Short Communication

Effects of Novel Phenylretinamides on Cell Growth and Apoptosis in Bladder Cancer[1]

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Abstract

Superficial bladder cancer is a major target for chemoprevention. Retinoids are important modulators of epithelial differentiation and proliferation and are effective in the treatment and prevention of several epithelial cancers. One class of compounds, the retinamides, is structurally similar to other retinoids but have the added feature of being potent apoptosis inducers. Among these, fenretinide (*N*-[4-hydroxyphenyl]retinamide), or 4HPR, has promise for bladder cancer chemoprevention and is currently under Phase III study in this setting. In addition to 4HPR, there are several new structurally related phenylretinamides bearing hydroxyl, carboxyl, or methoxyl residues on carbons 2, 3, and 4 of the terminal phenylamine ring [designated *N*-(2-hydroxyphenyl)retinamide, *N*-(3-hydroxyphenyl)retin amide, *N*-(2-carboxyphenyl)retinamide, *N*-(3-carboxyphenyl)retin amide, *N*-(4-carboxyphenyl)retinamide, and *N*-(4-methoxyphenyl)retinamide, respectively]. The objective of this study was to compare the growth inhibitory and apoptotic effects of these phenylretinamides with 4HPR in human bladder transitional cell cancer-derived cell lines of varying histological grade (RT4, grade 1; UM-UC9 and UM-UC10, grade 3; and UM-UC14, grade 4) by cell counting, cell cycle fluorescence-activated cell sorter analysis and a dual stain apoptosis assay. All of the seven phenylretinamides reduced cell number, altered the cell cycle distribution, and induced apoptosis when administered at a concentration of 10 μM, which is within the pharmacologically achievable range. Although the relative potencies of the phenylretinamides varied depending on the cell line, *N*-(3-hydroxy phenyl)retinamide was the most active with significantly greater growth inhibition than 4HPR in all of the four cell lines. These *in vitro* findings warrant further study of these novel phenylretinamides, which may have potential as preventive or therapeutic agents in transitional cell cancer.

Introduction

TCC[3] of the bladder is predicted to result in 53,200 new cancer cases for 2000 (1). Approximately 70% of TCC cases are superficial, low grade, noninvasive papillary tumors (2, 3). However, of these cases as many as 80% will recur. Patients require frequent reexamination involving cystoscopy, often followed by transurethral resection or, in some cases, cystectomy for invasive cancer (2). It is hypothesized that bladder cancer cells, like other cancer cell types, may have undergone a defect in the differentiation process, which results in dysregulated proliferation and the ability to invade and metastasize (3). Unfortunately, the urothelial differentiation process is not well delineated, making it difficult to assess changes in differentiation during malignant progression. As with other epithelial tissues, retinoids are likely to play a role in urothelial differentiation.

Retinoids are a class of chemical compounds that include active metabolites of vitamin A (retinol), as well as a diverse array of synthetic derivatives. Retinoids have been shown to modulate a wide variety of cellular processes, including proliferation, differentiation, homeostasis, malignant transformation, and apoptosis (4). 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, RARs and retinoid X receptors, both members of the nuclear steroid hormone receptor superfamily (4, 5). Fenretinide, or 4HPR, is one of the most promising retinoids for chemoprevention (6–8). It has a favorable toxicity profile, potent apoptosis-induction activity, biological activity in several preclinical systems, including ATRA-resistant and 9-*cis* retinoic acid-resistant neoplastic cells, and significant clinical chemopreventive activity in randomized trials for breast cancer and oral carcinogenesis (9, 10). Like other retinoids, 4HPR can up-regulate the expression of one of the RARs, RARβ (11, 12), and activate transcription of retinoic acid response elements by RARs (13, 14). Also, RARβ expression was associated with the antiproliferative action of 4HPR in ovarian cancer cells (12).

