Identification of Retinamides That Are More Potent than *N*-(4-Hydroxyphenyl)retinamide in Inhibiting Growth and Inducing Apoptosis of Human Head and Neck and Lung Cancer Cells[1]

Shi-Yong Sun,[2] Ping Yue, Gary J. Kelloff, Vernon E. Steele, Scott M. Lippman, Waun K. Hong,[3] and Reuben Lotan[4]

Departments of Thoracic/Head and Neck Medical Oncology [S-Y. S., P. Y., W. K. H., R. L.] and Clinical Cancer Prevention [S. M. L.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Division of Cancer Prevention, National Cancer Institute, NIH, Bethesda, Maryland 20892 [G. J. K., V. E. S.]

Abstract

The synthetic retinoid, *N*-(4-hydroxyphenyl)retinamide (4HPR), which is currently being evaluated in clinical trials for cancer prevention and therapy, inhibits the growth of a variety of malignant cells through induction of apoptosis. However, in the majority of tumor cells, this inhibitory effect of 4HPR requires high concentrations (>1 μM), which exceed the peak plasma level measured in humans. In the present study, we compared and contrasted the effects of several synthetic retinamides on the growth of human lung and head and neck cancer cells *in vitro*. We found that some retinamides, especially *N*-(2-carboxyphenyl)retinamide (2CPR), exhibited better growth inhibitory effects than 4HPR in some of the cell lines. 2CPR exerted potent growth inhibitory effects in 5 of 10 head and neck cancer cell lines and in 1 of 10 lung cancer cell lines (IC_{50}, <0.8 μM). 2CPR (1 μM) induced apoptosis ranging from 10 to 60% in four of five cell lines, whereas 4HPR was ineffective at the same concentration. Unlike 4HPR, 2CPR (up to 10 μM) failed to induce reactive oxygen species production in these sensitive cell lines but could activate caspases 3 and 7 as well as increase poly(ADP-ribose)polymerase cleavage. Interestingly, the effect of 2CPR on cell growth could be suppressed by the specific retinoic acid receptor pan antagonist AGN193109. Our results suggest that 2CPR acts via retinoic acid receptors and may be a good candidate for prevention and treatment of some head and neck and lung cancers.

Introduction

The synthetic retinoid 4HPR[5] was found to inhibit the growth of breast, prostate, and ovarian cancers in animal models (1–4). This retinoid is being evaluated clinically for prevention of various malignancies (5) and has already shown a potential for preventing ovarian (6) and breast (7) cancer in women. 4HPR is less toxic than most other retinoids in rodents (8). Clinical trials also indicate that 4HPR shows minimal toxicity and has a favorable pharmacokinetic profile (9, 10). Recent *in vitro* studies (11) have shown that 4HPR induces apoptosis in malignant hematopoietic cells, neuroblastoma, cervical, breast, ovarian, prostate, head and neck, and lung cancer cell lines, including those exhibiting resistance to the effects of the natural vitamin A metabolite all-*trans*-retinoic acid.

The induction of apoptosis by 4HPR *in vitro* requires high concentration (up to 10 μM). However, it was reported (9, 12) that the peak plasma level in patients receiving 200 mg of 4HPR daily was between 0.2 and 1 μM. Therefore, at 200 mg/day 4HPR may not be effective in inducing apoptosis *in vivo*.

In the present study, we compared and contrasted the growth inhibitory effects of a series of 4HPR analogues (retinamides) on human lung and head and neck cancer cells. We found that one of the retinamides, 2CPR, was at least 10-fold more active that 4HPR in inhibiting the growth of some cancer cell lines.

Materials and Methods

Retinamides. 4HPR and other retinamides (Table 1) were provided by the Chemoprevention Branch, National Cancer Institute (Bethesda, MD). AGN193109 was provided by Dr. Roshantha A. S. Chandraratna (Allergan, Irvine, CA). They were dissolved in DMSO at a concentration of 10 mM and were stored under N_2 in the dark at −80°C. Stock solutions were diluted to the designed concentrations with growth medium just before use.

Cell Lines and Cell Culture. The human HNSCC and NSCLC cell lines used in this study were grown in monolayer culture in a 1:1 (v/v) mixture of DMEM and Ham's F-12

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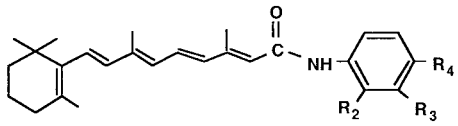
[2] To whom requests for reprints should be addressed, at Department of Thoracic/Head and Neck Medical Oncology, Box 432, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745-5062; Fax: (713) 794-0209; E-mail: ssun@mdanderson.org.
[3] W. K. H. is an American Cancer Society Clinical Research Professor.
[4] R. L. is the incumbent of the Irving and Nadine Mansfield and Robert David Levitt Cancer Research Chair.

