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ARTICLES

Differential Regulation of Protein Expression, Growth and Apoptosis by Natural and Synthetic Retinoids

M.A. Christine Pratt,* Minying Niu, and Dawn White

Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

Abstract All-*trans* retinoic acid (ATRA) can down regulate the anti-apoptotic protein Bcl-2 and the cell cycle proteins cyclin D1 and cdk2 in estrogen receptor-positive breast cancer cells. We show here that retinoids can also reduce expression of the inhibitor of apoptosis protein, survivin. Here we have compared the regulation of these proteins in MCF-7 and ZR-75 breast cancer cells by natural and synthetic retinoids selective for the RA receptors (RARs) α, β, and γ then correlated these with growth inhibition, induction of apoptosis and chemosensitization to Taxol. In both cell lines ATRA and 9-*cis* RA induced the most profound decreases in cyclin D1 and cdk2 expression and also mediated the largest growth inhibition. The RARα agonist, Ro 40-6055 also strongly downregulated these proteins although did not produce an equivalent decrease in S-phase cells. Only ATRA induced RARβ expression. ATRA, 9-*cis* RA and 4-HPR initiated the highest level of apoptosis as determined by mitochondrial Bax translocation, while only ATRA and 9-*cis* RA strongly reduced Bcl-2 and survivin protein expression. Enumeration of dead cells over 96 h correlated well with downregulation of both survivin and Bcl-2. Simultaneous retinoid-mediated reduction of both these proteins also predicted optimal Taxol sensitization. 4-HPR was much weaker than the natural retinoids with respect to Taxol sensitization, consistent with the proposed requirement for reduced Bcl-2 in this synergy. Neither the extent of cell cycle protein regulation nor AP-1 inhibition fully predicted the antiproliferative effect of the synthetic retinoids suggesting that growth inhibition requires regulation of a spectrum of RAR-regulated gene products in addition even to pivotal cell cycle proteins. J. Cell. Biochem. 90: 692–708, 2003.

Key words: all-*trans* retinoic acid; 9-*cis* retinoic acid; synthetic retinoids; protein regulation; cell cycle; apoptosis; chemosensitization

Retinoic acid receptors (RARs), like other members of the steroid/thyroid family of nuclear receptors, are ligand-activated transcription factors. Binding of ligands including the naturally occurring retinoids, ATRA and 9-*cis* RA results in a conformational change in the receptor which activates the transcription of genes containing RAR response element enhancer regions [Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995]. There are three known RARs; α, β, and γ. These receptors bind to response elements as heterodimers with a member of the RXR family [Mangelsdorf and Evans, 1995]. There are also three subtypes of RXR; α, β, and γ which can form homodimers and activate transcription in the presence of 9-*cis* RA. The natural retinoid ATRA binds exclusively to the RARs while 9-*cis* RA activates both RARs and RXRs. In the past few years, several synthetic retinoids have been generated with different retinoid receptor profiles. These include compounds from Hoffmann-La Roche, Ro 40-6055 (RARα-specific), Ro 44-4753 (RARγ-specific), Ro 26-4456 (RXR-specific) [Bollag et al., 1997 and references therein] and the RARβ-specific retinoid CD-417 produced by Galderma, Inc., France [Schneider et al., 2000]. Another synthetic retinoid, 4-(hydroxyphenyl)-retinamide (4-HPR) is thought to act through a receptor-independent mechanism although there is some evidence that it may act through the RARγ [Fanjul et al., 1996]. Recent work suggests that receptor-selective retinoids do not

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*Correspondence to: M.A. Christine Pratt, Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Ontario, Canada K1H 8M5.
E-mail: cpratt@uottawa.ca

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activate RAR-response elements or modulate the transcription of the same spectrum of endogenous genes in a manner identical to the natural retinoids [Mouchon et al., 1999; Brand et al., 2002].

Natural and synthetic retinoids are currently used or under investigation for treatment or chemoprevention in a number of different cancers [McBurney et al., 1993; Arnold, 1997; Lippman et al., 1997; Budd et al., 1998] and display several activities depending on the cell type ranging from inhibition of cell growth to induction of differentiation and apoptosis. In several estrogen receptor, positive breast cancer cell lines including MCF-7 and ZR-75 which express several RARs, RARα agonists have been shown to be capable of mediating differentiation and/or the inhibition of cell growth while other tested RAR-specific ligands reportedly cannot [Bollag et al., 1997; Toma et al., 1997]. In attempts to identify apical targets for retinoid-induced growth inhibition of breast cancer cells, various groups have suggested that individual factors such as induction of the RARβ [Liu et al., 1996], inhibition of AP-1 [Fanjul et al., 1994], or activation of the RARα [Schneider et al., 2000] are the key mediators of this activity. AP-1-mediated transcriptional activity contributes to the proliferative response of cells to stimulation by growth factors and other pathways since targets of the Jun/Fos complex include many genes involved in the cell cycle as well as survival functions. By contrast, all RAR selective agonists have been shown to mediate the induction of apoptosis in these breast cancer cells to different extents suggesting that these receptors differentially control gene expression programs which might converge on apoptotic pathways.

Retinoids have also shown synergy with certain chemotherapeutic agents. Wang et al. [2000] have demonstrated that pre-treatment with ATRA markedly reduces the ED50 for inhibition of colony formation following treatment with Taxol or Adriamycin. New retinoids with dual selectivity for both the RARα and β have shown synergy with Taxol and other agents which modify tubulin in certain breast, ovarian, and oral keratinocyte cancer cell lines [Vivat-Hannah et al., 2001].

Consistent with the anti-proliferative and proapoptotic effects of retinoids we and others have identified several retinoid-responsive gene products which are germane to both processes including cyclin D1 and cdk2 [Teixeira and Pratt, 1997; Zhou et al., 1997], and the anti-apoptotic protein, Bcl-2 [Benito et al., 1995; Raffo et al., 2000]. Here we demonstrate that retinoids also downregulate the inhibitor of apoptosis family member, survivin.

In the current study, we have evaluated the hypothesis that the ability of both natural and synthetic retinoids to regulate the cellular levels of these important gene products can be correlated with effects on growth, apoptosis, and synergy with the chemotherapeutic drug, paclitaxel.

MATERIALS AND METHODS

Cell Culture

MCF-7 and ZR-75 ER-positive breast cancer cells were maintained in α-minimal essential medium (αMEM) supplemented with non-essential amino acids, 0.3% glucose and 5% fetal bovine serum. ATRA, 9-*cis* RA and 4-HPR (Sigma), Ro 40-6055 (AM580) (RARα agonist); Ro 44-4753 (RARγ agonist); Ro 26-4456 (Targretin)(RXR agonist) (Hoffman LaRoche, Basel, Switzerland) and CD-417 (Galderma, Inc., Valbonne, France) were dissolved in ethanol at a stock concentration of 1 mM and were used at a final concentration of 1 μM for the indicated times. The IC50 values calculated for inhibition of ATRA binding to the target RAR/RXR for all ligands are less than 30 nM and relative activation (EC 50 nM) of a standard retinoic acid response element has been previously reported [Boehm et al., 1994; Schneider et al., 2000; Raffo et al., 2000]. For trypan blue exclusion analysis, both floating and adherent cells were collected and pooled. The live and dead cells were gently centrifuged at 1,000 rpm for 5 min on a hematological centrifuge. Trypan blue was added to a final concentration of 0.2% and both clear (live) and blue (dead) cells were enumerated on a haemacytometer. For each determination a minimum of 1,000 cells were counted.

Immunoblot Analysis

Protein extracts were prepared from cultures and subjected to SDS–PAGE and transfer to PDVF Polyscreen membranes (DuPont NEN, Boston, MA) as previously described [Pratt and Niu, 2003]. Immunoreactive proteins were visualized using the ECL chemiluminescence

system (Dupont NEN). Densitometry was performed using a Kodak Image Station.

Antibodies

Monoclonal antibodies against human cdk2 (D12) and cyclin D1 (HD11) were purchased from Santa Cruz Biotech, CA. Anti-survivin (AF886) was from R&D Systems (Minneapolis, MN). Anti- Bcl-2 was the gift of Dr. J.C. Reed (La Jolla, CA). Anti-α-actin was purchased from Sigma (Oakville, ON, Canada). Monoclonal antibody against the NH2-terminus of Bax (6A7) was purchased from Trevigen (Gaithersburg, MD).

