Retinoid Receptor-Dependent and Independent Biological Activities of Novel Fenretinide Analogues and Metabolites

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ABSTRACT

Fenretinide (4-HPR) is a retinoid analogue with antitumor and chemopreventive activities. In addition to 4-HPR, there are several other new phenylretinamides bearing hydroxyl, carboxyl, or methoxyl residues on carbons 2, 3, and 4 of the terminal phenylamine ring (N-(2-hydroxyphenyl)retinamide (2-HPR), N-(3-hydroxyphenyl)retinamide, N-(2-carboxyphenyl)retinamide, N-(3-carboxyphenyl)retinamide, N-(4-carboxyphenyl)retinamide, and N-(4-methoxyphenyl)retinamide (4-MPR)). It is hypothesized that these agents can act independent of the nuclear retinoid receptor pathway. To test this hypothesis directly, we have analyzed the activity of these phenylretinamides in vitro on a panel of F9 murine embryonal carcinoma cell lines, which includes wild-type (F9-WT) and mutant cells that have disrupted genes for both retinoid X receptor α and retinoic acid receptor γ retinoid receptors (F9-KO). The F9-KO cells lack almost all measurable response to all-trans-retinoic acid, the primary biologically active retinoid. Two distinct effects of retinamides were identified. The first is a rapid, dose-dependent induction of cell growth inhibition (reduced cell viability), and the second is a slower induction of differentiation and accumulation of cells in the G1 phase of the cell cycle that was observed with a concentration of 1 μM, for only those phenylretinamides bearing charged (hydroxyl or carboxyl) groups on the terminal phenylamine ring. The induction of differentiation and G1 accumulation was only observed in the F9-WT cells, indicating that this effect is receptor-dependent. 4-MPR, a major metabolite of 4-HPR, lacks a charged group on the terminal phenylamine ring and did not induce retinoid receptor-dependent effects, but did induce cell growth inhibition. Thus, 4-MPR may play a role in the clinical activity of 4-HPR. This study further reveals the mechanism of action of these novel phenylretinamides and supports continued investigation into their development as chemopreventive drugs.

INTRODUCTION

Retinoids are a class of chemical compounds, which include active metabolites of vitamin A (retinoic acid) as well as a diverse array of synthetic derivatives. Retinoids modulate a wide variety of cellular processes, including proliferation, differentiation, homeostasis, malignant transformation, and apoptosis (1). Retinoids also act pharmacologically to restore regulation of differentiation and growth in certain premalignant and malignant cells in vitro and in vivo. It is now well established that retinoids exert their effects primarily through nuclear retinoid receptor proteins. Retinoid receptors comprise two families of ligand-dependent, DNA-binding, transcriptional transactivators, RXRs and RARs, both members of the nuclear steroid hormone receptor superfamily (1, 2). Fenretinide (4-HPR) is one of the most promising retinoids for chemoprevention (3–5). It has a favorable toxicity profile, potent apoptosis-induction activity, biological activity in several preclinical systems, including all-trans- and 9-cis-retinoic acid-resistant neoplastic cells, and significant clinical chemopreventive activity in randomized trials for breast cancer and oral carcinogenesis (6, 7). In addition to 4-HPR, there are several other new phenylretinamides bearing hydroxyl, carboxyl, or methoxyl residues on carbons 2, 3, and 4 of the terminal phenylamine ring (2-HPR, 3-HPR, 2-CPR, 3-CPR, 4-CPR, and 4-MPR). One of these, 4-MPR, is a principle metabolite of 4-HPR in rodents and humans (8–10) and is the only one of these that lacks a charged group on the terminal ring. To date there have been few studies aimed at determining the activity of these compounds, with the exception of 4-CPR, which has been used extensively in Phase II clinical trials in China (11). The activity of this group of phenylretinamides for the growth inhibition of human oral epithelial cells (12), bladder transitional cell carcinoma cells (13), head and neck squamous cell carcinoma cells and non-small cell lung cancer cells (14),