[5] The abbreviations used are: HPR, hydroxyphenylretinamide; CPR, carboxyphenylretinamide; HNSCC, head and neck squamous cell carcinoma; NSCLC, non-small cell lung cancer; ROS, reactive oxygen species; RAR, retinoic acid receptor, PARP, poly(ADP-ribose)polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; MPR, methoxyphenylretinamide.

Table 1 Chemical structures and names of retinamides used in this study



R_2	R_3	R_4	Chemical name	Abbreviation
OH	H	H	*N*-(2-Hydroxyphenyl)retinamide	2HPR
H	OH	H	*N*-(3-Hydroxyphenyl)retinamide	3HPR
H	H	OH	*N*-(4-Hydroxyphenyl)retinamide	4HPR
COOH	H	H	*N*-(2-Carboxyphenyl)retinamide	2CPR
H	COOH	H	*N*-(3-Carboxyphenyl)retinamide	3CPR
H	H	COOH	*N*-(4-Carboxyphenyl)retinamide	4CPR (RII)
H	H	CH_3O	*N*-(4-Methoxyphenyl)retinamide	4MPR

medium containing 5% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2.

Cell Treatment and Determination of Cell Number. These procedures were performed as described in detail previously (13).

Apoptosis Assay. Cells were plated on 10-cm diameter dishes 1 day before treatment. After a 24 h-treatment with a retinamide, apoptosis was evaluated by examination of DNA fragments with 3′-hydroxyl ends using an APO-DIRECT TUNEL kit (Phoenix Flow Systems, Inc., San Diego, CA) following the manufacturer's protocol. In addition, apoptosis was evaluated by examination of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) using a Cell Death Detection ELISA^{Plus} kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions.

Measurement of Intracellular ROS Production. The intracellular generation of ROS was measured using the oxidation-sensitive fluorescent dye 2′7′-dichlorofluorescein diacetate as described previously (14). An equal number of cells (1×10^5/well) for each of the cell lines was seeded in 48-well cell culture plates.

Western Blot Analysis. Whole cell lysates from both attached and detached floating cells were prepared as described previously (15), and protein concentration was determined using the Protein Assay Kit (Bio-Rad, Hercules, CA). Cell lysates (50 μg) were electrophoresed through 7.5–12% denaturing polyacrylamide slab gels and transferred to a PROTRAN nitrocellulose transfer and immobilization membrane (Schleicher & Schuell, Inc., Keene, NH) by electroblotting. The blots were probed or reprobed with the antibodies, and then antibody binding was detected using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) according to the manufacturer's protocol. Mouse monoclonal anticaspase-3 (clone 31A1067) was purchased from IMGENEX (San Diego, CA). Mouse monoclonal anticaspase-7 (clone B94-1) was purchased from PharMingen (San Diego, CA). Rabbit polyclonal anti-PARP (VIC 5) and anti-β-actin antibodies were purchased from Roche Molecular Biochemicals and Sigma Chemical Co. (St. Louis, MO), respectively.

Results

Effects of Retinamides on the Growth of Human HNSCC Cells. To observe the maximum growth inhibitory activity of the retinamides on human HNSCC cells, we treated cells for 6 days with concentrations ranging from 0.001 to 10 μM. Dose-response curves were plotted, and the concentration required for IC_{50} was obtained by interpolation. Fig. 1 shows the dose-response curves of different retinamides used to treat 10 human HNSCC cell lines. At concentrations above 1 μM, 4HPR was very effective against the growth of all of the 10 HNSCC cells, and at 10 μM, it inhibited the growth of HNSCC cells completely. The IC_{50}s for 4HPR were around 2.5 μM in all of the cell lines. 3HPR, 2HPR, and 4CPR exhibited growth inhibitory activities that were comparable with 4HPR (Fig. 1). 3CPR showed weaker growth inhibitory effect than 4HPR, and 4MPR was the least potent in inhibiting the growth of HNSCC cells. 2CPR was a much more potent growth inhibitor than 4HPR in 5 of 10 HNSCC cell lines (22A, 38, 183A, SqCC/Y1, and TR146) with IC_{50}s lower than 1 μM. In the rest of the HNSCC cell lines, 2CPR was as active as 4HPR (Fig. 1). In addition, we found that SqCC/Y1 cells were more sensitive than other HNSCC cell lines to all of the retinamides, especially to 3HPR, 2HPR, 4CPR, 3CPR, and 2CPR (Fig. 1). The IC_{50}s for 3HPR, 2HPR, 4CPR, 3CPR, and 2CPR in SqCC/Y1 cells ranged from 0.01 to 0.1 μM in comparison with the IC_{50} 1.14 μM for 4HPR.