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[3] The abbreviations used are: TCC, transitional cell carcinoma; RAR, retinoic acid receptor; 4HPR, *N*-(4-hydroxyphenyl)retinamide; 2CPR, *N*-(2-carboxyphenyl)retinamide; 3CPR, *N*-(3-carboxyphenyl)retinamide; 4CPR, *N*-(4-carboxyphenyl)retinamide; 2HPR, *N*-(2-hydroxyphenyl)retinamide; 3HPR, *N*-(3-hydroxyphenyl)retinamide; 4MPR, *N*-(4-methoxyphenyl)retinamide; ATRA, all-*trans* retinoic acid; PI, propidium iodide; DAPI, 4′,6-diamidino-2-phenylindole.

However, the ability of 4HPR to induce apoptosis in cells that are resistant to ATRA suggests that this activity may not involve retinoid receptors in some cell types (15–19).

Several retinoid analogues have shown chemopreventive activity in rat and murine bladder carcinogenesis models, presumably by restoring the normal differentiation state of premalignant and malignant cells (20, 21). Whereas mixed clinical results have been obtained for TCC therapy, some success has been achieved with retinoids in TCC chemoprevention. Currently, there is an National Cancer Institute-sponsored multicenter Phase III trial of 4HPR in the prevention of superficial bladder cancer (2). Recently, several new retinamides bearing hydroxy, carboxy, or methoxy substitutions on the terminal phenylamine ring in human TCC cell lines have been isolated. With the exception of 4CPR, which has been used extensively in Phase II clinical trials in China, there have been few studies aimed at determining their activity (22). Recently, these retinamides have been compared with a panel of potential chemopreventive agents for the growth inhibition of human oral epithelial cells (23). Among the phenylretinamides, 2CPR showed the greatest selectivity for growth inhibition of premalignant and malignant cells (23). The objective of this study was to compare the biological activity of several of these novel phenylretinamides with 4HPR in cultured TCC cells.

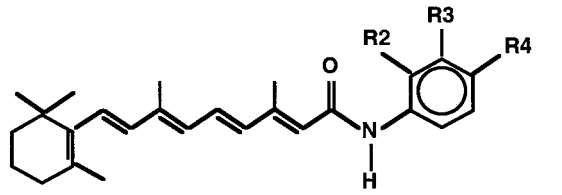
Materials and Methods

Cell Culture and Retinamides. UM-UC (24, 25) and RT4 cells were cultured in 50% DMEM low glucose/50% F12 medium containing 10% fetal bovine serum at 37°C in the humidified atmosphere of 5% CO_2/95% air. ATRA and 4HPR were obtained from Sigma Chemical Co. 2HPR, 3HPR, 2CPR, 3CPR, 4CPR, and 4 MPR were obtained from the National Cancer Institute. Stock solutions were made in DMSO at a concentration of 10 mM and diluted to the appropriate concentrations in culture media.

Cell Counting Assay. Cells were plated at identical densities in triplicate culture wells of a 24-well plate, treated the following day with retinoids along with addition of fresh media, trypsinized after 72 h of treatment, resuspended to appropriate dilutions, and counted on a Coultronics Particle Counter (Coultronics, Inc.). The percentage growth inhibition by retinoids was calculated using the equation: $(1 - R/C) \times 100$, where R and C represent the number of cells in retinoid-treated and control culture, respectively.

Cell Cycle Determination. The cell cycle profile of untreated and retinamide-treated cells was determined by cell cycle flow cytometry based on cellular DNA content using an Epics Profile II cell sorter (Coulter Electronics, Inc.), equipped with a 15-W argon laser at a wavelength of 488 nm and an emission wavelength of 575 nm. Subconfluent cultures of untreated cells or cells treated for 3 days with 10 μM of the indicated retinamides or 1 μM ATRA were trypsinized, resuspended, combined with their supernatants, and fixed in 70% ethanol at 4°C. After two washes in PBS, the cells and subcellular particles were incubated in 1 mg/ml RNase A (59 Kunitz units/mg; Sigma Chemical Co.) for 30 min at 37°C, centrifuged, and resuspended in PBS at a concentration of approximately 10^6 cells/ml. PI was added to a final concentration of 50 μg/ml immediately before sample analysis. The percentage of cells in the different phases of the cell cycle was determined from the raw data using the Epics (R) Elite Flow Cytometry software.