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\[ ^{2} \text{The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoid} \]

\[ \text{X receptor; 4-HPR, N-(4-hydroxyphenyl)retinamide; 2-HPR, N-(2-hydroxyphenyl)retinamide; 3-HPR, N-(3-hydroxyphenyl)retinamide; 2-CPR, N-(2-carboxyphenyl)retinamide; 3-CPR, N-(3-carboxyphenyl)retinamide; 4-CPR, N-(4-carboxyphenyl)retinamide; 4-MPR, N-(4-methoxyphenyl)retinamide; RA, all-trans retinoic acid; WT, wild-type; F9-KO, F9 RXRa}\text{-}\text{KO; DAPI, 4',6-diamidino-2-phenylindole; KO, knockout; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.} \]
has been determined recently. These studies have shown that all of the novel phenylretinamides can induce cell death and suppress proliferation to various degrees, depending on the cell type, and that these effects were often comparable with 4-HPR, indicating that they may also have chemopreventive potential (13).

Previous studies have shown that 4-HPR could induce apoptosis in cells that are resistant to the most biologically active retinoid, all-trans-RA, suggesting that this activity may not involve retinoid receptors in some cell types (15–19). To directly test the hypothesis that 4-HPR can act through a receptor-independent mechanism, we used the F9 murine embryonal carcinoma retinoid receptor KO system previously. The F9 cell line is a well-established model system for the study of RA-induced differentiation (20). Investigators have used homologous recombination-mediated gene targeting to generate F9 cells that lack expression of RARs α and γ, RXRα, and pairwise combinations of RARα/RXRα and RARγ/RXRα (Refs. 21, 22; reviewed in Refs. 23, 24). It was determined that knocking out the most abundant RXR (RXRα) and the most abundant RAR (RARγ) in the same cell, abolishes almost all of the measurable retinoid receptor-mediated effects (22). By comparing the effects of 4-HPR on cell growth and differentiation between the F9-WT and F9-KO cells, our group could show that 4-HPR had two distinct effects (25). The first was a rapid induction of apoptosis requiring a high concentration (10 μM), which was observed in both the F9-WT and F9-KO cells. The second was a slower induction of differentiation, accompanied by an accumulation of cells in the G1 phase of the cell cycle, which is seen with a lower concentration (1 μM). This effect was only observed in the F9-WT cells, leading to the conclusion that the rapid high concentration effects are receptor-independent and the delayed low concentration effects are receptor dependent.

In this study we have used the F9 retinoid receptor KO system to determine whether the cell growth inhibition and other biological effects of the novel phenylretinamides involve activation of the nuclear retinoid receptors. We have also determined which of the phenylretinamides can induce receptor-dependent differentiation and growth arrest.

MATERIALS AND METHODS

Cell Lines and Cell Culture. F9-WT and F9-KO cells were cultured and treated with retinoids as described previously (21). RA and 4-HPR were obtained from Sigma Chemical Co. 2-HPR, 3-HPR, 2-CPR, 3-CPR, 4-CPR, and 4-MPR were obtained from the National Cancer Institute. Stock were made in DMSO to a concentration of 1 mM and diluted to the appropriate final concentrations in culture medium. Cells were photographed using a phase contrast microscope at a magnification of ×200.

Cell Viability and Cell Cycle Determination. Cell viability was determined using the MTT assay (26). Cells were plated in triplicate wells (1.5 × 10^4 cell/well) in 100 μl growth medium in 96-well plates and treated for various times with a range of retinoid concentrations (1–10 μM). At the appropriate times, MTT (Sigma Chemical Co.) was added to each well to a final concentration of 2 mM and the plates incubated at 37°C for 2 h. Medium was removed, and 100 μl DMSO was added to each well followed by mild shaking to dissolve the insoluble dark blue crystals. Absorbance was determined for each well using a Biolumin 960 multwell plate reader (Molecular Dynamics Inc.) at an absorbance wavelength of 570 nm. Standard curves for cell number were generated for the F9-WT and F9-KO cells by plating a broad range of known cell numbers (10^5–10^4 cells/well) and performing the MTT assay in the same day. Cell number for the treated cell samples was calculated from these curves. The percentage growth inhibition was calculated using the equation: [1 - (R/C)] × 100, where R and C represent the number of cells in retinoid-treated and control cultures, respectively. The cell cycle profile of F9-WT and F9-KO cells treated for 4 days with 1 μM retinoids was determined by cell cycle flow cytometry based on cellular DNA content, using an Epics Profile II cell sorter (Coulter Electronics, Inc.), as described previously (13). The percentage of cells in different phases of the cell cycle was determined from the raw data using the Epics Elite Flow Cytometry software.