Effects of Retinamides on the Growth of Human NSCLC Cells. The growth inhibitory effects of the retinamides in 10 human NSCLC cell lines are shown in Fig. 2. In contrast to the results obtained with HNSCC cell lines, no retinamide had a better activity than 4HPR in inhibiting the growth of most NSCLC cells, except 2CPR, which is more effective than 4HPR in H292 cells. Similar to HNSCC cells, all of the 10 NSCLC cell lines were inhibited effectively by 4HPR at concentrations above 1 μM. Likewise at 10 μM, 4HPR was able to inhibit the growth of NSCLC cells completely. The IC_{50}s for 4HPR were also around 2.5 μM in all of the cell lines. 3HPR, 2HPR, and 4CPR were almost as potent as 4HPR in inhibiting the growth of most NSCLC cell lines. 3CPR and 2CPR exhibited relatively weaker growth inhibitory effects than 4HPR, whereas 4MPR had the weakest growth inhibition (IC_{50}, >5.7 μM). 2CPR was exceptionally effective against the growth of H292 cells, where the IC_{50} was 0.7 μM.

Comparison of Apoptosis-inducing Effects of 2CPR and 4HPR in HNSCC and NSCLC Cells. The above experiments clearly indicated that 2CPR is more potent than 4HPR in inhibiting the growth of 5 of 10 HNSCC cell lines and 1 of 10 NSCLC cell lines. To determine whether 2CPR induces apoptosis in these sensitive cell lines, we examined the apoptosis-inducing activities of 2CPR at a low concentration. As shown in Fig. 3, 2CPR at 1 μM exhibited more potent growth inhibitory effects than 4HPR (Fig. 3*A*) in the six cell lines. The TUNEL-flow cytometry assay was not able to detect apoptosis in the six cell lines treated for 3 days with 1 μM 4HPR (Fig. 3*B*). However, we did detect increased apoptotic cell populations in five of six cell lines treated with 1 μM 2CPR for 3 days (Fig. 3*B*). Although 2CPR suppressed the growth of TR146 cells, we failed to detect increased apoptosis in 2CPR-treated TR146 cells (Fig. 3). Using a more sensitive ELISA method, we were able to detect a dose-dependent increase in induction of apoptosis after 2CPR treatment in H292 cells even at early time points (24 h and 48 h; Fig. 3*C*).

2CPR Acts Like 4HPR to Activate Caspases and Increase PARP Cleavage. Because caspase activation plays a central role in induction of apoptosis (16), we began to elucidate the mechanism of 2CPR-induced apoptosis by examining its effect on activation of effector caspases and cleavage of their substrate PARP. After treatment with 2 μM 2CPR, a decrease in the level of procaspase-3 was observed as early as 24 h and became