Immunocytochemistry

Cells were cultured in 35-mm dishes on coverslips in the presence of retinoid for 72 h, rinsed and fixed with 3% formaldehyde in PBS at room temperature (RT) then permeabilized for 2 min with 0.2% CHAPS in phosphate-buffered saline. Mouse anti-Bax monoclonal 6A7 was used at a 1:300 dilution in 3% bovine serum albumen in PBS and incubated with coverslips for 1 h at RT. Immunostained cells were detected following a one hour incubation at RT with CY3-labeled goat anti-mouse secondary antibody. Coverslips were mounted with anti-fade (glycerol containing 1 mg/ml *p*-phenylene-diamine). Slide evaluations were performed using a Zeiss Axiophot fluorescence microscope equipped with Northern Eclipse image analysis software (EMPIX Imaging, Inc., Mississauga, ON, Canada). All histogram bars represent results from evaluation of at least 1,000 cells from counted in multiple microscopic fields and are representative of at least two separate experiments.

Cell Transfection

Exponentially growing cultures of MCF-7 cells in 60-mm dishes were transfected with 2 μg of a plasmid containing from −43 to −72 of the collagenase gene enhancer region driving chloramphenicol acetyltransferase (coll-tk-CAT) and 1 μg of CMV-LacZ in 6 μl of Fugene Transfection reagent (Roche Diagnostics, Haval, Canada) according to the manufacturer's directions. After removal of the Fugene complexes, cells were fed with 10% serum-containing growth medium containing 1 μM of the specified retinoid or ethanol vehicle for 48 h prior to harvest. The additional 5% serum was used to increase the activity of the transfected promoter–reporter construct. Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were performed as previously described [Pratt et al., 1996]. Values for LacZ activity from each sample were essentially equivalent indicating consistent transfection efficiency.

Flow Cytometric Analysis

Cells were collected by centrifugation at 4C in PBS with 1 mM EDTA then resuspended in 1 ml of ice-cold PBS/EDTA and fixed by the addition of 3 ml of 80% ethanol. An aliquot of cells was pelleted at 500*g* for 5 min, washed with PBS/EDTA then resuspended in PBS/EDTA containing 100 μg/ml RNase A for 20 min at RT then made 32 μM with propidium iodide. DNA content from approximately 3×10^5 cells per sample were subjected to flow analysis performed on a Coulter Epics Altra cytometer (Hialeah, FL) equipped with an Argon laser and EXPO II software (Applied Cytometry Systems, Sheffield, UK).

Clonogenic Assay/Taxol Sensitization

Freshly plated 2,000 cells were treated for 3 days with 1 μM of the indicated retinoid then exposed to vehicle or Taxol for 3 h. Taxol-containing media was removed and replaced with fresh medium without drug but containing the same retinoid followed by culture for an additional 7 days prior to fixing and staining with methylene blue. For each experiment, triplicate plates were enumerated. Only colonies containing a minimum of 50 cells were counted.

RESULTS

Differential Regulation of Survival Proteins by Retinoid Ligands

In order to determine whether retinoid ligands display differential effects on genes involved in the regulation of apoptosis, we considered their effects on Bcl-2 in MCF-7 and ZR-75 human breast cancer cell lines. In MCF-7 cells (Fig. 1a,b, panel 1), Ro-40 and 9-*cis* RA produced the strongest decrease in Bcl-2 down to less than 20% of control levels. The results in Figure 2a,b (panel 1) show that in ZR-75 cells 9-*cis* RA, ATRA and Ro-40 most strongly decreased the level of Bcl-2 to less than 30% of control levels with the effects being maximally observed at 96 h post-treatment. CD-417 also substantially reduced levels of Bcl-2 by at least

60%. The RARγ agonist Ro-44 and 4-HPR had a much weaker effect on Bcl-2 protein expression compared with the other ligands. Thus both the RARα and β are capable of down-regulation of the Bcl-2 protein. The activity of each depends on the cell. Whereas the RARα agonist was more potent than the RARβ agonist in MCF-7 cells, the reverse was true in ZR-75 cells. We recently

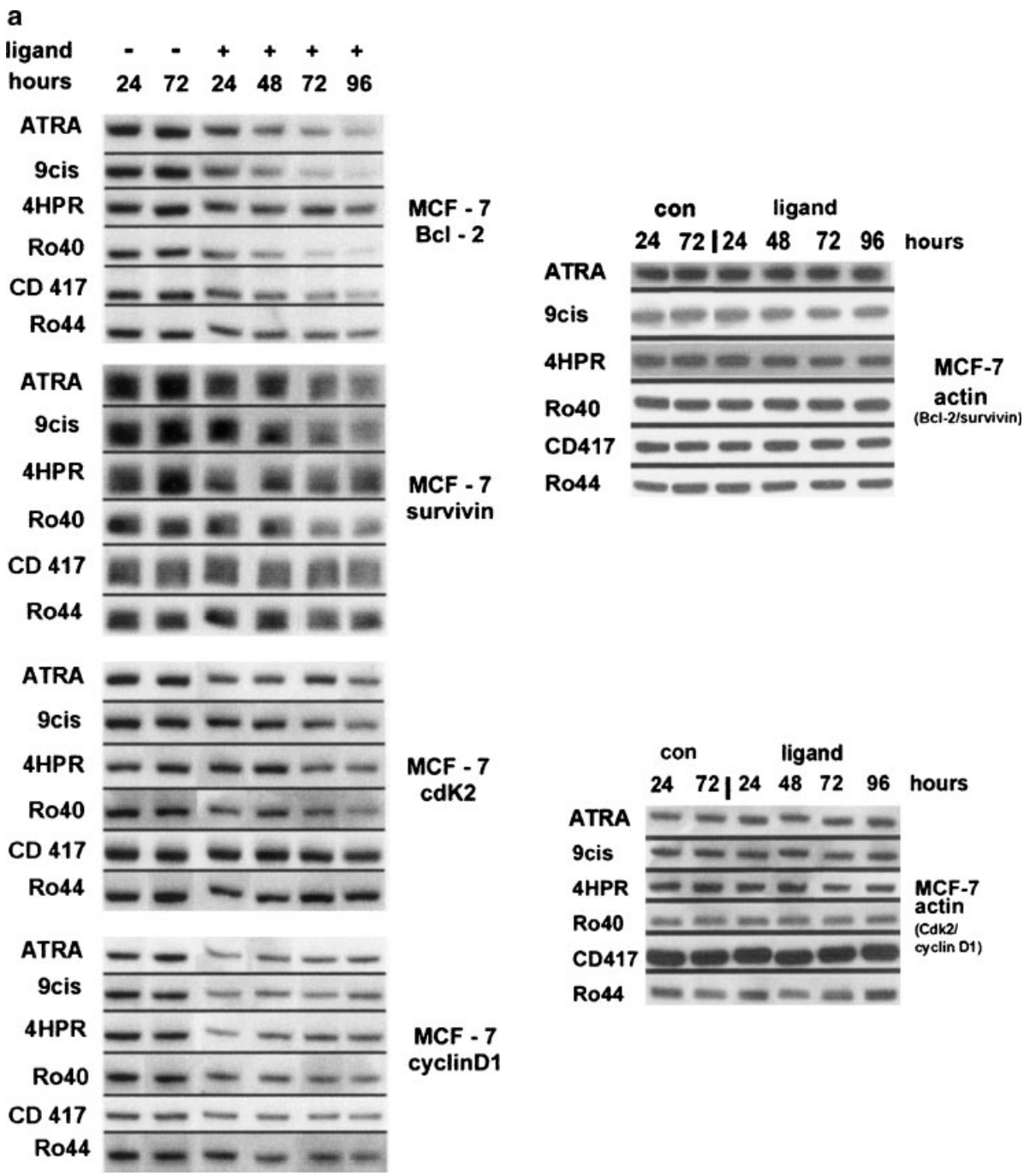

Fig. 1. Retinoid regulation of protein expression in MCF-7 cells. **a**: Twenty micrograms of whole cell extract from MCF-7 cells cultured in the presence of 1 μM of the indicated retinoid for 24–96 h or vehicle (24 and 72 h) was separated by PAGE and immunoblotted with antibodies to either Bcl-2 (**panel 1**), survivin (**panel 2**), cdk2 (**panel 3**), or cyclin D1 (**panel 4**). Equivalency of gel loading was assessed using an anti-α-actin antibody shown to the right of the respective panels. **b**: Histograms derived from densitometric values in arbitrary units for each of the time points presented as a percentage of the control values. Control values were calculated as the average of vehicle treated density values at 24 and 72 h for each time course. All values were normalized to density values for α-actin on the same blots. Data are representative of two separate experiments.

