retinoid *trans*-RA [3] inhibits retinoid-sensitive cancer cells, AHPN significantly activated RARβ. At 1.0 μM AHPN, reporter activation by RARα, RARβ, and RARγ was 9, 37, and 54%, respectively, of that caused by 1.0 μM *trans*-RA (Fig. 3). Unlike 1.0 μM *trans*-RA or 9-*cis*-RA, 1.0 μM MM11453 did not adequately activate any RAR subtype or RXRα to induce even modest $(TREpal)_2$-*tk*-CAT transcription. MM11453 did not activate RARα or RXRα and only activated RARβ and RARγ to 5 and 10%, respectively, of that of *trans*-RA or 13 and 19%, respectively, of that of AHPN. Thus, MM11453 is an analogue with substantially reduced capacity for RAR activation.

Retinoid Receptors Bound MM11453. Competitive ligand binding was used to determine whether MM11453 bound directly to RARs and RXRα. MM11453 at 1.0 μM displaced 61 ± 6% of $[^3H_2]$9-*cis*-RA bound to RARγ, whereas displacement from other receptors was lower [RARα (11 ± 2%), RARβ (25 ± 5%), and RXRα (18 ± 5%); Fig. 4].

MM11453 Did Not Induce an Agonist-bound RAR Conformation. DPSA on 9-*cis*-RA-bound RARα, RARβ, and RARγ produced 27-kDa PF27α, 35-kDa PF35β, and 32-kDa PF32γ, respectively (*Lane 3* in Fig. 5, *A–C*). DPSA on AHPN-bound RARs produced the same fragments (data not shown). These PFs were not observed on incubation with ethanol or MM11453 (*Lanes 2* and *4*, respectively, in Fig. 5, *A–C*). Unlike 9-*cis*-RA, neither MM11453 nor AHPN altered the proteolytic sensitivity of RXRα (data not shown). The lack of PFs from RAR-MM11453 complexes suggests that MM11453 does not

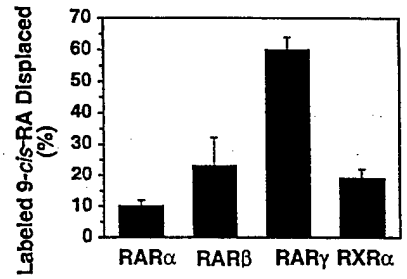

Fig. 4. Binding affinity of MM11453 to recombinant RAR and RXRα. Competition radioligand binding was conducted as described in "Materials and Methods." The data represent the means (n = 3) of the percentages of [11,12-$^3H_2$]9-*cis*-RA bound that were inhibited by 1.0 μM MM11453; *bars*, SE.

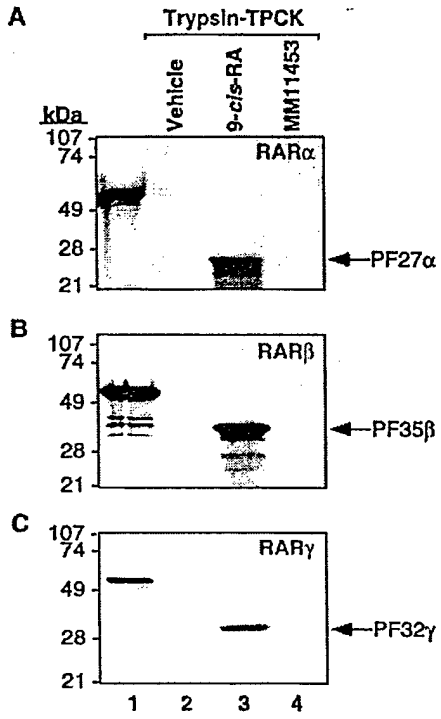

Fig. 5. MM11453 is not a RAR agonist. *A*, DPSA on [^{35}S]Met-labeled RARα in ethanol or 1 μM 9-*cis*-RA or MM11453. The migration of the 9-*cis*-RA-induced PF27α of RARα is indicated. In *B* and *C*, DPSA on RARβ and RARγ, respectively, were conducted as in *A* and "Materials and Methods." Positions of RARβ PF35β and RARγ PF35β are indicated. *Left*, marker migration (molecular mass).

promote an agonist-bound conformation. Similar to RARγ-selective antagonist MM11253,[5] MM11453 did not prevent RAR/RXR agonist 9-*cis*-RA from inducing this conformation in RARα, RARβ, or RARγ (data not shown).

