Involvement of Rac in Fenretinide-Induced Apoptosis

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Abstract

The synthetic retinoid N-(4-hydroxyphenyl)retinamide (4HPR) has shown potential as a chemopreventive and therapeutic agent. The ability of 4HPR to enhance production of reactive oxygen species (ROS) leading to apoptosis has been suggested as a possible mechanism underlying these effects. We explored the possibility that ROS induction by 4HPR involves the small GTPase Ras-related C3 botulinum toxin substrate (Rac), a regulatory subunit of the NADPH oxidase complex. Rac was activated in human head and neck squamous cell carcinoma (HNSCC) cells as early as 5 minutes following 4HPR exposure. Moreover, inhibition of Rac activity or silencing of its expression by RNA interference decreased ROS generation in human head and neck, lung, and cervical cancer cells and murine melanoma cells. In HNSCC UM122 cells, this decrease correlated with reduction in apoptosis induction by 4HPR. Expression of a constitutive active mutant Rac increased basal and 4HPR-induced ROS generation and poly(ADP-ribose) polymerase cleavage. In addition, the metastatic DM14 cells exhibited higher Rac activation following 4HPR treatment compared with the primary Tu167-C2 cells. Furthermore, the metastatic cancer cells tested exhibited higher ROS generation and growth inhibition due to 4HPR exposure compared with their primary cancer cell counterparts. These findings show a preferential susceptibility of metastatic cells to the proapoptotic retinoid 4HPR through Rac activation and support the use of ROS-inducing agents such as 4HPR against metastatic cancer cells. [Cancer Res 2008;68(11):4416–23]

Introduction

Programmed cell death or apoptosis is a natural process for elimination of defective cells such as those bearing detrimental mutations or alterations in important cellular processes (1). It is now well appreciated that evasion of apoptosis represents a major mechanism that drives tumor growth and is considered a hallmark of most, if not all, cancers (2). Therefore, induction of apoptosis is increasingly recognized as a desired effect for chemopreventive and therapeutic agents because it results in the elimination of premalignant or malignant cells (3, 4).

One promising anticancer agent, N-(4-hydroxyphenyl)retinamide (4HPR), also known as fenretinide, which exhibits a substitution of an amide-linked 4-hydroxyphenyl) group for the carboxyl group of all-trans retinoic acid, was first shown to prevent breast cancer in rats (5). In addition, fenretinide displayed markedly reduced adverse side effects such as liver toxicity compared with the natural retinoid all-trans retinoic acid (6). Moreover, 4HPR showed efficacy as a chemopreventive and therapeutic agent in various experimental models and clinical trials (7). In addition, 4HPR was effective in clinical trials aimed at chemoprevention of oral leukoplakias recurrence and new incidence in postsurgical patients (8) as well as patients resistant to natural retinoids (9). 4HPR was also shown to significantly reduce the risk of second breast cancer in premenopausal women, the effect of which persisting for several years after treatment cessation in a 15-year-long randomized phase III trial for breast cancer prevention (10). In addition and more recently, 4HPR was shown to significantly delay onset of ovarian cancer (11).

The proposed mechanism for the above-mentioned 4HPR-induced effects was induction of apoptosis based largely on earlier studies, which have shown that 4HPR can induce apoptosis in a variety of cell lines including head and neck squamous cell carcinoma (HNSCC) cells (12). Apoptosis induction by 4HPR is mediated by various mechanisms including ceramide induction (13), triggering of the mitochondrial pathway and modulating mitochondrial membrane permeability and cytochrome c release (14), activation of lipoxigenase 12 (15), nitric oxide production (16), and reactive oxygen species (ROS) generation (12, 17–19). The increase in ROS generation can cause sustained activation of c-Jun NH2-terminal kinase (JNK) and other mitogen-activated protein kinases (MAPK; refs. 20–22), triggering of the mitochondrial pathway (19), and, more recently, induction of endoplasmic reticulum stress (23), all of which seem to be downstream of ROS and to contribute to cell death induction. Whereas these studies have highlighted the pivotal role of the downstream effects of ROS, many gaps still exist in our knowledge of upstream mechanisms responsible for ROS generation by 4HPR.