Western Blotting. Whole cell extracts were purified and Western blotting performed as described previously (21). Blots were probed with a polyclonal goat antibody to mouse laminin B1 (Santa Cruz Biotechnology Inc.), stripped, and reprobed with a mouse monoclonal antibody to β-actin (Amersham). Secondary antibodies were horseradish peroxidase-conjugated anti-goat or antimouse (Zymed). Chemiluminescence detection was performed according to the manufacturer’s instructions (Amersham Life Science Inc.) followed by autoradiography.

Immunocytochemical Staining. F9 cells were grown on gelatinized glass coverslips in the presence or absence of the indicated retinoids for 4 days. After fixation in 1% paraformaldehyde, coverslips were incubated with Superblock blocking solution (Pierce) for 1 h at room temperature to block nonspecific antigen sites. After washing three times in PBS, samples were incubated overnight at 4°C with a rabbit polyclonal antibody specific for mouse type IV collagen (Sigma). Secondary antibody detection was performed using an Alexa 546-conjugated goat antirabbit antibody (Molecular Probes, Inc.). Cells were then stained with DAPI and mounted with coverslips. Images were captured with a QuantiX digital camera on a fluorescence microscope at ×600 magnification under oil immersion. Sequential captures were made with red and blue filters, and then merged to allow visualization of DAPI-stained nuclei and Alexa 546-stained (red) type IV collagen.

RESULTS

All of the Novel Phenylretinamides Induce Retinoid Receptor-Independent Cell Growth Inhibition. To compare the biological activity of the novel phenylretinamides (Fig. 1A) with the well-studied structural analogue 4-HPR, we first tested their growth-inhibitory effect on F9-WT and F9-KO cells in a cell viability assay. Treatment with 10 μM 4-HPR, a pharmacologically achievable concentration in target tissue (5), was shown previously to cause an equal growth-inhibitory effect on both F9-WT and F9-KO cells, indicating that that this effect is retinoid receptor independent (25). Treatment of F9-WT and F9-KO cells with the novel phenylretinamides for 2 days resulted in a dose-dependent growth inhibition (Fig. 1B). The magnitude of growth inhibition varied among the retinoids, with
Mechanism of Action of Novel Phenylretinamides

Barnes and their abbreviations used in Fig. 1 are shown. The hydroxyl, carboxyl, or methoxyl groups, respectively. The chemical names and their abbreviations used in Fig. 1B, and Figs. 3–5 are shown. B, F9-WT and F9-KO cells were treated with 1, 5, or 10 μM of the indicated retinamides for 2 days, and the cell viability of triplicate cultures was measured by the MTT assay. □ and ■ represent the mean percentage of inhibition of cell viability, relative to untreated controls, for F9-WT and F9-KO cells, respectively. Error bars ± SE for triplicate cultures.

3-HPR, 2-CPR, and 4-MPR being nearly as effective as 4-HPR at the highest concentration (10 μM). The effect was also retinoid receptor-independent because it was similar for F9-WT and F9-KO cells in most cases (Fig. 1B, compare white and black bars). In contrast, the growth-inhibitory effect of RA was greater for the F9-WT cells, particularly at the 5 μM and 10 μM concentrations, as has been shown previously (27).

Retinamides Bearing Charged Moieties on the Phenylamine Ring Induce Retinoid Receptor-Dependent G1 Accumulation. RA induces a substantial retinoid receptor-dependent increase in the percentage of cells in G1 in F9 cells (21, 27), consistent with its function as a suppressor of cell growth and inducer of differentiation (20). Because we have shown previously that a subtoxic concentration of 4-HPR (1 μM) can induce retinoid receptor-dependent differentiation in F9 cells in a manner similar to RA (25), we next determined whether 4-HPR or the other phenylretinamides could also alter the cell cycle distribution. Flow cytometric analysis revealed that a 4-day treatment of cells with a 1 μM concentration of all of the phenylretinamides bearing a charged group on the terminal phenylamine ring (charged phenylretinamides), and not 4-MPR, which has an uncharged group on the ring, increased the G1 fraction of cells (Fig. 2; Table 1). As was shown previously for RA, this effect was only observed in the F9-WT and not F9-KO cells, indicating the requirement for retinoid receptors for this effect (compare Fig. 2, top panels with bottom panels). Also of note is the higher percentage of cells in S phase for the F9-KO cells compared with F9-WT. This has been a consistent observation (27) and may result from a deregulation of proliferation control in the F9-KO cells (see “Discussion”).