Apoptosis/Cell Death Assays. The quantitation of apoptosis and necrosis was performed using a modified PI and DAPI double staining assay essentially as described previously (19).



Chemical name	Abrev.	R2	R3	R4
N-(2-hydroxyphenyl)retinamide	2HPR	OH	H	H
N-(3-hydroxyphenyl)retinamide	3HPR	H	OH	H
N-(4-hydroxyphenyl)retinamide	4HPR	H	H	OH
N-(2-carboxyphenyl)retinamide	2CPR	COOH	H	H
N-(3-carboxyphenyl)retinamide	3CPR	H	COOH	H
N-(4-carboxyphenyl)retinamide	4CPR	H	H	COOH
N-(4-methoxyphenyl)retinamide	4MPR	H	H	CH_3O

Fig. 1. Structure of retinamides. The numbers *R2, R3,* and *R4* represent chemical groups at the ortho, meta, and para ring positions, respectively. OH, COOH, and CH_3O represent the hydroxy, carboxy, or methoxy groups, respectively. The chemical names corresponding to ring positions occupied by chemical groups are shown.

Cells were plated at a density of 4×10^5 cells/10-cm dish and allowed to attach overnight before treatment with 10 μM 4HPR for 3 days. PI was added to the growth medium to a final concentration of 20 μg/ml 1 h before the end of the assay. The cells were trypsinized and combined with the culture supernatant, followed by resuspension in growth medium containing 5 μM DAPI dye and incubated at 37°C for 15 min. Approximately 3×10^4 cells were attached to slides using a Cytospin centrifuge (Shandon and Lipshaw, Inc.) and observed visually for quantitation of apoptosis and necrosis. Cells exhibiting the DNA condensation characteristic of apoptosing cells, as revealed by DAPI staining but which did not stain intensely with PI, were scored as apoptotic. Necrotic cells were scored as those staining substantially above background levels with PI. The percentage of apoptotic cells was calculated for at least 150 cells/sample, in three or more separate fields. Cytospun cells were photographed on a fluorescence microscope, sequentially using filters for both DAPI dye and PI, at a magnification of 400×.

Results

To determine whether 4HPR and the new phenylretinamides (Fig. 1) could inhibit the growth of TCC cells, we conducted cell-counting assays for a range of concentrations and treatment times. The extent of reduction in cell number is expressed as a percentage of growth inhibition compared with untreated controls. A significant percentage growth inhibition required 3 days of continuous treatment at a concentration of at least 1 μM (Fig. 2). At a concentration of $\leq$0.1 μM, none of the phenylretinamides studied had significant effects on cell growth or apoptosis (data not shown). The growth inhibition varied between the four cell lines, which were derived from tumors of varying histological grade: RT4, grade 1; UM-UC14, grade 2; and UM-UC9 and UM-UC10, grade 3. No correlation between grade and sensitivity to the phenylretinamides was observed.

To determine the relative contribution of cell cycle alterations to the observed growth inhibitory effects, the cell cycle profile of the TCC cell lines was determined after a 3-day treatment with a 10 μM concentration of the same phenylretinamides (Table 1; 4HPR results are highlighted in *bold* for comparison). One anticipated effect of retinoids on the cell cycle is a G_1-G_0 arrest, as evidenced by an increase in the