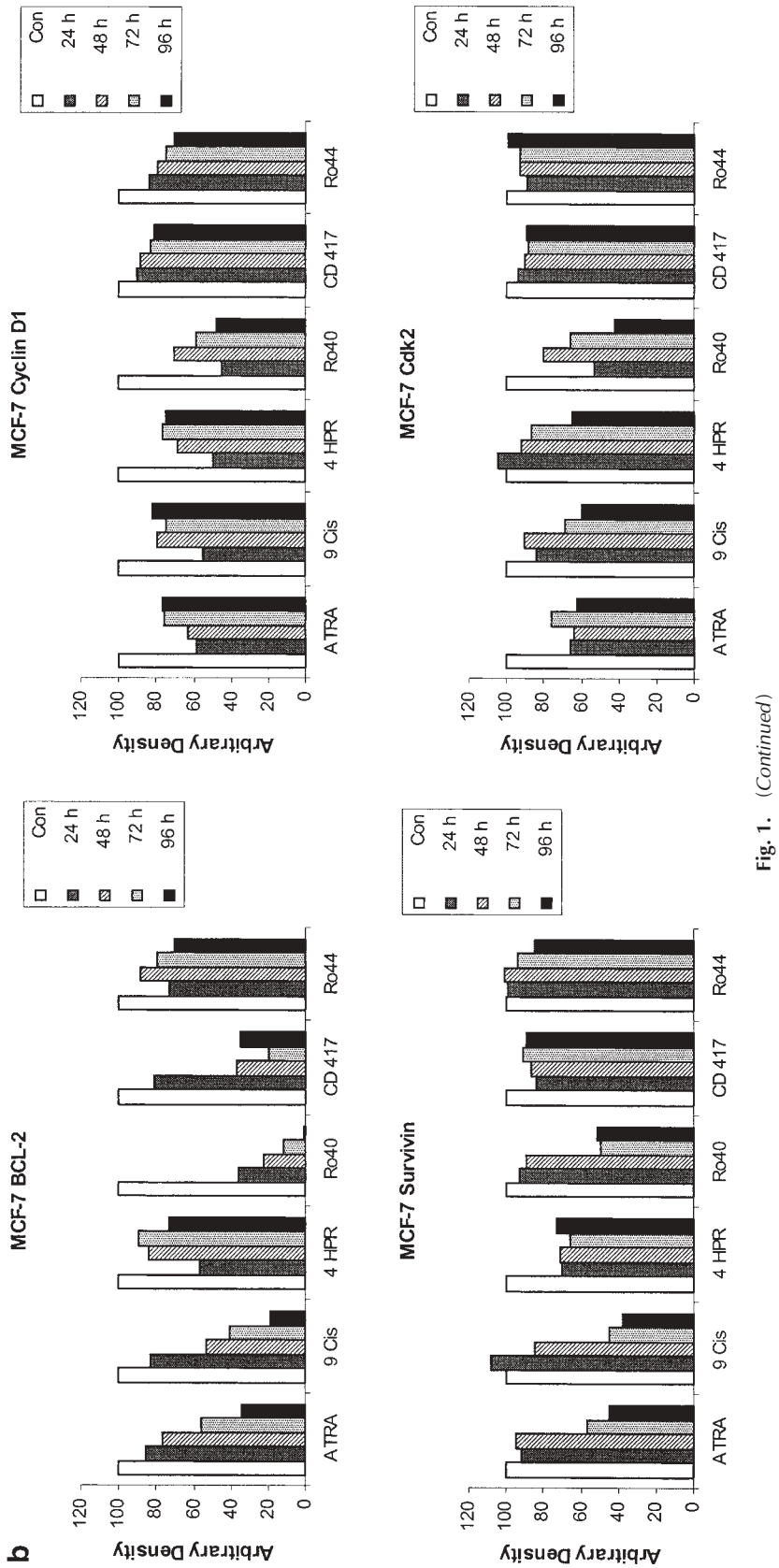

Fig. 1. *(Continued)*

identified the inhibitor of apoptosis (iap) protein family member, survivin, as a target of retinoic acid downregulation (unpublished data). The results in Figure 1a,b (panel 2) show that compared with control vehicle-treated cells, ATRA, 9-*cis* RA and Ro-40 produced the strongest repression of survivin levels in MCF-7 cells reducing the levels by 30–50% of control within 96 h after retinoid treatment. 4-HPR had less effect while CD-417 and Ro-44 had almost no effect on survivin levels. Similar results were obtained in ZR-75 cells (Fig. 2a,b, panel 2) except that Ro-40 was relatively less effective in these cells compared with MCF-7 cells. Given that all three of the most potent ligands with regard to survivin down-regulation interact with the RARα, the data suggest that this receptor is primarily responsible for retinoid effects on survivin protein expression in breast cancer cells.