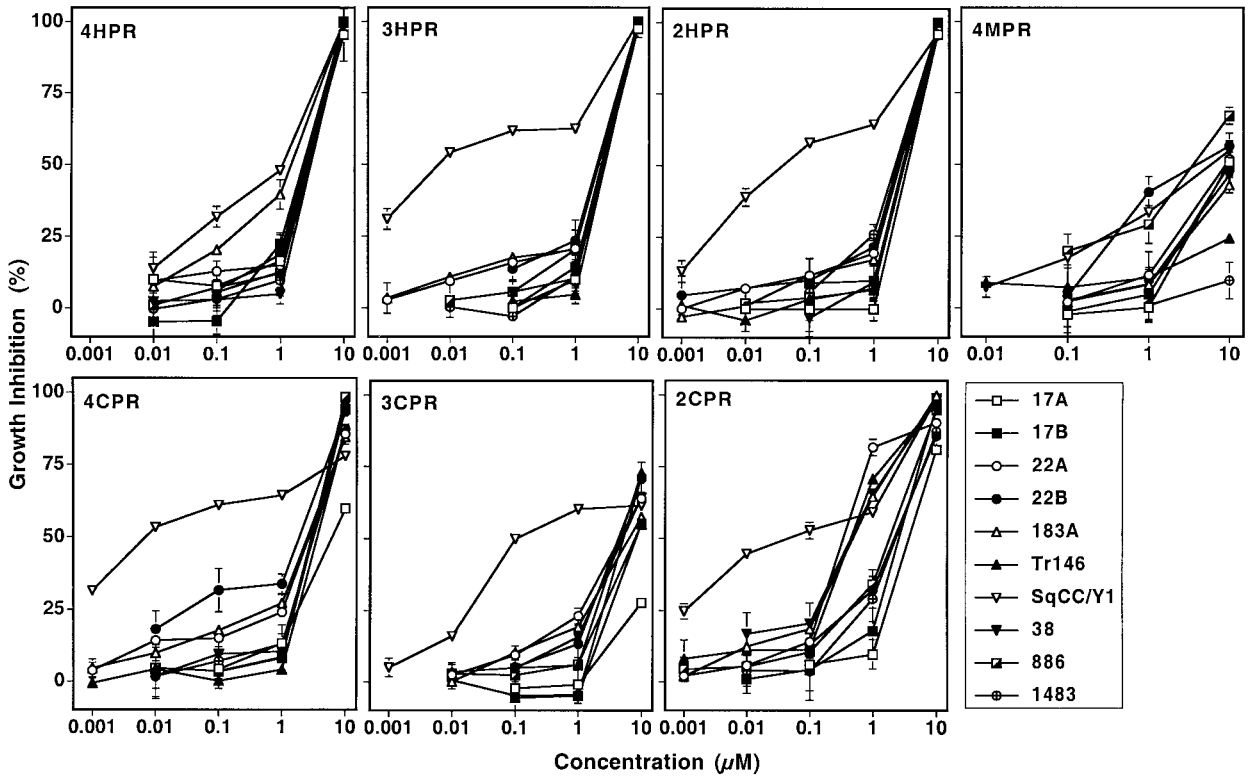


Fig. 1. Growth inhibitory effects of different retinamides on human HNSCC cells. Cells were seeded at densities of 2–5 × 10^3 cells/well in 96-well culture plates. At day 2, cells were treated with different concentrations of retinamides. The medium was replaced with fresh medium containing the same concentrations of retinamides on day 3. After 6-day treatment, cell numbers were determined as described in "Materials and Methods." *Point*, the mean of four replicate determinations; *bars*, SD.

more pronounced with increased incubation time, as evidenced by the appearance of cleaved forms of caspase-3. In a similar fashion, another effector caspase (caspase-7) was also activated, as evidenced by the decrease in the level of procaspase-7 protein. Concomitantly, PARP was cleaved as indicated by the appearance of a cleaved form of PARP at 48 and 72 h (Fig. 4*A*). 4HPR was reported previously (17) to activate caspase-3 and increase PARP cleavage. Therefore, we further compared the effects of 2CPR with 4HPR on caspase activation. As shown in Fig. 4*B*, both 2CPR and 4HPR could increase cleavage of procaspase-3 and PARP. However, 4HPR failed to decrease the level of procaspase-7 protein, suggesting that 4HPR may not activate caspase-7.

Comparison of Effects of 2CPR and 4HPR on ROS Production in 2CPR-sensitive Cells. Our previous study (14) has demonstrated that ROS generation plays a role in mediating 4HPR-induced apoptosis in some cancer cell lines. To determine whether 2CPR also induces ROS generation, we compared the effects of 2CPR and 4HPR on ROS production in these 2CPR-sensitive cell lines. As shown in Fig. 5, 4HPR was able to induce the generation of different levels of ROS in all of the cell lines. In contrast, 2CPR failed to induce ROS generation in any of these cell lines even at 10 μM. Two antioxidants, butylated hydroxylanisole and vitamin C, had no effects on 2CPR-induced apoptosis (data not shown). These results clearly indicate that 2CPR does not have any prooxidant property and that ROS production is not involved in 2CPR-induced apoptosis. It should be pointed out that although 4HPR could induce ROS production in all of the tested cell lines, the level of ROS production initiated by 4HPR was overall low (a less than 2-fold increase even at 10 μM).

Involvement of RARs in 2CPR-mediated Growth Inhibition and Apoptosis. In a few cell lines, RARs play a role in 4HPR-induced apoptosis (14, 15). Therefore, we performed an experiment to address the question of whether RARs are involved in 2CPR-induced growth inhibition and apoptosis. As shown in Fig. 6, the RAR-selective pan antagonist AGN193109 suppressed the growth inhibitory effect of 2CPR in both H292 and SqCC/Y1 cell lines, suggesting that RARs are involved in mediation of 2CPR-induced apoptosis.

Discussion

Previous *in vitro* studies (14, 15, 18–20) and our current study indicate that 4HPR exerts its growth inhibitory and apoptosis-inducing activity at high concentrations (1 to 10 μM). Because patients in chemoprevention trials who receive 4HPR 200 mg/day show peak plasma levels in the range of 0.2 to 1.0 μM (9, 12), it is plausible to assume that these levels were insufficient to induce apoptosis in premalignant or malignant cells *in vivo*. Furthermore, the poor clinical activity of 4HPR in a few recent studies (12) may be a consequence of the less than optimal *in vivo* plasma levels. One way to address this problem is to identify 4HPR analogues that have better efficacy than 4HPR in inhibiting cell growth and in inducing apoptosis in cancer cells. The present study describes our efforts in this direction.