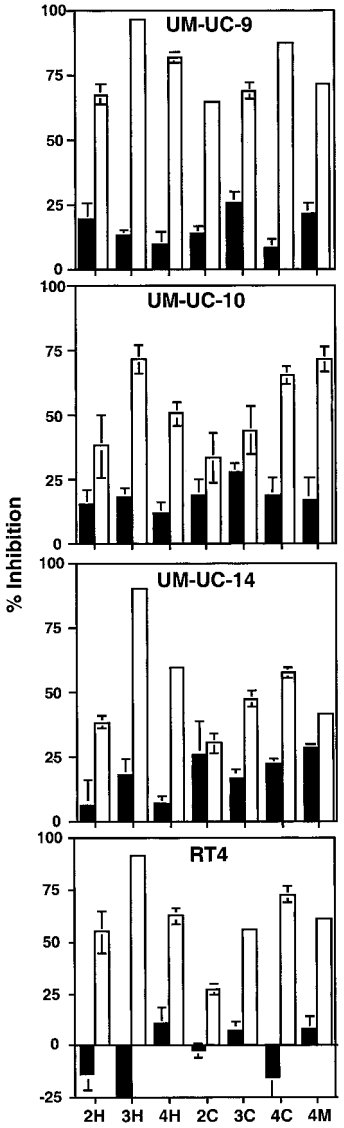


Fig. 2. Several new retinamides have cell growth inhibitory activity that is comparable with 4HPR. TCC cell lines were treated with 1 μM (■) or 10 μM (□) of the retinamides for 3 days, and the cell viability was measured by cell counting. The percentage growth inhibition by the drug treatments is calculated using the equation: $(1 - R/C) \times 100$, where R and C represent the number of cells in treated and control cultures, respectively. *Error bars* represent the SE for triplicate samples.

Table 1 Cell cycle distribution after 3 days treatment with 10 μM retinamides[a]

Cell line	Treatment	% G_1-G_0	% S	% G_2	% Growth Inh
UM-UC9	Control	42	36	23	
	2HPR	45	35	20	68
	3HPR	58	19	23	97
	4HPR	**44**	**35**	**21**	**83**
	2CPR	14	68	18	65
	3CPR	42	34	23	69
	4CPR	66	24	10	88
	4MPR	31	38	31	72
UM-UC10	Control	43	33	24	
	2HPR	45	32	23	38
	3HPR	49	32	19	72
	4HPR	**50**	**29**	**21**	**51**
	2CPR	25	55	20	34
	3CPR	41	33	26	44
	4CPR	62	19	19	66
	4MPR	39	31	31	72
UM-UC14	Control	40	34	26	
	2HPR	53	29	18	39
	3HPR	56	25	19	91
	4HPR	**55**	**28**	**17**	**60**
	2CPR	23	68	9	31
	3CPR	50	31	20	48
	4CPR	63	24	13	58
	4MPR	41	30	29	42
RT-4	Control	57	27	17	
	2HPR	67	18	15	55
	3HPR	72	15	14	92
	4HPR	**65**	**19**	**16**	**63**
	2CPR	57	32	12	28
	3CPR	60	23	17	57
	4CPR	76	10	14	73
	4MPR	61	23	16	62

[a] Cell cycle profile of TCC cell lines treated with several retinamides. Numbers shown are the percentage of live cells in each phase of the cell cycle (G_1-G_0 phase, S phase, and G_2 phase) as determined by fluorescence-based flow cytometry. The last column on right indicates the percentage growth inhibition of cells after treatment with 10 μM retinamides, corresponding to the graphed data in Fig. 2, *white bars*. The results for 4HPR are in **bold** to facilitate comparison.

percentage of cells in the G_1-G_0 phase. This could account for the observed growth inhibition. A comparison of the change in percentage of G_1-G_0 from controls to treated cells (Table 1, column 3, % G_1-G_0) with the percentage growth inhibition for each treatment (Table 1, column 6, % Growth Inh) indicates that G_1-G_0 arrest alone cannot account for the growth inhibition. Whereas the increased percentage of G_1-G_0 observed for many of the treatments is likely to contribute to growth inhibition, particularly for the 3HPR and 4CPR treatments for all of the four cell lines, the high growth inhibition for other treatments in the absence of elevated G_1-G_0 (*e.g.,* 2HPR treatment for UM-UC9 and UM-UC10 cells) strongly suggests that these phenylretinamides also induce cell death. Treatment with 3HPR and 4HPR resulted in a mild increase in percentage of G_1-G_0 for all of the four cell lines, whereas 2HPR only increased the percentage of G_1-G_0 for UM-UC14 and RT4 cells. 4CPR treatment caused the greatest increase in percentage of G_1-G_0 for all of the four cell lines (Table 1, column 3). An interesting exception to the other phenylretinamides was the decrease in percentage of G_1-G_0 for 2CPR treatment with concomitant increase in percentage S phase for UM-UC9, UM-UC10, and UM-UC14 cells. This potentially indicates a block in the S phase to G_2 phase transition. Cells were also treated with 1 μM ATRA, a dose that can activate the retinoid receptor-mediated signaling pathway in many cell types, for comparison. This produced little or no effect on growth inhibition or cell cycle distribution for any of the cells, indicating a relative lack sensitivity to retinoids (data not shown).