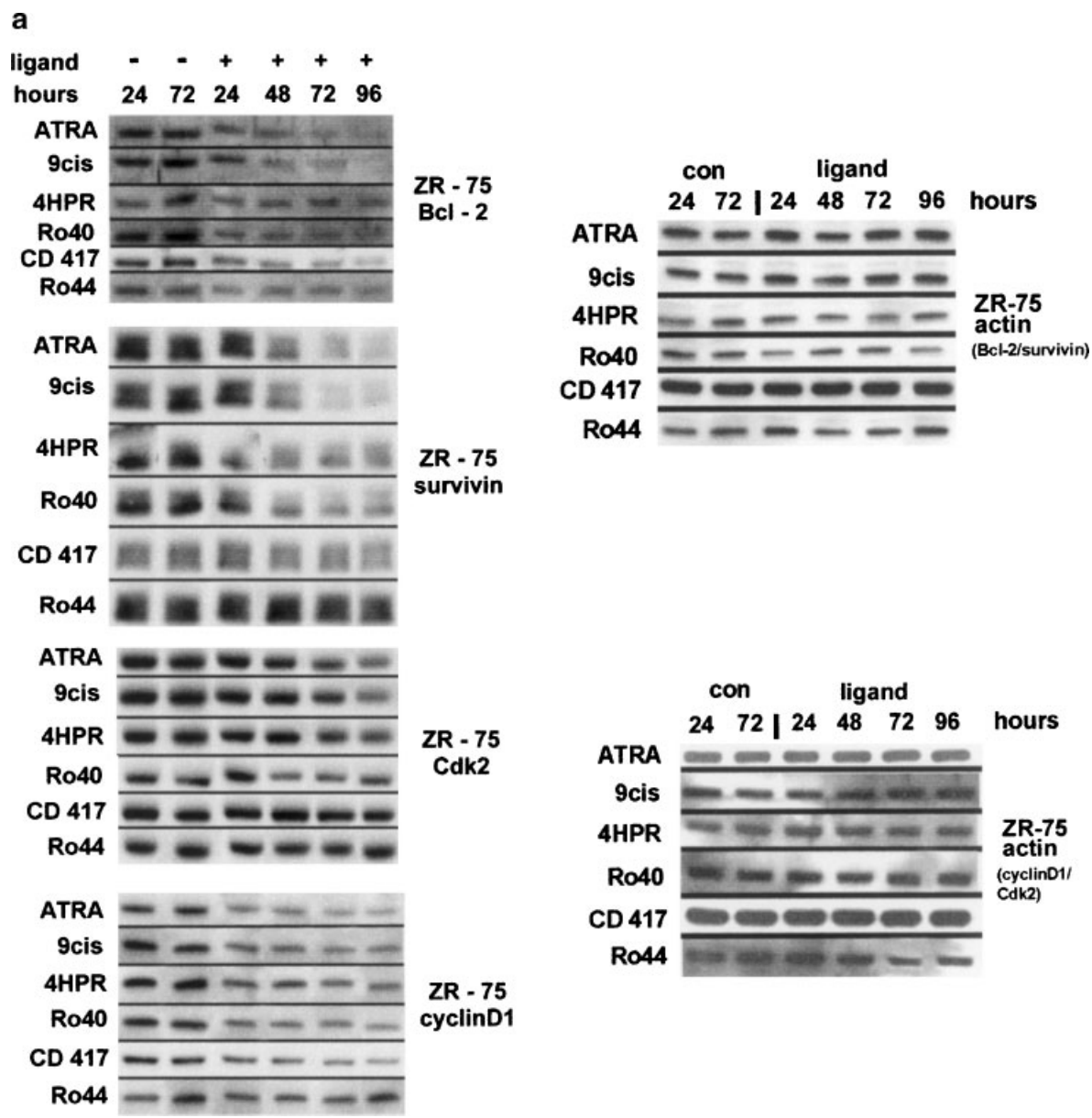

Fig. 2. Retinoid regulation of protein expression in ZR-75 cells. **a**: Twenty micrograms of whole cell extract from ZR-75 cells cultured in the presence of 1 μM of the indicated retinoid for 24–96 h or vehicle (24 and 72 h) was separated by PAGE and immunoblotted with antibodies to Bcl-2, survivin, cdk2, or cyclin D1 in **panels 1–4**, respectively. Equivalency of gel loading was assessed using an anti-α-actin antibody shown to the right of the immunoblots. **b**: Histograms representing densitometric analysis of these gels were derived as described for Figure 1.

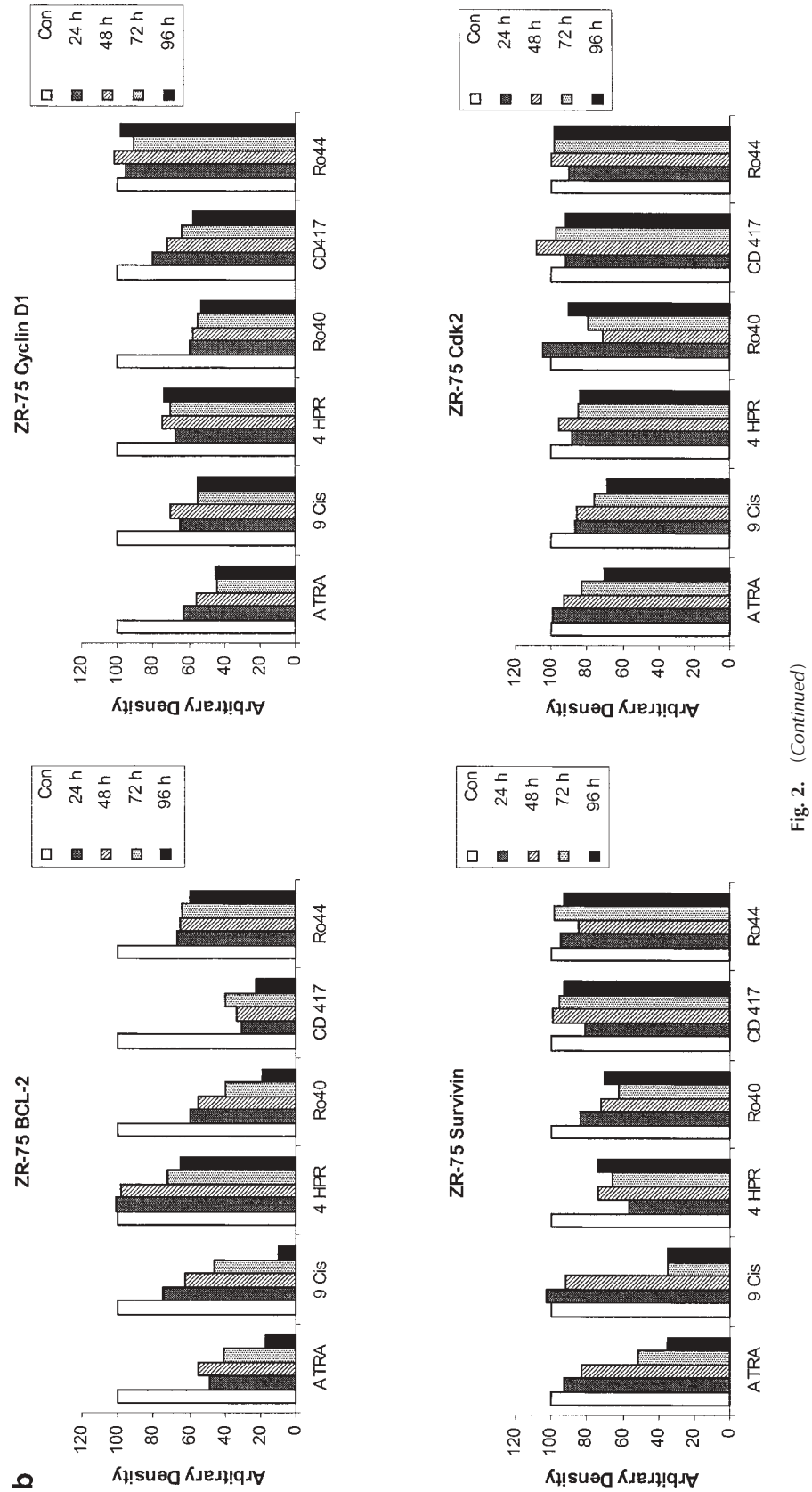

Fig. 2. (*Continued*)

Differential Regulation of Cell Cycle Proteins by Retinoid Ligands

We and others have identified cyclin D1 and cdk2 [Teixeira and Pratt, 1997; Zhou et al., 1997] as cell cycle protein targets for down-regulation by ATRA. Since these are key proteins involved in G_1-phase progression we assayed the ability of the various retinoid ligands to decrease the levels of these proteins in the breast cancer cell lines under study. Most retinoid ligands were capable of decreasing cdk2 levels in MCF-7 cells from 50% (Ro-40) to 70% (ATRA, 9-*cis* RA and 4-HPR) of control levels (Fig. 1a,b, panel 3). On the other hand, Ro-44 and CD-417 had minimal effects on cdk2 levels. In ZR-75 cells (Fig. 2a,b, panel 3), ATRA and 9-*cis* RA induced the maximal 35% decrease in cdk2 expression while Ro-40 had a transient effect at 48hrs post treatment. 4-HPR produced a weaker down-regulation of cdk2. Both Ro-44 and CD-417 had virtually no effect on cdk2 expression. Unlike the regulation of the apoptosis regulatory proteins, cyclin D1 regulation proved to be not only retinoid-dependent but also cell-dependent. The immunoblot in Figure 1a (panel 4) and histogram in Figure 1b shows that in MCF-7 cells all ligands except CD-417 and Ro-44 were able to reduce cyclin D1 levels to at least 60% of control. With the exception of Ro-44 and CD-417, the maximal decrease in protein was observed with 24 h after exposure to the ligand. The RARα agonist Ro-40 was a strong inhibitor of cyclin D1 expression maximally reducing levels to just over 40% of control levels at both the 24 and 96 h time-points. In contrast to MCF-7 cells, the strongest inhibition of cyclin D1 expression in ZR-75 cells was induced by ATRA which maximally decreased levels by 70% compared with controls (Fig. 2a,b, panel 2). 9-*cis* RA and Ro-40 also substantially reduced cyclin D1 to under 40% of control levels. These effects were strongest after 96 h of treatment making the kinetics of inhibition slower in ZR-75 cells than in MCF-7 cells. The exception to this was 4-HPR and Ro-44 which both produced a weaker maximal inhibition of cyclin D1 protein expression of about 25% compared to control. Thus in both MCF-7 and ZR-75 cells, the strongest regulation of cyclin D1 occurred in the presence of a ligand which could interact selectively or as a pan-agonist with the RARα.

Taken together the results suggest that activation of the RARα is important for inhibition of Bcl-2, survivin, cyclin D1, and cdk2 expression by retinoids. The RARβ ligand had its strongest, most consistent effect on Bcl-2 expression and, while it also downregulated cyclin D1 protein levels, it had little effect on survivin or cdk2. 4-HPR had its strongest effects on the expression of Bcl-2 and cyclin D1, however, for all proteins considered, 4-HPR was only a moderate regulatory influence compared with the natural and synthetic RARα-inducing ligands. The RARγ ligand Ro-44 did decrease expression of Bcl-2 and cyclin D1 albeit with less magnitude than the other ligands and similar to CD-417, had virtually no effect on protein levels of cdk2 and survivin.