ROS are produced from the reactions of unpaired electrons of oxygen molecules from various intracellular sources such as the mitochondrial electron transport chain and the NADPH oxidase complex, a multicomponent electron transfer complex composed of the membrane-bound cytochrome b558 (gp91phox and p22phox) and the cytosolic components [p67phox, p47phox, p40phox, and Ras-related C3 botulinum toxin substrate (Rac)-1; ref. 24]. On activation, the cytosolic components of the enzyme complex translocate to the plasma membrane where they associate with cytochrome b558, forming an active NADPH oxidase (25, 26). Because NADPH-mediated generation of ROS is involved in various apoptosis signaling pathways (27–30), we hypothesized that it may be a target through which 4HPR induces ROS generation and apoptosis.

In this study, we found that 4HPR activates the NADPH oxidase regulatory subunit Rac, and that this activation results in excessive ROS levels in cancer cells that lead to apoptosis induction apparently by exceeding the endogenous antioxidant capacity. Moreover, we found that 4HPR caused a higher activation of Rac in metastatic cells compared with their corresponding primary cancer cells, which correlated with increased ROS generation and cell growth inhibition.
Rac Mediates ROS Induction by 4HPR

Materials and Methods

Retinoid and reagents. The synthetic retinoid 4HPR was obtained from Dr. James A. Zwiebel (Cancer Therapy Evaluation Program, National Cancer Institute, Bethesda, MD) and dissolved in DMSO at a concentration of 10 mmol/L and stored in dark containers at −80°C under N2 atmosphere. Stock solutions were diluted to the final concentration with growth medium just before use. DMSO, Krebs-Ringer buffer, tert-butyl hydroxyl anisole, sulforhodamine B, and crystal violet were purchased from Sigma Chemical Co. The Rac inhibitor NSC-23766 was obtained from Calbiochem. Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes. The RacV12 expression vector was obtained from Dr. Kaikobad Irani (University of Pittsburgh Medical Center, Pittsburgh, PA).

Cell culture. The HNSCC cell lines UMSCC-22A and UMSCC-22B, derived from pharyngeal primary tumors and lymph node metastasis, respectively, and isolated originally from the same patient, were obtained from Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). The primary and metastatic isogenic HNSCC cell line pair Tu167-C2 and DMM1 was obtained from Dr. Jeffrey Myers (The University of Texas M. D. Anderson Cancer Center, Houston, TX). Muriene melanoma B16-F1 and B16-F10 cells were obtained from Dr. Isaiah Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX), and bronchioalveolar cancer H522 cells and squamous cervical cancer C33A cells were purchased from the American Type Culture collection. The cells were grown and maintained in monolayer culture in a 1:1 (v/v) mixture of DMEM and Ham’s F12 medium. The medium was supplemented with 5% fetal bovine serum and the cells were incubated at 37°C in an atmosphere consisting of humidified air with 5% CO2.

Treatment with 4HPR and the Rac inhibitor NSC-23766. The Rac inhibitor NSC-23766 was prepared as a 50 mmol/L stock solution in distilled water and stored at −80°C. Cells were seeded in 10-cm-diameter dishes for Rac activation assays and Western blot analysis, and in 24-well plates for assessment of ROS levels. After 24 h, cells were serum starved for 24 h before treatment with 4HPR in serum-free medium for the indicated time points. The control cells were treated with the same final concentration of DMSO as the retinoid-treated cultures.

Measurement of intracellular ROS levels. Intracellular ROS levels were measured with the oxidation-sensitive fluorescent dye DCFH-DA, with the conversion of DCFH-DA to dichlorofluorescin (DCF) assessed as previously described (31). Briefly, cells were electroporated with small interfering RNA (siRNA) or expression vectors and/or seeded at a density of 104 per well in 24-well plates. After 1 d, cells were washed twice with PBS and incubated in either serum-free medium alone or in serum-free medium containing NSC-23766 at different doses before treatment with 5 µmol/L 4HPR for different time points. The control cells were treated with the same final concentration of DMSO as the retinoid-treated cultures.