In this study, we found that among the HNSCC cell lines examined, only SqCC/Y1 showed a clear dose-response rela-

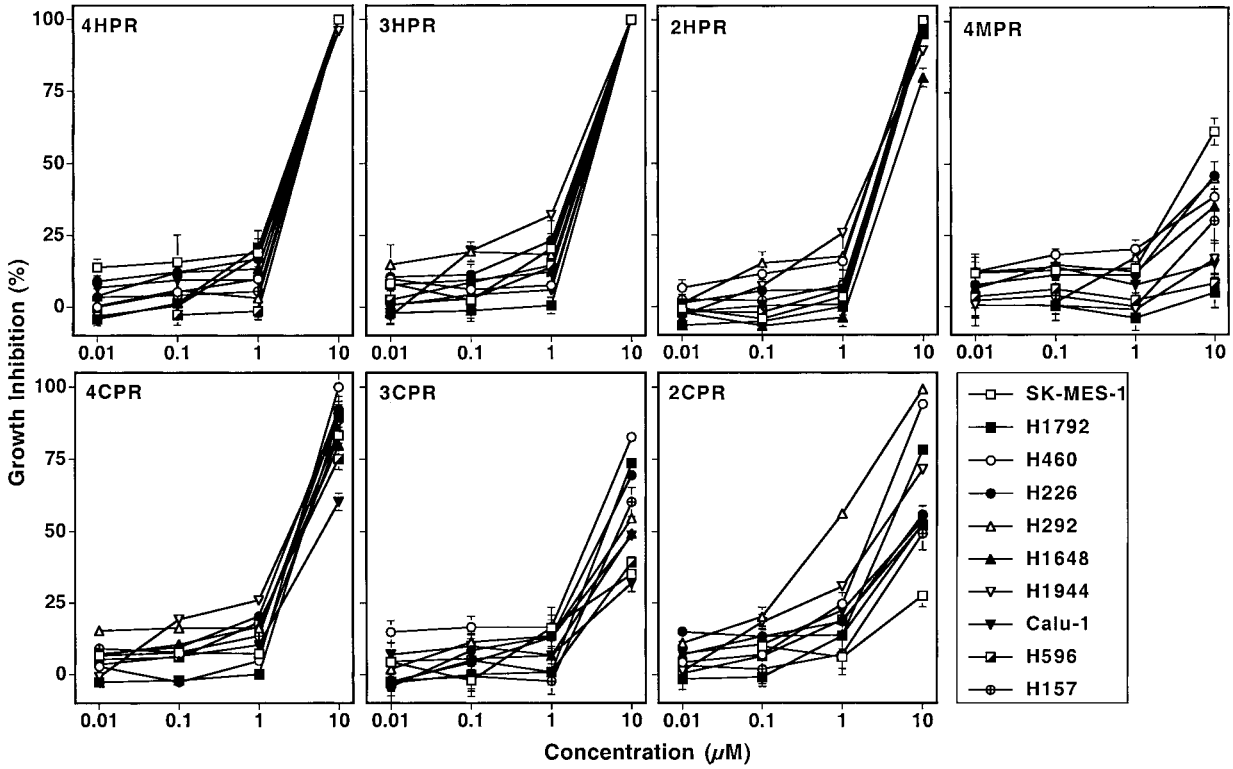


Fig. 2. Growth inhibitory effects of different retinamides on human NSCLC cells. Cells were seeded at densities of 2–5 × 10^3 cells/well in 96-well culture plates. Cell treatment and determination of cell numbers were the same as described in Fig. 1. *Point*, the mean of four replicate determinations; *bars*, SD.

tionship when treated with retinamides at doses between 0.001 and 1 μM. Among the NSCLC cell lines, H292 showed dose-dependent inhibition to 2CPR but not to other retinamides between 0.001 and 1 μM. One interpretation of these findings is that most of the cell lines are resistant to retinamide concentrations up to 1 μM. On the basis of clinical studies with 4HPR, peak plasma levels of up to 1 μM could be obtained when 4HPR was administered at a 200-mg/day dose (9, 12). Thus, it appears that clinical activity of the most potent retinamides may require higher doses to have an effect *in vivo*. Recent dose escalation studies[6] have demonstrated that peak plasma levels of 10 μM 4HPR can be achieved by oral 4HPR administration at about 1-gram/day dose levels.

Among the analogues of 4HPR we analyzed in this study, 4CPR (RII) has been studied for its chemopreventive potential since the early 1980s (21, 22). 4CPR inhibited dimethylnitrosamine-induced forestomach carcinogenesis in mice, suppressed 7,12-dimethylbenz(*a*)anthracene-induced papilloma formation in mouse skin, and inhibited buccal pouch carcinogenesis in the Syrian golden hamster (21, 22). Phase II clinical trials demonstrated that this retinamide was effective in treating oral and vulvar leukoplakia and in treating myelodysplastic syndrome and dysplasia of the uterine cervix (21, 22). Our results indicate that 4CPR had comparable growth inhibitory effects with 4HPR in most of HNSCC and NSCLC cell lines. However, 4CPR had better activity than 4HPR in inhibiting the growth of SqCC/Y1, 1 of 10 HNSCC cell lines.