MM11453 Failed to Dissociate Corepressor NCoR-RARγ *in Vitro*. GST-pulldown was used to test whether MM11453 dissociated NCoR (5) from RARγ, as 9-*cis*-RA does. As indicated (Fig. 6*A*), 9-*cis*-RA (*Lane 3*), but not MM11453 (*Lane 4*) or vehicle (*Lane 2*), disrupted the NCoR-RARγ complex.

MM11453 Failed to Recruit Coactivator p300 to RARγ. We compared the abilities of MM11453 and 9-*cis*-RA (36) to recruit p300 (43) to RARγ. Vehicle (*Lane 2* in Fig. 6*B*) or MM11453 (*Lane 4*) did not enhance p300 recruitment, whereas 9-*cis*-RA did (*Lane 3*). These findings, which agree with results on MM11453 in RARγ DPSAs (Fig. 5) and corepressor-dissociation experiments (Fig. 6*A*), confirm that MM11453 does not induce a RARγ-agonist conformation.

MM11453 Inhibited Cancer Cell Growth. Increasing evidence, including retinoid-resistant cancer cell growth inhibition (13, 27), suggests that AHPN action is independent of retinoid receptors (21, 44). Cell counting and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were conducted to show that MM11453 inhibited growth similarly. MM11453 inhibited HL-60R and MDA-MB-231 growth with IC_{50}s of 0.17 and 0.32 μM (Fig. 7, *A* and *B*), respectively, compared with AHPN values of 0.15 and 0.30 μM, respectively. Inhibition by 1.0 μM *trans*-RA was ≤5% (13). The effects of MM11453 on H460, H292, LNCaP, and Jurkat cells were then examined. As shown (Fig. 7*C*), 1.0 μM MM11453 significantly reduced growth by 70, 46, 64, and 70%, respectively, whereas 1.0 μM *trans*-RA reduced H460 growth by 15% and had no evident effect on the other lines (0–3%). Some of us reported previously that 1.0 μM AHPN for 48 h inhibited the growth of H460, H292, and LNCaP cells by 62 ± 6% (21), 53 ± 5% (21), and 100 ± 5% (25), respectively, whereas Jurkat growth was inhibited by 84% and 80 ± 3% after 24 and 96 h, respectively (22). Thus, both AHPN and MM11453 similarly retard the growth of these cell lines.

MM11453 Induced Cancer Cell Apoptosis. The MM11453 EC_{50}s for inducing nuclear fragmentation in HL-60R and MDA-MB-231 cells were 0.12 and 0.13 μM, respectively (Fig. 7, *D* and *E*), which are similar to AHPN EC_{50}s of 0.07 and 0.35 μM, respectively (13). HL-60R apoptosis inhibition by 1.0 μM MM11453 and AHPN was 82 ± 3% and 91 ± 4%, respectively, and MDA-MB-231 apoptosis

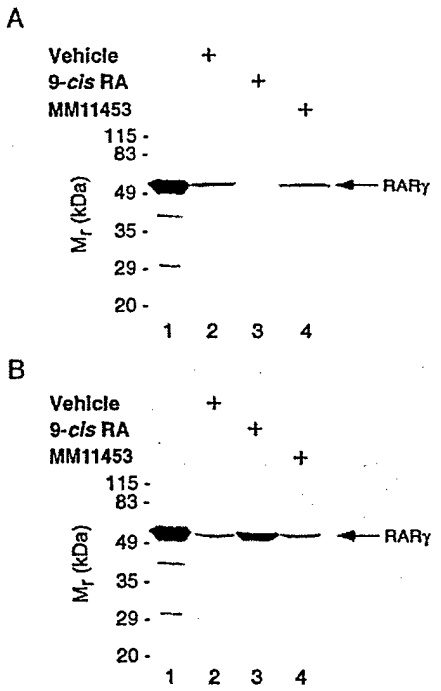

Fig. 6. MM11453 does not induce NCoR corepressor dissociation from RARγ or coactivator p300 recruitment to RARγ. *A*, NCoR-RARγ dissociation using [^{35}S]Met-RARγ and GST-NCoR 2110–2453 is as described in "Materials and Methods." Only 9-*cis*-RA induced NCoR-RARγ dissociation (*Lane 3*). In *B*, RARγ coactivator recruitment using GST-p300 (1–450) and [^{35}S]Met-RARγ is as in "Materials and Methods." Only 9-*cis*-RA enhanced binding of p300 to RARγ. *Lane 1* in *A* and *B* represents ~15% of [^{35}S]Met-RARγ. *Left*, marker migration (molecular mass).