Measurement of Annexin V–positive cells. Apoptotic cell death was quantitatively measured by measuring externalized phosphatidylserine with the Annexin V-FLOUS Staining Kit (Roche) according to the manufacturer’s instructions. Briefly, cells were harvested at the indicated time points after treatment and were washed twice with PBS before staining with and incubation in 100 µL of prediluted Annexin V-FLOUS labeling agent for 15 min at room temperature in the dark. Cells were then washed once with the manufacturer’s incubation buffer and finally resuspended in 400 µL of the same buffer. Propidium iodide solution was added to the Annexin V-FLOUS–labeled cells just before analysis with the BD Flow cytometer for quantification of Annexin V–positive cells.

Cell growth inhibition assay. The cells were seeded at calculated cellular densities that allow them to reach near-confluent states 24 h after seeding, at which time the cells were incubated in serum-free medium containing either DMSO or 4HPR. After 1 or 2 d of treatment, cell number was estimated by the sulforhodamine B assay as previously described (32). Briefly, after treatment of cells for the indicated time points, the media were poured on the cells gently, then cells were fixed by adding 100 µL of cold 10% trichloroacetic acid (from Sigma) and incubated for 60 min at 4°C. Following fixation, the supernatants were discarded and plates were washed five times with deionized water and then air-dried or stored in a warm chamber until use. After drying, 50 µL of 0.4% (w/v) sulforhodamine B solution in 1% acetic acid were added to each well, and the plates were then incubated with the stain for 10 min at room temperature. Following staining, plates were washed five times with 1% acetic acid and air-dried at 37°C, after which 100 µL of 10 mmol/L unbuffered Tris base (pH 10.5) were added to each well to solubilize the dye. Plates were shaken for 5 min on a rocker plate shaker and optical densities were read on an automated spectrophotometric plate reader at a wavelength of 510 nm. The error bars represent the SE of four replicate measures.

Colony formation assay. Following transfection with RacV12, cells were seeded in six-well plates at a seeding density of 5,000 per well. Ten days later, the medium was decanted and cells were gently washed with PBS to remove residual medium. Cells were then incubated with Carney’s fixative (3 parts methanol to 1 part acetic acid) at room temperature for 10 min. The cells were then gently washed with distilled water several times and stained with crystal violet for 10 min. The cells were then washed with distilled water several times, after which excess water was gently shaken off and the plates were left to dry.

Western blot analysis. Cell monolayers were washed twice with ice-cold PBS, harvested, and processed for immunoblotting as previously described (12). The antibodies used for immunoblotting included antihuman Rac1 and anti-myc tag (Upstate); HSP70/HSPA1A (Stressgen Bioreagents); poly(ADP-ribose) polymerase (PARP), p21-activated kinase (PAK)-1, JNK, phospho-JNK, c-Jun, and phospho-Jun (Ser63) (Cell Signaling Technology); and β-actin (Sigma Chemical Co.). Antibody binding was detected by enhanced chemiluminescence (Amersham Biosciences Corp.). Equality of loading and transfer (internal controls) were estimated by probing membranes with β-actin or staining the blots with the protein stain Ponceau S.

Rac activation assay. To test for Rac activation by 4HPR, cells were seeded at −104 cells in serum-containing medium in 10-cm dishes. The next day, the cells were switched to serum-free medium and serum starved for 48 h before treatment with 4HPR in serum-free medium for the indicated time points. Samples were handled for the Rac activation assay according to the manufacturer’s instructions (Cytoskeleton). Briefly, the medium was decanted, dishes were washed with 5 mL of PBS at room temperature, and 0.5 mL of the manufacturer’s ice cold lysis buffer was added. Cells were scraped off the plates into lysis buffer and cell lysates were clarified by centrifugation. Samples of the cell lysates were incubated with 20 µg of PAK-p21 binding domain (PBD) beads and 1× of the manufacturer’s protease inhibitor cocktail in a total volume of 1 mL and were incubated at 4°C on a rocker for 1 h. The beads were then pelleted by centrifugation and washed thrice with the manufacturer’s 1× wash buffer, after which they were resuspended in Laemmli buffer and subjected to SDS-PAGE and Western blotting analysis. Membrane blots were incubated with the manufacturer’s antibody raised against Rac and antibody binding was detected as described before.