2CPR was much more potent in inhibiting the growth of five of HNSCC cell lines ($IC_{50} \leq 0.5$ μM) than was 4HPR. In the rest of the cell lines, 2CPR had comparable growth inhibitory effects with 4HPR. Although 2CPR exhibited less activity than 4HPR against the growth of most NSCLC cell lines, it exerted better growth inhibitory effect than 4HPR in one of the NSCLC cell lines, H292 cells. 4HPR inhibits the growth of certain cancer cells including human head and neck and lung cancers through induction of apoptosis (14, 18, 20). However, induction of apoptosis required high concentration (2–10 μM; Refs. 14, 17–20). In this study, we found that 2CPR induced apoptosis in five cell lines at 1 μM, whereas 4HPR failed to do so at the same concentration. Therefore, 2CPR may have an advantage over 4HPR for prevention and/or treatment of cancer, especially for some types of head and neck cancers. 2CPR failed to induce apoptosis in TR146 cells, although it effectively inhibited their growth, indicating that 2CPR may inhibit cell growth through an apoptosis-independent mechanism. We noted that cell line TR146 was inhibited by about 20% after exposure to 1 μM 4HPR for 3 days, and further treatment for an additional three days failed to improve the response. The reason for the refractoriness of the TR146 cells is not clear at this time; however, it may indicate that certain HNSCC may be resistant to 4HPR therapy *in vivo*. In contrast, this cell line was still sensitive to 2CPR because its growth inhibitory effect increased as the treatment time increased (71% for 6 days *versus* 44% for 3 days). This suggests that some HNSCC cells that are resistant to 4HPR may be still sensitive to 2CPR treatment.

2CPR has been reported to inhibit tumor promotion as indicated by suppression of ornithine decarboxylase induction in rat epithelial cells at nontoxic concentrations (23). In addition, 2CPR was also reported to prevent the production of

[6] C. Patrick Reynolds, personal communication.

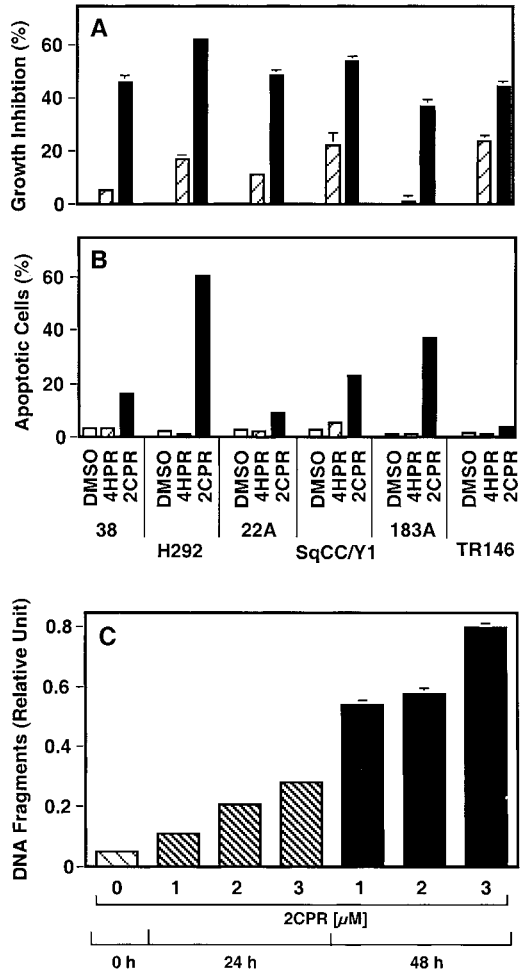


Fig. 3. Effects of 2CPR and 4HPR on growth inhibition (*A*) and induction of apoptosis (*B* and *C*) in some human HNSCC and NSCLC cells. *A* and *B*, cells at densities of 5000/well (*A*) and 1×10^6 (*B*) were seeded in 96-well plates (*A*) and in 10-cm diameter dishes (*B*), respectively. After 3-day treatment with 1 μM 2CPR or 4HPR, cells were harvested and used for determination of growth inhibition using SRB assay (*A*) and for detection of the DNA fragmentation using TUNEL-flow cytometry assay (*B*). *C*, cells were seeded in 10-cm diameter dishes and treated on day 2 with the indicated concentrations of 2CPR for the indicated times. After cells were harvested and counted, 5000 cells were used from each treatment group for evaluation of apoptosis using the ELISA method. *Column*, the mean of four replicate (*A*) or triplicate (*C*) determinations; *bars*, SD.