Effects of Retinoids on S-Phase Percentages

We next considered the associations between cell cycle protein regulation in response to the various retinoids and their relative abilities to reduce proliferation as determined by changes in the proportion of cells in S-phase. Flow cytometric analysis of the cell cycle in MCF-7 and ZR-75 cells treated with the panel of retinoids for 48 and 72 h is shown in Figure 3. Because the RARγ agonist Ro-44 had produced only weak gene regulation, while the RAR/RXR agonist 9-*cis* had strongly regulated the protein expression studied, we decided to use an RXR-specific ligand, Ro-26 and compare the biological effects of activating only the RXR with those of 9-*cis* and the other retinoids. All retinoids mediated a clear decrease in S-phase after 48 h compared with vehicle treated controls. With the exception of 4-HPR these reductions in S-phase were greater after a further 72 h exposure to ligand. S-phase percentages were maximally lowered by 9-*cis* RA and ATRA (8 and 11%), Ro-40 was moderately repressive (14%) while the RXR agonist Ro-26, CD-417, and 4-HPR were the least effective at reduction of S-phase proportions with maximal levels being 20, 24, and 23% (at 48 h), respectively. A similar pattern of S-phase inhibition was obtained in ZR-75 cells except that Ro-26 and Ro-40 displayed almost equivalent potency. Thus breast cancer cell lines may present with different retinoid-responsive profiles with respect to growth inhibition. Clearly the natural ligands ATRA and 9-*cis* RA are the strongest inhibitors of cell cycle progression while both Ro-40 and Ro-26 are between 1.8- and 2.5-fold less potent

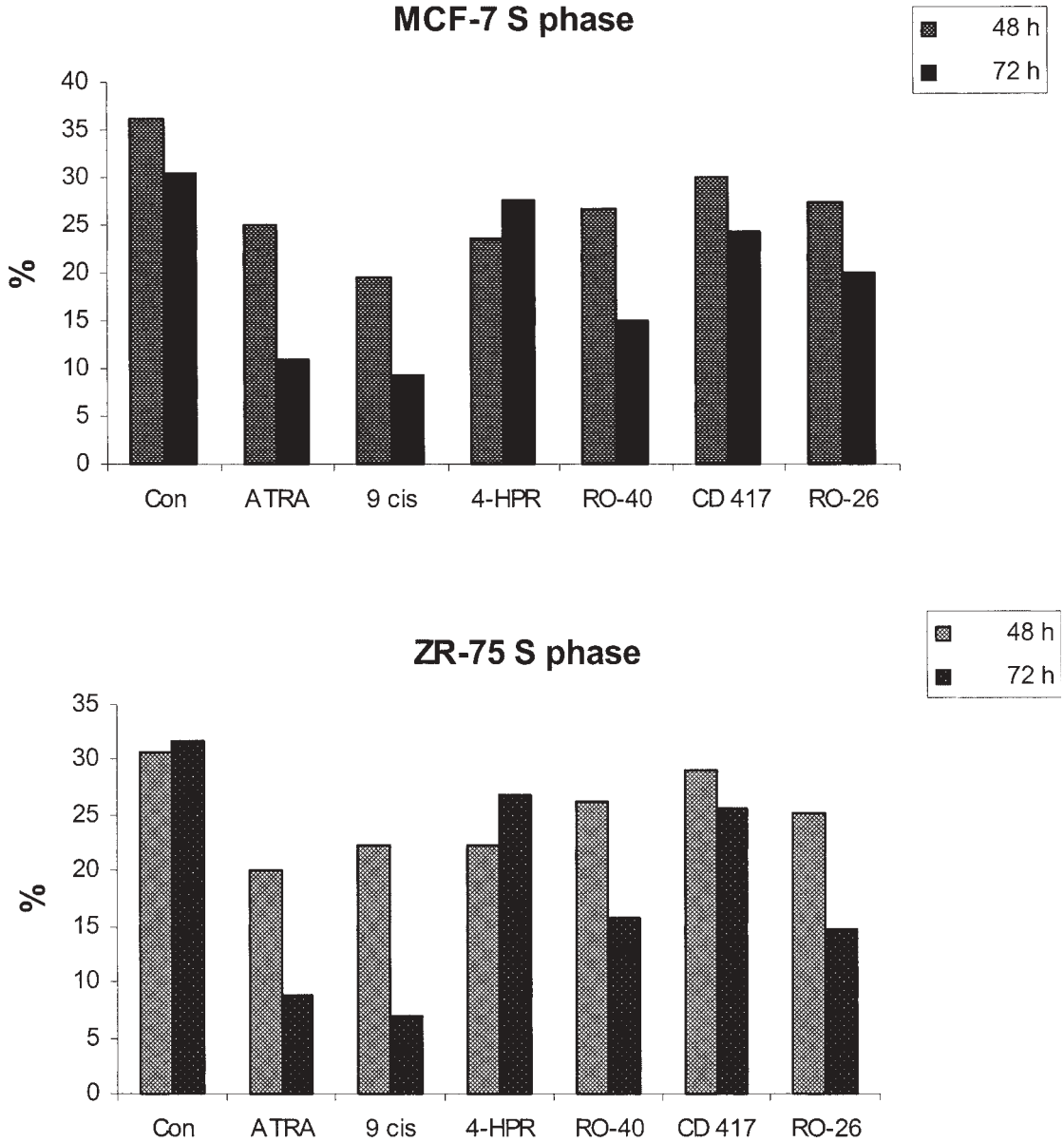

Fig. 3. Effects of retinoids on S-phase populations in breast cancer cells. Cell cycle analysis was performed as described in "Materials and Methods." The percentages of cells in S-phase after a 48 and 72 h culture period in the presence of vehicle or the indicated retinoid is shown. Data are representative of two separate experiments which differed by less than 5% for all values.

in this respect. CD-417 has a small but measurable effect on the cell cycle as does 4-HPR although the latter drug acts in a more transient manner than do any of the other compounds.

All Retinoid Ligands Significantly Inhibit AP-1 Activity

The mechanism by which retinoids inhibit cellular proliferation is thought in large part to be due to the inhibition of the JUN:FOS AP-1 complex and this activity has clearly been distinguished from the transactivational properties of the retinoic acid receptor [Fanjul et al., 1994; Benkoussa et al., 2002]. To assess the relative abilities of the retinoids to inhibit activation of an AP-1 responsive element, we transfected MCF-7 cells with a reporter construct containing the AP-1 responsive region of the collagenase gene driving chloramphenicol acetyltransferase (coll-tk-CAT) previously shown to be inhibited by natural and synthetic retinoids [Guerin et al., 1997] and treated

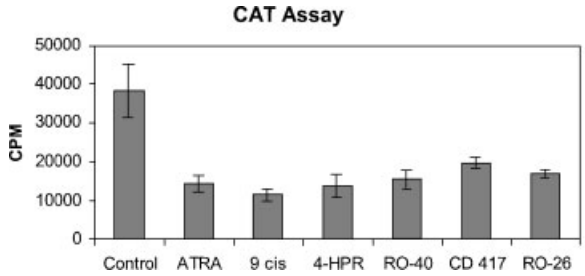

Fig. 4. Retinoid inhibition of AP-1 activity. MCF-7 cells were transiently transfected with a reporter plasmid containing the collagenase AP-1 response element and cultured for a further 48 h in the presence of vehicle or retinoid. CAT assays were performed as described in ''Materials and Methods'' and transfection efficiencies were equivalent as determined by measurement of β-galactosidase activity from a cotransfected CMV-LacZ plasmid (data not shown). The graph depicts triplicate values ± standard deviations for [^{3}H]-acetylated chloramphenicol for each treatment.

cultures with either retinoid or vehicle. The resulting activity of the collagenase AP-1 response element is shown in Figure 4. Clearly all retinoids had a significant repressive effect on AP-1 activity ranging from 60–70% reduction compared to control. 9-*cis* RA had the strongest effect while 4-HPR and ATRA had a slightly smaller inhibitory effect. The synthetic ligands Ro-40 and Ro-26 inhibited AP-1 with respectively less magnitude although there were relatively small differences between Ro-40 and Ro-26. CD-417 was the weakest retinoid in this capacity. Given the relative effects on the cell cycle of the individual retinoids, these data suggest that retinoid ligand-mediated repression of AP-1 activity is not fully commensurate with the ability to inhibit cell growth.