Charged Phenylretinamides Induce Retinoid Receptor-Dependent Differentiation. We next determined whether the phenylretinamides could induce receptor-dependent primitive endodermal differentiation, as was shown previously for 4-HPR (25). For this experiment it was necessary to treat cells with 1 μM phenylretinamides for 5 days. All of the charged phenylretinamides, and not 4-MPR, induced a morphological differentiation in the F9-WT cells that was similar to RA-treated cells (Fig. 3, compare F9-WT panels labeled 2H, 3H, 4H, 2C, 3C, and 4C to C and 4M). Cells became flattened, separated from one another, and bore extended branched processes, all characteristics of the primitive endodermal phenotype (20). As expected, none of the retinoids induced morphological differentiation in the F9-KO cells, indicating the requirement for retinoid receptors for this effect (Fig. 3, F9-KO). The absence of differentiation observed with 4-MPR treatment is consistent with its lack of effect on cell cycle distribution.

To further confirm that the charged phenylretinamides induce primitive endodermal differentiation, we determined the expression of two well-established primitive endodermal markers, laminin B1 and type IV collagen (28), by Western blotting and immunohistochemistry, respectively. All of the charged phenylretinamides, and not 4-MPR, induced laminin B1 expression in the F9-WT cells to an extent similar to RA (Fig. 4). None of the retinoids induced laminin B1 expression in the F9-KO cells, in agreement with the absence of morphological differentiation. Similarly, only the charged phenylretinamides caused an increase in expression of type IV collagen over existing expression levels, and this was observed only in the F9-WT cells (Fig. 5, compare RA, 2H, 4H and 4C to C and 4M on the left side of the diagram). Note that the effect of RA on type IV collagen expression was more pronounced than for the charged phenylretinamides and that a very low level of type IV collagen expression could be detected in the RA treated F9-KO cells (Fig. 5 RA on right side). This is likely because of activation of the low levels of retinoid receptors (RARα, RXRβ, and RXRγ), which are still expressed in the F9-KO cells (22).

DISCUSSION

In this study we demonstrate that a panel of phenylretinamides bearing hydroxyl, carboxyl, or methoxyl substitutions...
at the 2, 3, and 4 positions of the terminal phenylamine ring can, like 4-HPR, induce rapid retinoid receptor-independent cell growth inhibition in F9 embryonal carcinoma cells. All but one of these phenylretinamides, 4-MPR, have a charged group on the terminal phenylamine ring at the approximate location of the C15 position of RA and can induce retinoid receptor-dependent primitive endodermal differentiation, along with G1 phase accumulation. The correlation between the presence of a charged terminal group at this position with biological activity is well established for other retinoids (29). This provides a plausible explanation for the inability of 4-MPR to stimulate retinoid receptor-dependent actions and is in agreement with a previous study, which showed that 4-MPR could not activate retinoid receptors in cotransfection reporter assays, whereas 4-HPR could (30). In the same study it was demonstrated that a 4-day treatment with 20 μM 4-MPR did not cause substantial growth inhibition for several cell lines (30), in contrast with our finding of an ~75% growth inhibition induced by 4-MPR in both the F9-WT and F9-KO cells using a 10 μM concentration (Fig. 1B). This discrepancy suggests a cell line-specific sensitivity to 4-MPR.

4-MPR is the most abundant metabolite of 4-HPR detected in circulation and in breast adipose tissue of patients treated with 4-HPR (5, 9, 10). Because of this, studies were undertaken previously to determine whether 4-MPR could have tumor-suppressive activity. It was demonstrated that sensitivity to 4-HPR correlated with the appearance of 4-MPR as a metabolite among a panel of breast carcinoma and melanoma cell lines (31). These authors also observed a similar growth-inhibitory effect for 4-MPR to the one we demonstrate here, for cells treated with the highest concentration (10 μM), supporting the idea that 4-MPR could contribute to the antitumor effects of 4-HPR. More comprehensive studies with animal models will be necessary to better determine whether 4-MPR has tumor-suppressive activities on its own.