Transfection of cells with siRNA and plasmids. siRNAs against Rac1 and PAK1 were synthesized by a proprietary design as SMARTpool siRNA, which consists of four pooled SMARTselection-designed siRNAs that are 21 nucleotides forming a 19-bp duplex core with symmetrical two nucleotide 3’-UU overhangs (Dharmacon, Inc.). Cells were electroporated with the siRNAs and plasmids as previously described (23). Briefly, 1× to 106 cells were reconstituted in 100 µL of electroporation transfection solution or Nucleofector Solution V from Cell Line Nucleofection Kit V (Amaxa Biosystems). siCONTROL or siGenome SMARTpool siRNA against Rac1 and PAK1 was added to the cells at a final concentration of 200 pmol per sample, and the mixtures were transferred to electroporation cuvettes and subjected to electroporation according to the manufacturer’s programs and instructions. The electroporated and transfected cell suspensions were
immediately mixed with 500 μL of prewarmed RPMI medium supplemented with 5% fetal bovine serum (FBS). The cells were then transferred to six-well plates for Western blot analysis or to 24-well plates for measurement of ROS levels containing prewarmed DMEM/F12 medium supplemented with 5% FBS. Cells were incubated for 24 h, after which they were incubated in serum-free medium for another 24 h before treatment with 4HPR. Cells were also electroporated with the expression vector encoding the mutant Rac, RacV12, and were subjected to analysis in the same manner as described above following transfection with siRNAs.

Results

Activation of Rac in HNSCC cells by 4HPR. Treatment of UMSCC-22B HNSCC cells with 4HPR increased the amount of GTP-Rac compared with control cells (Fig. 1A). This effect was inhibited by pretreating the cells with NSC-23766, a Rac GTPase inhibitor that targets Rac activation by guanine nucleotide exchange factors (Fig. 1A; ref. 33). These results indicate that Rac is activated by 4HPR.

Inhibition of 4HPR-induced apoptosis by suppression of Rac expression or activity. 4HPR induced apoptosis in UMSCC-22B cells as indicated by increased PARP cleavage (Fig. 1B) and percentage of Annexin V–positive cells. This effect was suppressed by inhibition of Rac with NSC-23766 (Fig. 1B). Because up-regulation and phosphorylation of c-Jun by JNK (21) and up-regulation of HSPA1A/HSP70 (23) were found to be important for apoptosis induction by 4HPR, we examined whether they are affected by inhibition of Rac before exposure to 4HPR. Pretreatment of the cells with NSC-23766 decreased both phosphorylated and total levels of c-Jun, as well as HSPA1A levels, profoundly (Fig. 1B), suggesting that Rac is upstream of these changes. Further support for this conclusion has come from targeting Rac1 expression by RNA interference, which diminished the level of Rac1 (Fig. 1C) and decreased 4HPR-induced up-regulation of HSPA1A and PARP cleavage (Fig. 1D). These results showed that Rac activation contributes to 4HPR-induced apoptosis and Rac is upstream of JNK activation and HSPA1A induction.

Induction of ROS by 4HPR is downstream of Rac activation. Our group has previously shown that 4HPR induces ROS increase, which is abrogated by cotreatment of cells with the antioxidant