Retinoid Induction of RARβ Expression

The induction of expression of the RARβ gene by retinoids through a canonical DR-5 RA response element in the regulatory region of the RARβ promoter as been proposed to be a component of the antiproliferative action as well as the apoptotic effect of retinoids [Seewaldt et al., 1995; Liu et al., 1996]. We therefore wished to compare the induction of RARβ protein expression by the retinoids under study in both MCF-7 and ZR-75 cells. The immunoblot in Figure 5 depicts protein extracts from MCF-7 and ZR-75 cells following 72 h of treatment with retinoid ligands or vehicle. A low basal level of RARβ expression was detectable in both cell lines. Surprisingly, however, only ATRA was able to induce the expression of the RARβ protein and then only in MCF-7 cells. Therefore, in this instance the RARβ may contribute to the actions of ATRA. These results strongly suggest that significant retinoid induction of RARβ expression is not required for either antiproliferative or apoptotic responses of retinoids in MCF-7 and ZR-75 cells.

Induction of Cell Death by Retinoid Ligands

In order to determine whether retinoid regulation of apoptotic genes is commensurate with the levels of induced cell death we assayed dead cells by trypan blue exclusion staining. The graph in Figure 6 shows that, in both cell lines, 9-*cis* and ATRA produced the largest number of dead cells (8.5 and 7.5%, respectively). The ligands Ro-40, Ro-26, 4-HPR, and CD417 were virtually equivalent in this capacity resulting in death at about half of this percentage in MCF-7 cells. Statistical analysis of these data is shown in Table Ia. All data were significantly different from control. Both ATRA and 9-*cis* RA induced cell death to a significantly higher level compared with all other ligands ($P \leq 0.04$). In ZR-75 cells, Ro-40 was nearly as potent as was RA (8.0% vs. 8.5%) and significantly higher (see Table Ib for P values) than both 4-HPR and Ro26 in ZR-75 cells. CD-417 induced almost twice as much cell death than did 4-HPR in ZR-75 cells ($P = 0.055$). These results suggest that the involvement of individual RARs in transmitting a death signal varies in different cell lines.

The activation of the apoptotic protein Bax involving translocation of the protein to the mitochondrial membrane is one of the initial events in retinoid-induced cell death in breast cancer cells detectable using an antibody against the NH2-terminus of Bax (6A7) [Nechushtan et al., 1999]. The percentages of MCF-7 and ZR-75 cells reactive with the 6A7 antibody were enumerated following a 96 h treatment with retinoids and are presented in Figure 7. In MCF-7 cells, 9-*cis* RA and 4-HPR induced activation of Bax in nearly 3% of cells while all-*trans* RA produced half this level of Bax positive cells. CD-417 and Ro-44 did not result in result in significant 6A7 immunoreactivity compared with control cells (not shown). In contrast, both ATRA and 9-*cis* RA treatment resulted in similar levels of activated Bax (just under 2%) in ZR-75 cells while 4-HPR, CD-417, and Ro-44 produced Bax translocation at about half this level. Statistical analysis of these data are shown in Table IIa,b. The induction of 6A7

TABLE I. Statistical Analysis of Relative Cell Death Determined by Trypan Blue Exclusion Following Treatment of MCF-7 Cells; (a) and ZR-75 Cells; (b), With the Indicated Retinoids

	Control	ATRA	9-*Cis*	Ro-40	Ro-26	4-HPR
a: MCF-7						
ATRA	<0.0001					
9-*Cis*	<0.0001	NS				
Ro-40	<0.0001	0.04	0.003			
Ro-26	<0.0001	0.03	0.002	NS		
4-HPR	<0.0001	0.04	0.004	NS	NS	
CD-417	<0.0001	0.006	0.0003	NS	NS	NS
b: ZR-75						
ATRA	<0.0001					
9-*Cis*	<0.0001	NS				
Ro-40	<0.0001	NS	0.048			
Ro-26	<0.0002	0.006	<0.0001	0.014		
4-HPR	<0.0119	0.0002	<0.0001	0.0005	NS	
CD-417	<0.0001	0.074	0.0004	NS	NS	0.055

Proportions of dead cells from cultures were compared by contingency table analysis. Numbers represent *P* values calculated for differences between the data sets.
NS: not significant.

reactivity by 9-*cis* RA and 4-HPR was clearly significant in MCF-7 cells. In contrast 6A7 immunoreactivity induced by ligands in ZR-75 cells was considerably less than in MCF-7 cells resulting in lower levels of significance. The results suggest that the extent of apoptosis induced by ATRA, 9-*cis* RA and to a lesser extent Ro-40 can be associated with their relative abilities to decrease protein levels of Bcl-2 and survivin. In contrast, 4-HPR which has minimal effects on the expression of the latter proteins was an effective inducer of Bax activation in both MCF-7 and ZR-75 cells indicating that the mechanism of action of this ligand is dissociated from regulation of at least these regulators of apoptosis. Moreover, based on the numbers of dead cells versus the number of Bax 6A7-positive cells following 96 h 4-HPR treatment, it may be that this compound activates apoptotic events in a different manner or with different kinetics in MCF-7cells.

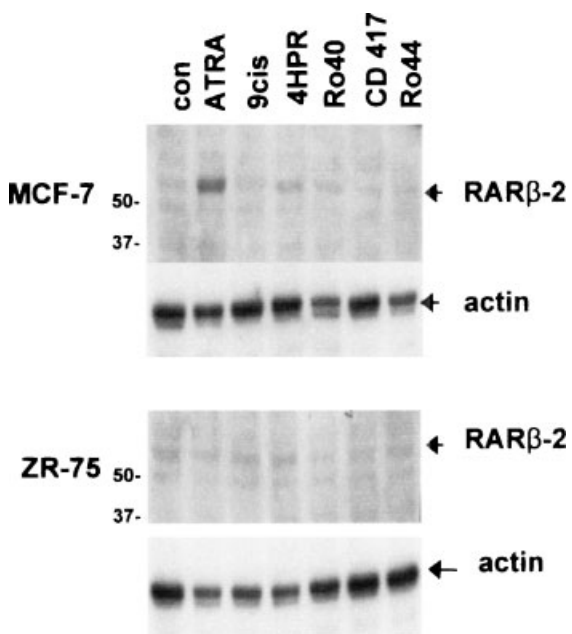

Fig. 5. Induction of RARβ expression. Immunoblot analysis for RARβ expression in 20 μg of MCF-7 and ZR-75 whole cell extract from cultures grown for 72 h in the presence of the indicated retinoid. Immunoblot with anti-α-actin was used as a loading control.

Effects of Retinoids on Clonogenicity and Sensitization to Taxol

We next studied the effects of retinoid treatment on long-term clonogenicity of MCF-7 breast cancer cells. To do this we evaluated colony formation following a 10-day exposure to retinoid. Since the retinoids lower levels of survival genes we also wanted to determine their relative abilities to sensitize cells to Taxol-mediated inhibition of colony formation correlated with survival gene expression. The results of retinoid inhibition of colony formation and Taxol sensitization are shown together in Figure 8. Alone, both ATRA and 9-*cis* RA strongly reduced the numbers of resulting colonies by about 80%. The RARα ligand Ro-40 was also able to decrease colony formation but to a lesser extent than the natural ligands. Both CD-417 and 4-HPR were much less efficient at reduction of colony formation although they still affected 45 and 58% reductions respectively of vehicle-treated colonies. All of the retinoids