The suppression of cell viability, or growth inhibition, induced by retinoid treatment could be because of an induction

Table 1  Cell cycle distribution after 4 days of treatment with 1 μM phenylretinamides

The percentage of cells in the indicated phases of the cell cycle for the histograms shown in Fig. 2 are shown.

<table>
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<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>% G1/G0</th>
<th>% S</th>
<th>% G2</th>
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<tr>
<td>F9-WT</td>
<td>Control</td>
<td>30</td>
<td>57</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>2HPR</td>
<td>46</td>
<td>41</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>3HPR</td>
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<td>39</td>
<td>14</td>
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<tr>
<td></td>
<td>4HPR</td>
<td>50</td>
<td>37</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>2CPR</td>
<td>46</td>
<td>42</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>3CPR</td>
<td>37</td>
<td>49</td>
<td>14</td>
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<tr>
<td></td>
<td>4CPR</td>
<td>45</td>
<td>40</td>
<td>15</td>
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<td>4MPR</td>
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<td>12</td>
</tr>
<tr>
<td></td>
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<td>11</td>
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<td>70</td>
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<tr>
<td></td>
<td>RA</td>
<td>20</td>
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<td>11</td>
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Fig. 2  Charged phenylretinamides induce retinoid receptor-dependent G1 accumulation. Subconfluent cultures of F9-WT (top panels) and F9-KO cells (bottom panels) were grown for 4 days in the presence of 1 μM of the indicated retinoids and analyzed by flow cytometry. The X-axis indicates the integrated fluorescence intensity and the Y-axis particle number. Fifteen to 20,000 particles are represented in each histogram.
of cell killing, a reduction in proliferation rate, or both. We have attempted to measure apoptosis induction after phenylretinamide treatment using two methods: a DAPI-direct DNA staining method and the fluorometric Annexin V staining assay. We found that apoptosis increased only slightly upon phenylretinamide treatment for F9-WT and F9-KO cells (data not shown). This is similar to our previous results for human bladder transitional cell carcinoma cells (13) and is not surprising, because apoptosis is a rapid process, taking as little as 4 h to complete (32). The F9 cells, like transitional cell carcinoma cells, disintegrate and disperse into the medium during later stages of apoptosis, making counting of all but the cells in early stages of apoptosis impossible. Although the percentage of cells captured in the process of apoptosing rarely exceeded 6%, this amount could represent a substantial amount of apoptosis, resulting in a large percentage of growth inhibition over time.

By flow cytometric analysis, we observed an increased proportion of cells in the G1 phase of the cell cycle after a 4-day treatment with the lowest concentration (1 μM) of only the charged phenylretinamides, indicating a reduction in proliferation rate. This effect was observed only in the F9-WT and not in the F9-KO cells. Because it is clear that the F9-KO cells are growth inhibited to a similar extent as the F9-WT cells by the phenylretinamides, we conclude that this growth inhibition is receptor independent and that it must be attributable mainly to

Fig. 3 Charged phenylretinamides induce retinoid receptor-dependent morphological differentiation. F9-WT and F9-KO cells were grown in monolayer culture for 24 h and then treated with a 1 μM of 2-, 3-, and 4-HPR (2, 3, and 4H, respectively), 2-, 3-, and 4-CPR (2, 3, and 4C, respectively) and 4-MPR (4M) or vehicle as control for 5 days with media change and new retinoids added at day 2. Cells were photographed on a phase contrast microscope at ×200 magnification.

Fig. 4 Charged phenylretinamides induce retinoid receptor-dependent expression of laminin B1. F9-WT and F9-KO cells were grown and treated as in Fig. 3. Whole cell extracts (25 μg) were Western blotted by SDS-PAGE and sequentially probed with anti-Laminin B1 (LnB1) and anti-β-actin antibodies. 2, 3, and 4H; 2, 3, and 4C; and 4M indicate 2-, 3-, and 4-HPR; 2-, 3-, and 4-CPR; and 4-MPR, respectively.
cell killing, by induction of apoptosis and/or necrosis. Efforts are currently underway to investigate the precise mechanisms of phenylretinamide-induced cell killing.