Figure 1. Apoptosis induction by 4HPR in HNNSC cells mediated by Rac activation. A, UMSCC-22B cells were seeded at 1 × 10⁶ per dish in a series of 10-cm dishes for 24 h. The cells were then incubated in serum-free medium for another 24 h before overnight pretreatment with the Rac inhibitor NSC-23766 (60 μmol/L). The cells were then incubated for 5 min in medium containing either DMSO (control) or 5 μmol/L 4HPR, harvested, and then scraped into lysis buffer. The cell lysates were processed for assessment of activated Rac (GTP-Rac) levels as described in Materials and Methods. Material adsorbed to PBD beads was analyzed by immunoblotting with anti-GTP-Rac antibodies after SDS-PAGE and samples of the nonadsorbed material were also analyzed by immunoblotting for total Rac levels and β-actin. B, cells seeded as in A were used for Rac inhibition by pretreating them with 60 μmol/L NSC-23766 overnight before incubation in medium containing DMSO, 60 μmol/L NSC-23766, 5 μmol/L 4HPR alone, or a combination of both NSC-23766 and 4HPR for 24 h. Cells were then harvested and used to extract total protein fraction. Samples of these extracts were subjected to immunoblotting analysis to assess PARP cleavage, HSPA1A levels, and total and phosphorylated levels of c-Jun. Membrane blots were probed with β-actin and stained with the dye Ponceau S to assess for equality of protein loading in the different lanes. Similarly treated cells were also subjected to analysis of Annexin V–positive cells as described in Materials and Methods. Columns, mean of two duplicate measurements; bars, SE. C, Western blot analysis depicting the effect of RNA interference on Rac1 protein levels. UMSCC-22B cells were transfected with siCONTROL or siRNA targeting Rac1 (siRac1) as described in Materials and Methods. Two days later, cells were harvested and used to extract total protein fraction. Samples of these extracts were subjected to Western blot analysis with anti-Rac1 antibody. Membrane blots were stained with the dye Ponceau S to assess for equality of protein loading in the different lanes. D, UMSCC-22B cells were transfected with siCONTROL and siRNA against Rac1, as described in Materials and Methods, and then transferred to six-well plates. After 24 h, cells were maintained in serum-free medium for another 24 h before treatment with 5 μmol/L 4HPR. Total protein lysates of these samples were prepared following 4HPR treatment and subjected to immunoblotting to assess PARP cleavage and HSPA1A levels. Membrane blots were stained with Ponceau S to compare loading in the different lanes.
Suppression of ROS induction by 4HPR by the Rac inhibitor NSC-23766. The findings that the Rac inhibitor NSC-23766 compromised the ability of 4HPR to induce apoptosis and that ROS generation was required for 4HPR-induced apoptosis and plating JNK activation downstream of ROS (Fig. 2A). In contrast, ROS scavenging by tert-butyl hydroxyl anisole failed to inhibit Rac activation by 4HPR (Fig. 2B). These results indicate that in 4HPR-treated cells, Rac activation is upstream of ROS generation.

Expression of a constitutively active RacV12 increased ROS generation and cell growth inhibition in HNSCC cells. Because we have shown that activation of Rac by 4HPR is important for ROS generation and apoptosis induction, we hypothesized that expression of RacV12, a constitutively active mutant form of Rac, will emulate the effects of 4HPR. The expression of RacV12 was confirmed by the differential detection of the Myc tag by Western blotting (Fig. 5A). RacV12 expression has led to a small increase in ROS (Fig. 5A) and a modest but reproducible increase in PARP cleavage (Fig. 5B). Moreover, RacV12 increased the sensitivity of cells to 4HPR-induced ROS and apoptosis, evident in the increased PARP cleavage in 4HPR-treated RacV12-expressing cells compared with control plasmid–transfected treated cells (Fig. 5A and B). In addition, expression of RacV12 inhibited colony formation by HNSCC cells compared with cells that were either nontransfected or transfected with a control plasmid (Fig. 5C and D).

4HPR induced higher levels of Rac activation, ROS, and cell growth inhibition in metastatic cancer cells compared with their primary cancer cell counterparts. Our finding that 4HPR can induce apoptosis by activating Rac and increasing ROS in cancer cells raised the question on whether metastatic cells are partially resistant to 4HPR because they are known to have elevated levels of both Rac and ROS, which are important for their proliferation, growth, and migration (35–37). Therefore, we compared and contrasted the effects of 4HPR on pairs of cell lines derived from primary and metastatic cancers (UMSCC-22A and UMSCC-22B, respectively) or selected in vivo for enhanced metastasis from less metastatic cells (DM14/Tu167-C2 and B16 F10/F1). 4HPR induced higher Rac activation in the metastatic DM14 HNSCC cancer cells relative to the primary cancer Tu167-C2 cells, evidenced by the increased Rac-GTP levels in DM14 cells (Fig. 6A). Interestingly, 4HPR induced higher levels of ROS in DM14 and B16-F10 cells compared with their primary cancer cell counterparts, Tu167-C2 and B16-F1 (Fig. 6B). ROS levels in all of these cell lines were inhibited by cotreatment of cells with the Rac inhibitor. The increase in ROS levels in 4HPR-treated cells correlated with increased cell growth inhibition in the metastatic cancer cells compared with their corresponding primary cancer cells (Fig. 6C). The growth inhibitory effect of 4HPR was decreased by cotreatment of cells with the Rac inhibitor NSC-23766 (Fig. 6D).