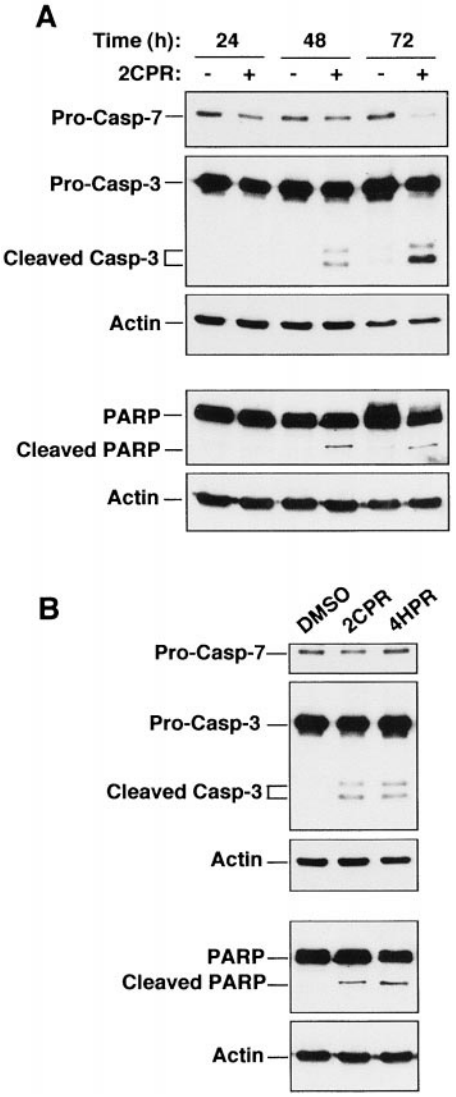


Fig. 4. Activation of caspases and cleavage of PARP by 2CPR in H292 cells. After cells were treated with 2 μM 2CPR for the indicated times (*A*) or with 2 μM 2CPR or 3 μM 4HPR for 48 h (*B*), both floating and attached cells were harvested and whole-cell protein lysates were prepared for Western blot analysis. *Casp*, caspase.

azoxymethane-induced aberrant crypt foci in the colon of rats more effectively than did 4HPR and 9-*cis* retinoic acid (24). However, unlike 4HPR and 9-*cis* retinoic acid, 2CPR enhanced rather than prevented azoxymethane-induced colon cancer when tumor formation was used as an end point (25). It is worth noting that 2CPR also failed to induce apoptosis in adenomas, unlike 4HPR (25). In contrast, we report here that 2CPR is a potent inducer of apoptosis in certain HNSCC and NSCLC cell lines in which 4HPR was much less effective. Thus, it appears that the effect of 2CPR on apoptosis may be cell-type specific. This is also noted in our study where most NSCLC cell lines were resistant to 2CPR-induced apoptosis, whereas most HNSCC cells were sensitive.

Recently, 4HPR was reported (14, 17, 26, 27) to induce apoptosis through ROS generation in some cancer cell lines. 2CPR induced apoptosis at 1 μM in some HNSCC and NSCLC cell lines but failed to induce ROS production in any of the five sensitive cell lines, whereas 4HPR was able to induce certain levels of ROS production in all of these cell lines. In the present report, 4HPR increased ROS production in a dose-dependent fashion when used at 5 and 10 μM for 180 min. The increase by 5 μM 4HPR was about 1.86-fold. In our previous study (14), 5 μM 4HPR caused a lower effect on ROS production (1.14-fold increase only). The difference may be attributable to the use of different frozen batches of the SqCC/Y1 cells thawed and used at several year intervals. Nonetheless, this difference is of no biological significance because we have demonstrated that apoptosis induction requires an increase of 4–8-fold in ROS levels; therefore, the 1.86-fold increase observed here with 5 μM 4HPR is not sufficient to induce apoptosis (14). In addition, we found that antioxidants did not have any suppressive effects on 2CPR-induced growth inhibition or apoptosis. Thus, it ap-