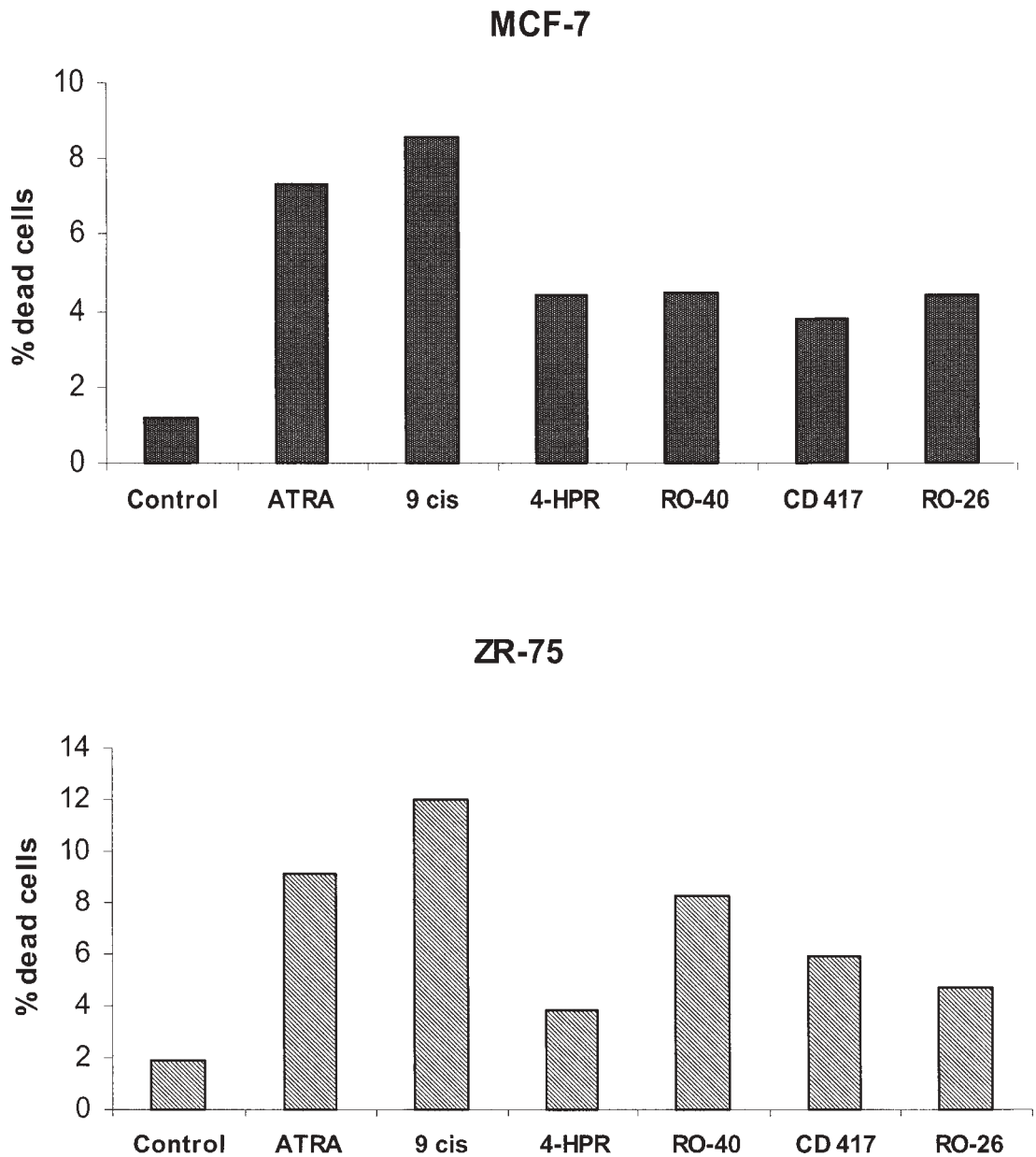

Fig. 6. Induction of cell death by retinoids. The percentage of dead cells in cultures of cells treated with retinoid or vehicle for 96 h was calculated using trypan blue exclusion staining. Numbers represent total counts including adherent and floating cells as described in ''Materials and Methods.'' The results are representative of two separate experiments. For each experiment, a minimum of 1,000 cells were enumerated in duplicate.

tested augmented Taxol inhibition of clonogenicity in the same rank order as they reduced clone formation when used alone without Taxol. Consistent with the growth inhibitory effects of retinoids, all ligand-treated colonies were smaller than those in control and Taxol only-treated cultures. Comparison with the protein expression studies indicated that inhibition of Bcl-2 and survivin expression by individual retinoids correlates well with their effects on Taxol sensitization.

DISCUSSION

Numerous studies have demonstrated the ability of retinoids to inhibit breast cancer cell growth and induce apoptosis. In terms of cell growth, three important targets have emerged as likely candidates for effectors of this outcome including the expression of both cyclin D1 and cdk2 and the transcriptional activity of AP-1. Given the importance of AP-1 regulated gene transcription in the proliferative response,

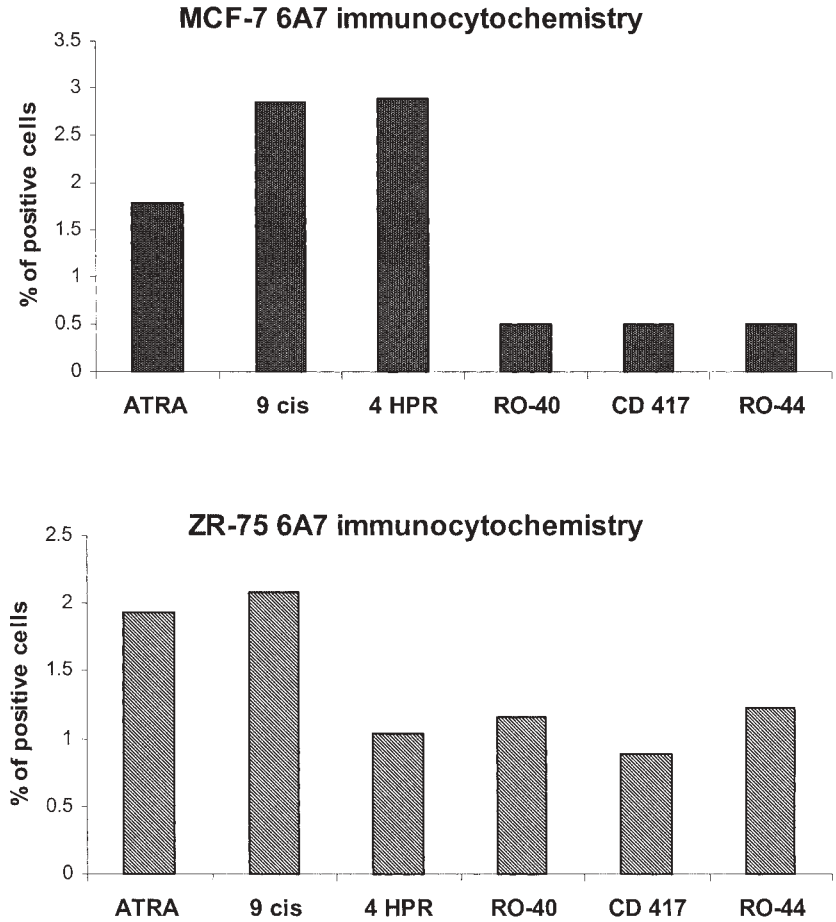

Fig. 7. Bax activation following retinoid treatment. The percentage of MCF-7 and ZR-75 cells containing mitochondrial translocated Bax protein detected using the 6A7 Bax monoclonal antibody were determined after a 72 h treatment with the indicated retinoids. Calculated percentages were derived from enumeration of a minimum of 1,000 cells on coverslips and are representative of two separate experiments.

TABLE II. Statistical Analysis of Relative 6A7 Immunohistochemistry in MCF-7; (a) and ZR-75 Cells; (b), Following Treatment With the Indicated Retinoids

	ATRA	9-*Cis*	4-HPR	Ro-40	CD-417
a: MCF-7					
9-*Cis*	0.09				
4-HPR	0.08	0.002			
Ro-40	0.006	<0.0001	<0.0001		
CD-417	0.004	<0.0001	<0.0001	NS	
Ro-44	0.005	<0.0001	<0.0001	NS	NS
b: ZR-75					
9-*Cis*	NS				
4-HPR	0.06	0.04			
Ro-40	NS	0.08	0.005		
CD-417	0.034	0.02	NS	NS	
Ro-44	NS	0.086	NS	NS	NS

Proportions of 6A7-positive cells on coverslips were compared by contingency table analysis. Numbers represent *P* values calculated for differences between the data sets.
NS: not significant.

notably its ability to activate the cyclin D1 promoter [Bakiri et al., 2000], regulation of AP-1 activity is thought to be a key feature the mechanism by which retinoids inhibit proliferation of breast cancer cells [Pfahl, 1993]. To this end new retinoids have been developed with the ability to block AP-1 activity but not induce transcription. These retinoids can inhibit AP-1 activity in vitro [Fanjul et al., 1994] and have been shown to prevent tumor promotion by TPA in DMBA-induced papillomas in mice [Huang et al., 1997]. Divergent reports have shown that inhibition could involve direct interactions between the AP-1 constituent c-*Jun* [Suzukawa and Colburn, 2002], prevention of JNK signaling pathway [Caelles et al., 1997] or extracellular regulated kinase [Benkoussa et al., 2002] both of which interfere with CBP recruitment to AP-1 response elements. In the present study,