The flow cytometry analysis also revealed a feature of the F9 KO system that may have bearing on proliferation control by retinoids. The F9-KO cells had a higher percentage of cells in S phase under normal growth conditions (Fig. 2; Table 1) in agreement with previous observations (27). The F9-KO cells were originally generated by knocking out the RARγ gene in an RXRα-KO cell clone (21, 22, 27). The parental RXRα-KO cells had a higher basal rate of proliferation than the parental F9-WT cells, which resulted in a 2–3 h shorter doubling time (21). The F9-KO cells used in the present study have retained a higher proliferation rate, similar to the parental RXRα-KO cell line (data not shown). The shorter doubling time and increase percentage of cells in S phase may reflect a perturbation of cell cycle control caused by disruption of the retinoid signaling pathway in the F9-KO cells and will be further investigated.

We have shown that the novel phenylretinamides bearing a hydroxyl or carboxyl group on their terminal phenylamine ring (2,3,4-HPR and 2,3,4-CPR) elicit retinoid receptor-dependent differentiation and accumulation of cells in G1. The one phenylretinamide analogue tested lacking a charged group at this position, 4-MPR, does not. Because RA-induced differentiation of F9 cells is clearly retinoid receptor-dependent (21, 23, 33), these findings are in agreement with previous studies by our group and others showing the apparent activation of retinoid receptors by 4-HPR (25, 30, 34, 35). It is known that 4-HPR and 4-MPR bind the RARs poorly (17, 36), and no information is yet available on retinoid receptor binding for the other phenylretinamides. Therefore, it is not clear how these compounds may act through the RAR/RXR pathway to induce differentiation. We have suggested previously four possible explanations for the ability of 4-HPR to induce retinoid receptor-dependent differentiation in this system. These can be extended to the novel phenylretinamides and are as follows: (a) direct binding to and activation of retinoid receptors; (b) displacement of RA or other bioactive retinoids from membrane-bound intracellular stores; (c) alteration of retinoid metabolism such that synthesis of bioactive retinoids is enhanced or catabolism is inhibited; and (d) hydrolysis to RA, which could directly activate the retinoid receptors (see the discussion in Ref. 25 for a more detailed explanation of the different possibilities). On the basis of our present findings, in which only the uncharged phenylretinamide 4-MPR is unable to induce differentiation and G1 accumulation, we favor the first explanation. It remains possible that for unknown reasons 4-MPR, and not the others, is defective for displacing bioactive retinoids, inducing changes in retinoid metabolism, or in conversion to a bioactive retinoid. Therefore, these other possibilities cannot be formally ruled out without additional studies.

4-HPR is less toxic than other retinoids and shows great promise as a cancer chemopreventive drug (37, 38). It is currently being tested in a number of chemoprevention trials for breast, prostate, cervical, skin, ovary, and lung cancer, as well as transitional cell carcinoma of the bladder (37). Although the present study was conducted with an in vitro system, we would propose that the cancer therapeutic and chemopreventive efficacy of 4-HPR, and by extension the other charged phenylretinamides, in cancer patients might be because of its dual role as both a chemotoxic agent (receptor-independent) and as a differentiating agent (receptor-dependent). Tumor cells that can escape toxic effects at initial stages of exposure to high concentrations of 4-HPR would then be susceptible to differentiating effects of lower concentrations. We additionally propose that 4-MPR, as a principle metabolite of 4-HPR in humans, may contribute to the

Fig. 5  Charged phenylretinamides induce retinoid receptor-dependent up-regulation of type IV collagen. F9-WT and F9-KO cells were grown and treated as in Fig. 3 with 1 μM of the indicated retinoids. Cells were fixed and probed with an anticollagen IV antibody followed by an Alexa546-conjugated secondary antibody and counterstained with DAPI as described in “Materials and Methods.”
chemotherapeutic effects of 4-HPR, possibly through the same retinoid receptor-independent mechanisms. Additional studies in animal models comparing the effects of 4-HPR, 4-MPR, and the other phenylretinamides on tumorigenesis will be necessary to establish an in vivo correlate with these results.

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