To better characterize the cell cycle alterations caused by the phenylretinamides, we have examined the expression of several cell cycle regulatory proteins, including Rb, p27, and p16, by Western blotting in the UM-UC9 and UM-UC10 cells. Thus far, we have observed no correlation between the expression of these proteins and the sensitivity to growth inhibition by any of the phenylretinamides and have observed no alterations in their expression upon phenylretinamide treatment (data not shown). We have also determined the level of Rb phosphorylation on serine 795 (with a phosphoserine 795 specific anti-

body) and see no alteration in response to phenylretinamide treatment (data not shown).

It has been shown previously (26) that 4HPR induces apoptosis in TCC cell lines, including UM-UC9 cells. We have also shown that the cell growth inhibitory effect of 4HPR in the F9 murine embryonal carcinoma cell line involves apoptosis induction, which was also accompanied by the induction of necrosis (19). To determine whether the new phenylretinamides also induce apoptosis and/or necrosis in this system, we have conducted a dual stain procedure with the fluorescent DNA-binding dyes DAPI and PI, which are membrane permeable and impermeable, respectively. Cells undergoing apoptosis exhibit a distinct morphological alteration characterized by condensation and fragmentation of nuclear DNA, which can be readily detected by intense staining with DAPI above the level normally seen for noncondensed DNA (Fig. 3*A*). Treatment of all of the four cell lines for 3 days with 10 μM phenylretinamides but not 10 μM ATRA resulted in the appearance of apoptosing cells (Fig. 3*B* and data not shown). The quantitation of phenylretinamide-induced apoptosis is shown for the UM-UC9 cells and is representative of the results observed for the other cell lines. In contrast to apoptosing cells, necrotic cells lose membrane integrity and leak their contents into the surroundings. Such cells are able to take up PI, which results in an intense red staining of DNA (Fig. 3*A*). Whereas some necrosis was observed in the phenylretinamide-treated cultures, it was very rare and contributed little to the percentage of dying cells. It should be noted that apoptosis is a rapid process, taking as little as 4 h to complete (27). In this cell culture system, most cells that have undergone apoptosis disintegrate, disperse into the media, and can no longer be counted. Although the percentage of cells captured in the process of apoptosing never exceeded 10% at the time of cell harvest, this amount could easily account for a dramatic percentage growth inhibition over time.