[5] V. J. Peterson, M. I. Deinzer, M. I. Dawson, K-C. Feng, A. Fields, and M. Leid. Mass spectrometric analysis of agonist-induced retinoic acid receptor γ conformational change, unpublished results.

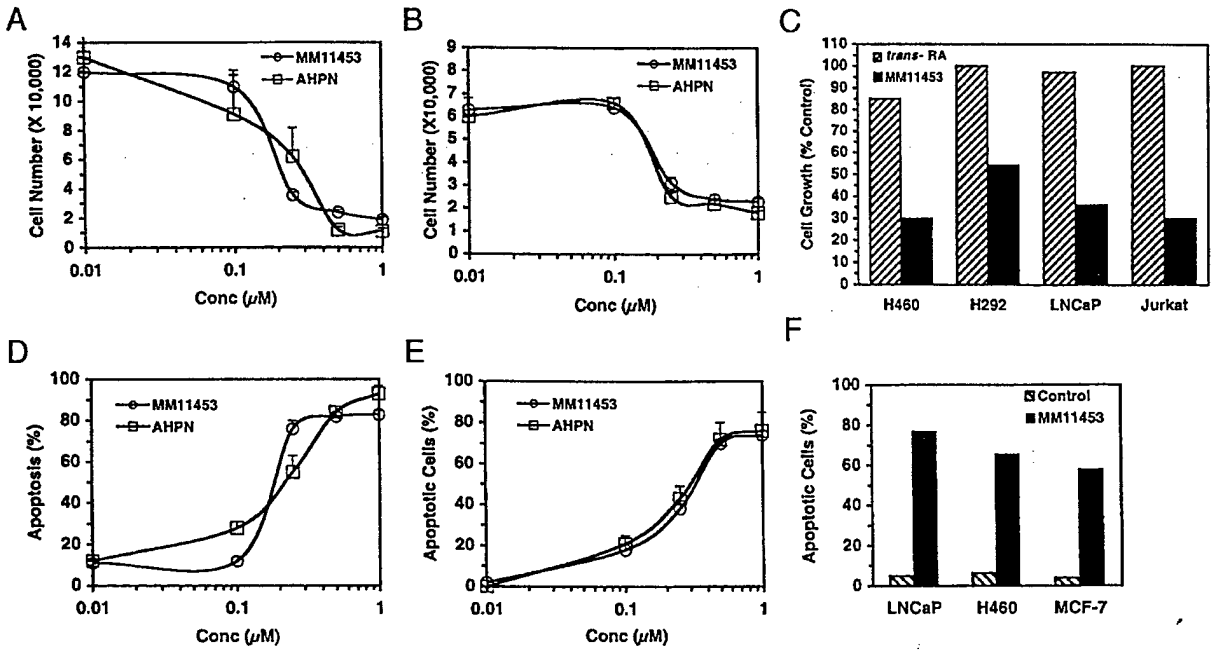

Fig. 7. MM11453 inhibits cell growth and induces apoptosis. HL-60R cells (*A* and *D*) and MDA-MB-231 cells (*B* and *E*) were treated with 10 nM to 1.0 μM MM11453, AHPN, or Me_2SO alone for 24 h and 120 h, respectively, as described in "Materials and Methods," and then harvested and counted (*A* and *B*) or assayed for apoptosis (*D* and *E*) as in "Materials and Methods." The results shown represent the means of three replicates; *bars*, SE. In *C* and *F*, H460, H292, LNCaP, and Jurkat cells were treated with ethanol alone, 1.0 μM *trans*-RA, or MM11453 for 48 h before viability was determined (*C*) or treated with 1.0 μM MM11453 or ethanol alone for 48 h before nuclear morphology was analyzed (*F*) as in "Materials and Methods." The experiments shown are representative of three triplicates.