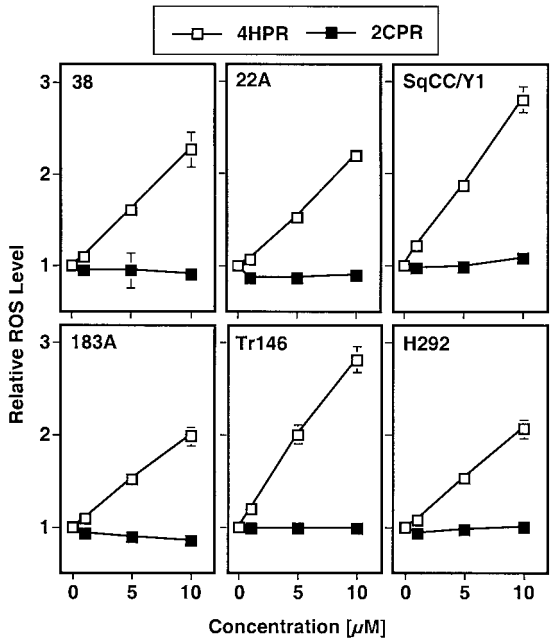


Fig. 5. Effects of 2CPR and 4HPR on intracellular ROS production in 2CPR-sensitive cell lines. Equal numbers of cells from each cell line were seeded in 48-well plates, and ROS generation was determined after 180 min of incubation with different concentrations of 2CPR or 4HPR on day 2 by the 2′7′-dichlorofluorescein diacetate assay. *Point*, the mean of four replicate determinations; *bars*, SD.

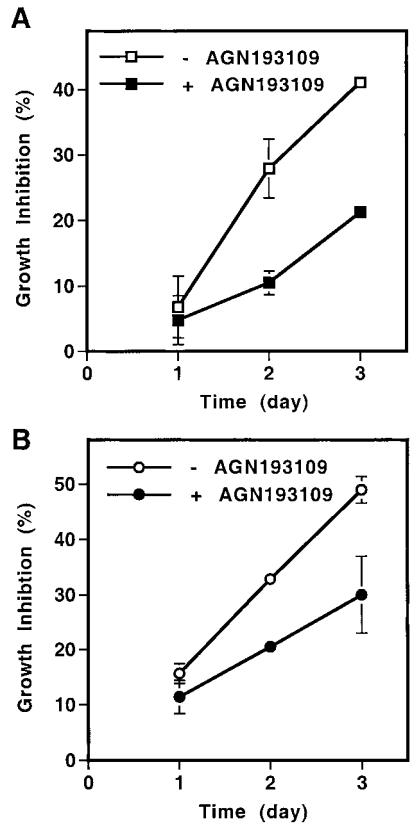


Fig. 6. Effect of RAR-specific pan antagonist AGN193109 on 2CPR-induced growth inhibition in SqCC/Y1 (*A*) and H292 (*B*) cells. Cells were seeded in 96-well culture plates and treated with 1 μM 2CPR and 2CPR in combination with 10 μM AGN193109 for 3 days. Cell number was determined as described in "Materials and Methods." AGN193109 alone at all of the time points caused <10% growth inhibition.

pears that 2CPR does not have any pro-oxidant property, and induction of apoptosis by 2CPR is independent of ROS generation.

Similar to 4HPR, 2CPR was able to activate caspase-3 as indicated by increased cleavage of procaspase-3 and its substrate PARP. This indicates that both 2CPR and 4HPR share a similar downstream apoptotic pathway (*i.e.* caspase-3 activation). However, 2CPR could activate caspase-7, another important effector caspase, whereas 4HPR could not. This suggests that the upstream apoptotic signaling pathway of 2CPR leading to activation of effector caspases may be different from that of 4HPR.

In our previous studies (14, 17), we demonstrated that 4HPR induces apoptosis in human cancer cells largely independently of RAR-signaling pathway. However, 2CPR-induced apoptosis in two tested cell lines could be suppressed in the presence of RAR-specific pan antagonist AGN193109, indicating that RAR-mediated signaling pathway is involved in 2CPR-induced apoptosis. Previously (14), we have shown that AGN193109 failed to suppress 4HPR-induced apoptosis in one of the cell lines (SqCC/Y1). This finding again supports the conclusion that the upstream apoptotic signaling mechanisms of 2CPR and 4HPR are different.

This is the first report on the growth inhibitory and apoptosis-inducing activities of a large series of retinamides using human cells. The results are consistent with a previous study (28) on structure-activity of these retinamides in a hamster tracheal organ culture, which analyzed reversal of keratinization induced by vitamin A deficiency. Newton *et al.* (28) found that 2CPR was more potent than 3CPR, 4CPR, 2HPR, 3HPR, and 4HPR on reversal of keratinization. Because the metabolic rates of retinoids affect their growth inhibitory potency (29), it is possible that differences in the apparent potency of growth inhibition of different retinamides may reflect differences in rates of metabolic or chemical degradation.

The present study has focused on the effects of retinamides on fully malignant cells. It is not clear whether premalignant cells will respond better or worse than the malignant ones to these retinamides. However, in a previous study (30) we found that immortalized premalignant cells were more sensitive to 4HPR than their tumorigenic counterparts. Furthermore, a recent report by D'Ambrosio *et al.* (31) has shown that among several retinamides, 2CPR exhibited the greatest selectivity in growth inhibition of both premalignant and malignant human oral epithelial cells.

In conclusion, our results suggest that 2CPR may be a better candidate than 4HPR for prevention and/or treatment of some cancers, especially head and neck cancer.

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