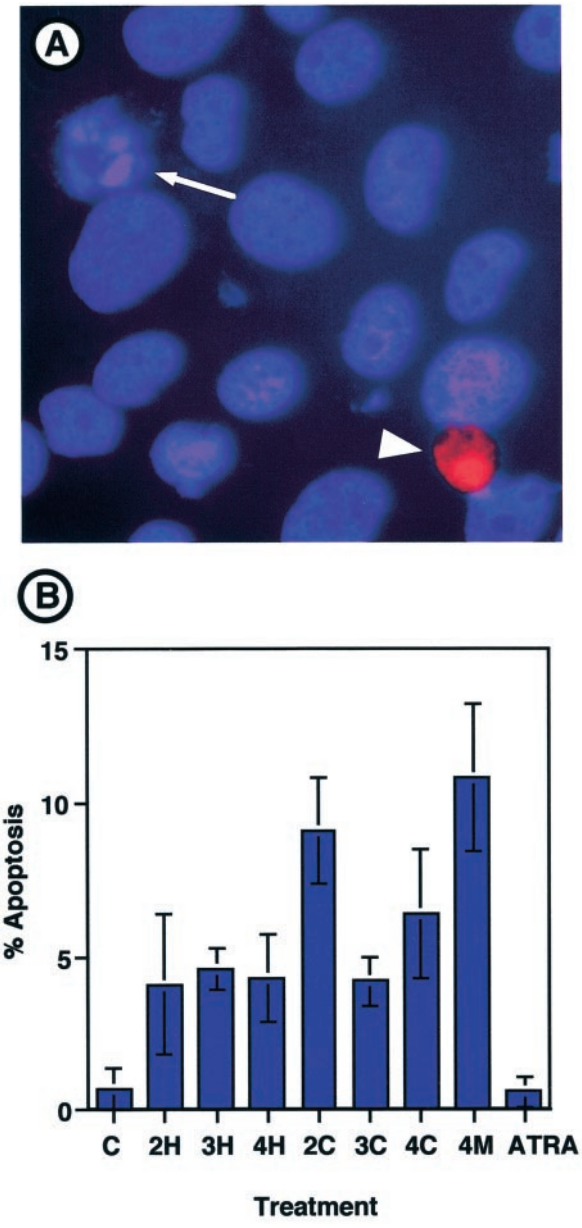


Fig. 3. All of the retinamides induce apoptosis in TCC cells. All of the four TCC cell lines were treated with 10 μM of the indicated retinamides or ATRA for 3 days and cytospun onto slides, and apoptosis was quantitated as described in "Materials and Methods." *A*, example of cytospun cells stained with DAPI and PI. Identical fields were photographed with filters revealing the *blue* fluorescence of DAPI and *red* PI fluorescence and merged electronically. *Small arrow*, DAPI-stained cells containing nuclei with features characteristic of apoptosis. A necrotic cell, which stains intensely with the membrane impermeable PI, is indicated by the *large arrow*. *B*, the percentage of UM-UC9 cells scoring positive for an apoptotic morphology after a 3-day treatment with the indicated retinamides is shown. *Error bars* indicate SE for triplicate fields of greater than 100 cells. The *P*s (two-sided Student's *t* test) comparing controls with treated samples were $\leq$0.05, except for 2HPR and 4CPR treatments, which had a *P* of 0.142 and 0.073, respectively.

Discussion

4HPR is less toxic than other retinoids and shows promise as a cancer chemopreventive drug (28, 29). 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 (28). Although 4HPR has been extensively studied in both clinical and laboratory settings, the activity of other phenylretinamides has not been as well studied. We determined the effects of seven phenylretinamides (including 4HPR) on cell growth and apoptosis in four TCC cell lines. The novel phenylretinamide 3HPR was the most active compound, with significantly greater growth inhibition than 4HPR in all of the four cell lines (Fig. 2). These data provide incentive to further explore the development of these novel phenylretinamides as preventive agents in TCC.

Through the use of retinoid receptor-null F9 cells, we have demonstrated previously (19) that 4HPR can have dual functions as both a differentiating agent and apoptosis inducer, which are mediated through retinoid receptor-dependent and -independent mechanisms, respectively. This led us to propose that the cancer therapeutic and chemopreventive efficacy of 4HPR in cancer patients might be because of its dual role as both an apoptotic agent and as a differentiating agent (retinoid). Tumor cells that can escape apoptotic effects at initial stages of exposure to high doses of 4HPR would then be subject to differentiating effects of lower doses of 4HPR. A similar mechanism may apply to the new phenylretinamides, and preliminary data using the same retinoid receptor-null F9 cell system indicate that several of them can also act as both retinoid receptor-dependent cytotoxic and retinoid receptor-independent differentiating agents (data not shown). It remains to be determined whether 4HPR and the new phenylretinamides exert

their effects on TCC cells through retinoid receptor-dependent or -independent mechanisms. The relative insensitivity of TCC cells to apoptosis induction by ATRA treatment, however, suggests that these cells may be more sensitive to retinoid receptor-independent actions of phenylretinamides (Fig. 3*B*). We also note that there was no correlation between the grade of tumor from which the cell lines were derived and the overall response to retinamides. We speculate that either the sensitivity to growth inhibition induced by the retinamides does not vary with tumor grade or the characteristics associated with tumor grade may have been lost during the derivation and passaging of the cell lines. Future studies are aimed at comparing the efficacy of the new phenylretinamides with 4HPR for chemoprevention in animal models.

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