was 75 ± 1% and 76 ± 7%, respectively. Thus, both MM11453 and AHPN are similarly apoptotic in retinoid-resistant cells. MM11453 at 1.0 μM induced apoptosis in LNCaP (38%), H460 (47%), and MCF-7 (57%) cells, as demonstrated by nuclear morphological changes (Fig. 7*F*). In other experiments using these cells, 1.0 μM AHPN was found to induce 21 (21), 37, and 42% apoptosis, respectively (data not shown). Thus, both MM11453 and AHPN also induce apoptosis in retinoid-sensitive cells.

MM11453 Induced TR3 Expression. TR3 expression must be induced for AHPN to cause lung cancer cell apoptosis (21). To determine whether 1.0 μM MM11453 had this capability, H460 and LNCaP cells were treated for 6 h. MM11453 strongly induced TR3 expression, whereas *trans*-RA did not (Fig. 8).

MM11453 Induced TR3 Mitochondrial Targeting. MM11453 at 1.0 μM induced the migration of transiently expressed TR3/ΔDBD-GFP to mitochondria in H460 cells, as indicated in Fig. 9 by colocalization of GFP fluorescence with that of immunostained Hsp60. Colocalization did not occur in vehicle-alone-treated cells (data not shown). Thus, both AHPN and MM11453 induce TR3 targeting to mitochondria.

MM11453 Altered Mitochondrial Membrane Potential. We found that MM11453 induced TR3 targeting to the mitochondrial outer membrane of breast and prostate cancer cells to initiate cytochrome *c* release and apoptosis (27). A loss of inner mitochondrial membrane potential or depolarization, which may signify outer membrane or permeability transition pore opening (45) and has been suggested as causing cytochrome *c* release (45), is associated with apoptosis. The effect of MM11453 on this process was explored using Rh123, which cells incorporate on depolarization. MM11453 increased MCF-7, MDA-MB-231, and LNCaP cell Rh123 fluorescence 2.2-, 1.9-, and 5.4-fold, respectively (Fig. 10). Again, MM11453 behaves similarly to AHPN (46).

Bcl-2 Attenuated Mitochondrial Membrane Depolarization by MM11453. Because overexpression of antiapoptotic, mitochondrial membrane-surface protein Bcl-2 is reported to block cancer cell apoptosis (47), its effect on apoptosis by MM11453 was explored in Jurkat cells transfected with an expression vector containing *bcl-2* or the vector alone. In MM11453-treated nontransfected cells and MM11453-treated vector alone-transfected cells, depolarized cell numbers increased 4.8- and 5.5-fold, respectively, over that of the nontreated control, whereas cell numbers increased only 2-fold in treated cells overexpressing *bcl-2* (Fig. 11). Thus, *bcl-2* modified the effect of MM11453 on mitochondrial membranes.

DISCUSSION

AHPN induces apoptosis in cancer cell lines (13, 14, 18–22, 24). How AHPN initiates this process remains to be completely defined. A report of the RARγ selectivity of AHPN (12) led to the hypothesis of an apoptotic role for RARγ in breast cancer, melanoma, and neuroblastoma cells (17, 23, 24). To support this, RARγ transcriptionally active AHPN derivatives and analogues were also reported to inhibit growth and induce apoptosis (28, 48, 49). Other reports present data strongly suggesting an RAR-independent pathway, such as growth inhibition and apoptosis of retinoid-resistant cancer cells (13, 14, 21, 27, 42, 44, 50).[5] Our results support the latter by showing that MM11453, although unable to activate retinoid receptors on a reporter with the efficacy of standard retinoids or AHPN, strongly inhibited growth and induced apoptosis in retinoid-resistant cancer cell lines.