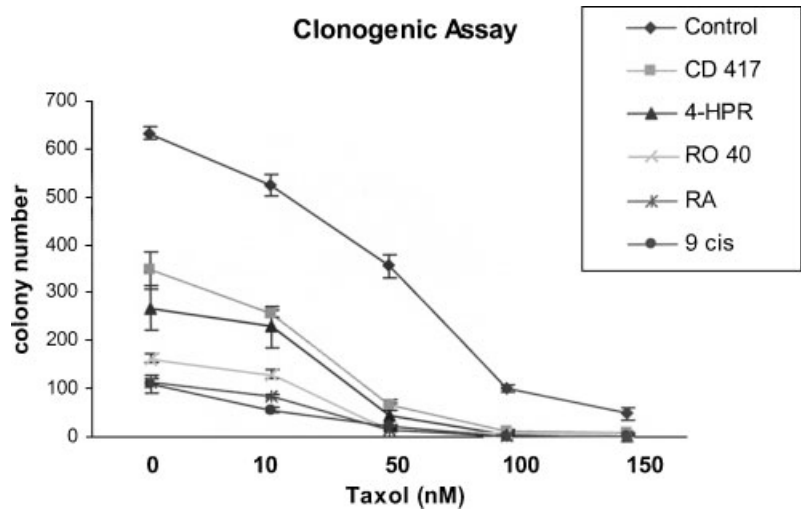

Fig. 8. Taxol sensitization by retinoids in MCF-7 cells. MCF-7 cells were pretreated with 1 μM of the indicated retinoid or vehicle for 3 days then cultured for 3 h in various concentrations of vehicle or Taxol. After removal of Taxol, retinoid was again added to the media and colony formation was allowed to proceed for a further 7 days. Colony numbers were evaluated in triplicate. Bars represent standard deviation.

all retinoids tested with the exception of the RARβ agonist were almost equally capable of inhibition of basal levels of AP-1 activity in transfected cells.

4-HPR produced distinct cell line-dependent effects. The mechanism of action of 4-HPR is not fully understood and while there are indications that it does not signal in a retinoid receptor dependent manner, there are at least two reports that suggest that 4-HPR interacts with the RARγ to activate transcription [Fanjul et al., 1996; Um et al., 2001] while its regulation of AP-1 is mediated by RARα, β, and RXRα [Fanjul et al., 1996]. While 4-HPR is reported to inhibit growth of several breast cancer cell lines through a mechanism involving downregulation of cyclin D1 and suppression of retinoblastoma protein phosphorylation [Panigone et al., 2000] its most pronounced effect in the present study was the induction of Bax translocation in MCF-7 cells. In some cells, 4-HPR is thought to induce apoptosis through the generation of reactive oxygen species (ROS) [Asumendi et al., 2002]. In addition others have shown that ATRA and 4-HPR produce distinctively different changes in the mitochondria [Sun et al., 1999; Poot et al., 2002]. The fact that 4-HPR had little effect on the two anti-apoptotic proteins studied here while still producing strong Bax activation indicates that its mechanism of action is dissociated from the regulation of these proteins. Unlike ATRA and 9-*cis* RA wherein Bax activation was commensurate with reduced levels of vital stain exclusion and determination of cell death, 4-HPR appeared to induce strong Bax translocation but a lower level of cell death. This could be a function of the difference in cell death programming induced by these ligands in which 4-HPR induces apoptosis with slower kinetics. It should be noted that the levels of apoptosis in our cultures never approached those reported by Poot et al. [2002] which may relate to the method of calculating total cell death as well as clonal differences in the cell lines.

It was of interest that the levels of expression of the RARβ were very low in both MCF-7 and ZR-75 cells in culture, albeit still detectable with the antibody employed, and yet CD417 was still significantly active in respect to some gene expression in both cell lines as well as AP-1 activity in MCF-7 cells. RARβ expression in the cells may be the result of low-level stimulation of the RARβ promoter by serum retinoids. Regardless, the level of RARβ required to mediate a response to this ligand is clearly very low. Of all the ligands tested, only ATRA was capable of inducing a robust RARβ expression and then only in MCF-7 cells. In contrast to other retinoids, the RARβ is thought to mediate its anti-tumor effect by inhibition of AP-1 activity in a ligand-independent manner [Lin

et al., 2000]. Based on this observation, the presence of higher levels of this receptor in response to ATRA in MCF-7 cells may have contributed to the maximal inhibition of AP-1 demonstrated by this ligand. Interestingly, Li et al. [1999] have reported AP-1 inhibition by a novel class of RARβ antagonists. Together with our results this suggests that even low levels of the RARβ can mediate ligand-inducible anti-proliferative responses without further auto-induction.

There are numerous reports that retinoids are capable of inducing programmed cell death as single agents or sensitizing cancer cells including breast and ovarian to death induced by other agents such as Taxol [Grunt et al., 1998; Wang et al., 2000; Vivat-Hannah et al., 2001]. In the present studies, we have examined the regulation of Bcl-2 and survivin as indicators of the ability of retinoids to either induce apoptosis alone or potentiate apoptosis induced by Taxol. In keeping with their abilities to sensitize cells to Taxol, both 9-*cis* RA and ATRA strongly downregulated Bcl-2 and survivin in both cell lines. Ro-40 also decreased expression of Bcl-2 and survivin predominantly in MCF-7 cells in a manner relative to Taxol sensitization. In contrast ligands which downregulated only one of these proteins had a reduced capability to sensitize to Taxol. Although we were unable to detect a phosphorylated species of Bcl-2, it has been postulated that the mechanism of synergy involves potentiation of the ability of paclitaxel to induce inactivating phosphorylation of Bcl-2 along with retinoid-mediated downregulation of Bcl-2 possibly as a result of degradation [Brichese et al., 2002]. Others have suggested that the synergy involves inhibition of AP-1 activity [Wang et al., 2000; Vivat-Hannah et al., 2001]. Taken together these data suggest that inhibition of both survivin and Bcl-2 expression predict optimal retinoid chemosensitization to Taxol. The fact that 4-HPR was as efficient in the activation of Bax as the natural ligands yet was less able to reduce Bcl-2 levels and synergize with Taxol clearly differentiates between mechanisms involved in the initiation of apoptosis and those responsible for sensitization to Taxol. Clonogenic inhibition in the presense or absence of Taxol also closely mirrored retinoid effects on S-phase percentages suggesting that growth inhibition contributed to reductions in the final numbers of colonies with more than 50 cells.

Our results show that the relative ability of ATRA and 9-*cis* RA to decrease levels of the cell cycle-associated genes considered here is generally commensurate with their ability to decrease S-phase percentages. However, the same is not true for the synthetic ligands which in some instances demonstrate anti-proliferative effects without strong regulation of these cell cycle genes or vice versa. Of the synthetic ligands used in this study, only the RARα ligand Ro-40 approached the effectiveness of the natural ligands in terms of both protein regulation and cell growth in MCF-7 and ZR-75 cells and Taxol synergy in MCF-7 cells. These results are in agreement with those of Schneider et al. [2000] who showed that the RARα was the pivotal receptor mediating growth inhibition and induction of apoptosis in SK-BR-3 and T47-D breast cancer cells. AP-1 inhibition and especially the induction of RARβ do not consistently predict the anti-proliferative or pro-apoptotic effects of a retinoid. The lack of correlation between retinoid induction of the RARβ and growth inhibition was also reported by Raffo et al. [2000]. Moreover, the extent to which a pan-agonist or receptor-specific ligand regulates target gene expression varies in a cell-dependent manner, the reasons for which could be multiple including coactivator/corepressor levels, relative receptor subtype levels, cellular metabolism of retinoids and even promoter access.

Together these results are consistent with the hypothesis that for each cell line a spectrum of genes must be turned on and or off by a given retinoid in order to achieve an outcome such as growth inhibition. The optimal combination includes genes that are regulated by variety of different receptor types since receptor specific ligands are not consistently able to regulate cell cycle genes or induce a comparable decrease in S-phase cells as do the natural ligands.

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