The near absence of RAR subtype and RXRα transcriptional activation by MM11453 was confirmed by limited proteolysis. DPSAs suggest that MM11453 is not a RAR or RXRα agonist. MM11453 did not induce a protease-resistant RARγ conformation, characteristic of binding a retinoid agonist, such as MM11254 [6], but behaved as the

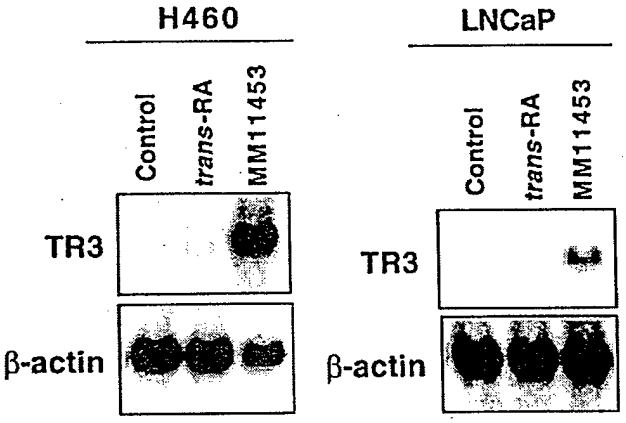

Fig. 8. MM11453 induces TR3 expression in H460 and LNCaP cells. Cells were treated with ethanol alone, 1.0 μM *trans*-RA, or MM11453 for 6 h. Total RNAs were prepared and analyzed for TR3 expression by Northern blotting. Expression of β-actin was the RNA-loading control.

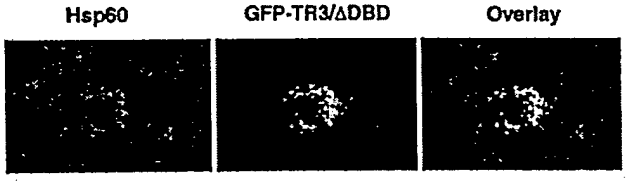

Fig. 9. MM11453 induces TR3 translocation to H460 mitochondria. Cells were transiently transfected with TR3/ΔDBD-GFP expression vector and then treated with 1.0 μM MM11453 for 6 h as in "Materials and Methods." Immunostained mitochondrial Hsp60 (*red*) and TR3/ΔDBD-GFP protein (*green*) were visualized by confocal microscopy, and images were overlaid (*Overlay*) to indicate colocalization (*yellow*).

RARγ-selective antagonist MM11253 [7], a dithiane (14, 39) of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)-2-naphthalenecarboxylic acid].[5] MM11453 did not detectably dissociate NCoR from RARγ or recruit p300 to RARγ, as agonists did. Thus, the behavior of MM11453 contrasts with that of RAR-agonist AHPN (12, 14). A retinoid receptor-independent pathway for anticancer activity has precedent in the mechanism of action of *N*-(4-hydroxy)phenyl retinamide, which inhibits the growth of cancer cells that resist standard retinoids (51, 52).

DPSA (data not shown) and molecular modeling (Fig. 2) suggest that AHPN does not induce a unique conformation in the RARγ LBD that could account for apoptosis-inducing activity. RARγ on binding AHPN, *trans*-RA, or MM11254 produced the same PFs,[5] whereas 1.0 μM MM11253 [7] did not induce this conformation[5] or transcriptionally activate RARγ (14). Both transactivation and DPSA show that the bulky 1-adamantyl group of AHPN (Fig. 2*A*) did not prevent an agonist-induced RARγ conformation, and modeling shows the 1-adamantyl group occupying the same region as the tetrahydronaphthalene rings of agonists MM11254 and BMS270394 [8] (Ref. 41; Fig. 2*B*). The three hydroxyl and carboxyl oxygen molecules are also close. Thus, on the basis of the strategy used by Klaholz *et al.* (41) that the low-energy conformation of a ligand approximates its bound form, our findings suggest that pharmacophoric AHPN groups are not responsible for inducing any unique conformation in RARγ. Only the 3-chloro group *ortho* to the COOH group distinguishes MM11453 from AHPN. How the chloro group inhibits transcriptional activation remains to be determined. Both its steric and electronic properties may perturb hydrogen bonding by the COOH group or shift van der Waals contacts of RARγ LBD pendant groups, thereby preventing the conformational changes in the receptor necessary for coactivator recruitment and transcriptional activation.

The inhibition of [^{3}H]9-*cis*-RA binding to RARs by MM11453 suggests direct binding, whereas transfection indicates minimal RAR or RXRα agonism. Thus, MM11453 may function as a moderately selective RARγ antagonist. Although how RARγ antagonism or that of another RAR or RXR subtype contributes to MM11453 activity remains to be defined, the lack of growth inhibition by antagonist MM11253 (data not shown) suggests that the contribution, if any, is small. Unlike *trans*-RA, both MM11453 and AHPN strongly inhibited HL-60R, MDA-MB-231, LNCaP, and H292 cell growth and induced apoptosis. EC_{50}s for inhibiting growth in HL-60R and MDA-MB-231 cells were comparable, and their apoptotic EC_{50}s were similar (Fig. 7). These results indicate that MM11453 functions independently of RARs and RXRα and strongly suggest a similar mode of action for AHPN. Both AHPN (21) and MM11453 (Fig. 8) induced TR3 expression in H460 and LNCaP cells and TR3 mitochondrial translocation (Ref. 27 and Fig. 9, respectively) and caused inner mitochondrial membrane depolarization in MCF-7, MDA-MB-231, LNCaP, and Jurkat cells (Figs. 10 and 11). These results demonstrate that

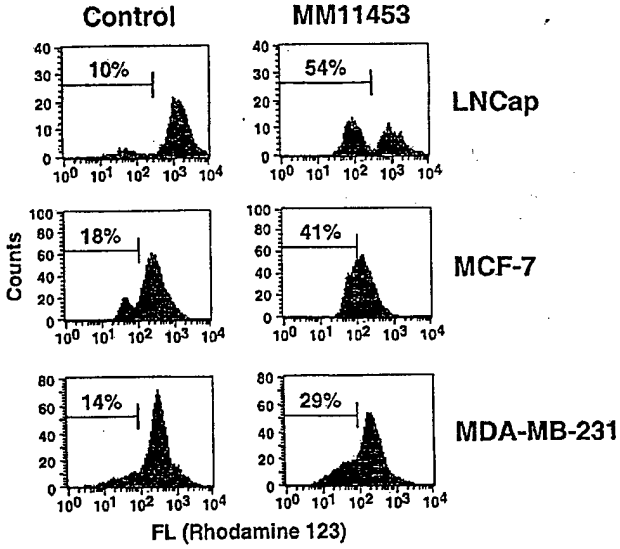

Fig. 10. Effect of MM11453 on LNCaP, MCF-7, and MDA-MB-231 mitochondrial membrane potential. Cells were treated with or without 1.0 μM MM11453 for 18 h and then with Rh123 as in "Materials and Methods." Rh123-fluorescencing cells are expressed as a percentage of the total.

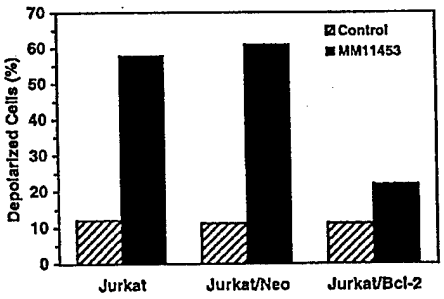

Fig. 11. Bcl-2 inhibits Jurkat mitochondrial membrane potential decrease by MM11453. Nontransfected cells stably expressing vector alone (*Jurkat/Neo*) and transfected cells stably expressing Bcl-2 (*Jurkat/Bcl-2*) were treated with 1.0 μM MM11453 or ethanol alone for 18 h and analyzed for change in mitochondrial membrane potential as in "Materials and Methods."

MM11453 retains the apoptotic properties of AHPN without behaving as a competent RARγ agonist and, thus, indicate that RARγ activation is not required for apoptotic activity. The recent report that AHPN induces apoptosis in RARγ-negative myeloma cells through a mitochondrial pathway (46) supports this conclusion. Reporter and limited proteolysis assays on MM11453 and AHPN suggest that their apoptotic activity does not involve RARα, RARβ, or RXRα activation.

Transactivation by liganded RARγ is reported to correlate with retinoid toxicity (53, 54). The lack of retinoid receptor activation activity by MM11453 suggests that toxic side effects characteristic of retinoid receptor activation (reviewed in Ref. 11) should be reduced in this class of apoptosis inducers, thereby affording more effective candidates for development as cancer chemotherapeutic agents.

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