Discussion

Several groups including our own have shown that 4HPR generates ROS and that this effect is important for apoptosis.
induction (12, 13). Various effects of 4HPR leading to apoptosis seem to be downstream of ROS induction, including activation of MAPKs (21). However, little is known about events or mechanisms by which 4HPR increases ROS generation. Although activation of the small GTPase Rac is often associated with cancer progression (36, 37), it also functions as a catalytic subunit of NADPH oxidase leading to ROS generation (24, 38) and has been implicated in apoptosis induction (39). Therefore, we hypothesized that Rac plays a role in 4HPR-induced ROS generation and subsequent apoptosis. Indeed, in this study, we present for the first time evidence that points to a novel role of Rac activation in 4HPR-induced ROS generation and subsequent apoptosis. Different experimental approaches provided this evidence. Chemical inhibition of the function of Rac or siRNA-mediated silencing of its expression resulted in decreased ROS generation. In addition, siRNA-mediated targeting of the downstream Rac effector kinase PAK1 has led to a decrease in ROS generation by 4HPR. All of these changes in Rac were associated with modulation of 4HPR-induced apoptosis. Furthermore, expression of the constitutively active Rac mutant RacV12 alone inhibited the growth of human head and neck cancer cells and sensitized them to 4HPR-induced ROS generation and apoptosis.

The finding that ROS generated following Rac activation can cause apoptosis, as shown in 4HPR-treated cells, is novel because the previous studies implicating Rac in apoptosis induction by bufalin and capsaicin did not explore the possible involvement of Rac-mediated ROS generation in this effect (39, 40).

The JNK MAPK is involved in c-Jun phosphorylation and activation as well as expression (41). In this study, we showed that inhibition of Rac activation by cotreating cells with 4HPR and a Rac inhibitor abrogated both 4HPR-induced c-Jun phosphorylation and expression, supporting the conclusion that Rac is involved in JNK activation. Interestingly, Rac has previously been shown to selectively activate JNK as well as c-Jun transcriptional activity (41). However, in this study, we also showed that ROS elevation in 4HPR-treated cells leads to sustained JNK activation and apoptosis evidenced by PARP cleavage, both of which were abrogated following scavenging of 4HPR-induced ROS by tert-butyl hydroxyanisole. Moreover, we have shown that this ROS increase by 4HPR is downstream and dependent on Rac activation by 4HPR. These findings indicate that ROS generation induced by Rac activation mediates 4HPR-induced apoptosis and not other signaling mechanisms possibly initiated by Rac activation alone.

In an attempt to elucidate the mechanism of Rac activation by 4HPR, we targeted signaling pathways that have been shown to activate Rac. For example, we inhibited epidermal growth factor signaling with the irreversible tyrosine kinase inhibitor EKB559 (42) and the phosphatidylinositol 3-kinase inhibitor LY129004 (43) and found no change in ROS generation by 4HPR in UMSCC-22B HNSCC cells (data not shown). Further studies are needed to determine how 4HPR activates Rac.

It is has been shown that transformed cells exhibit higher basal ROS levels compared with nontransformed cells. The higher ROS level is thought to be essential for stimulation of cell growth and sustenance of high metabolic rates in transformed cells (44). Furthermore, metastatic cells also exhibit high levels of ROS thought to be important for their growth and progression (37). In addition, elevated ROS levels confer a growth advantage to cells by activation of several mitogenic signaling pathways (45). We thought it plausible that elevated ROS levels, although capable of inducing...
apoptosis, can still instigate growth-promoting signals in the metastatic UMSCC-22B HNSCC cells we used in this study. We compared levels of ROS generation and cell growth inhibition by 4HPR in isogenic pairs of primary and metastatic cancer cells. Interestingly, 4HPR led to increased ROS generation, which correlated with increased cell growth inhibition in the metastatic cancer cells compared with their corresponding primary cancer cells. Moreover, Rac, which we also found in this report to be important for ROS generation by 4HPR, was activated by 4HPR to higher levels in the DM14 metastatic cancer cells compared with their corresponding Tu167-C2 primary cancer cells. It is worthwhile to note that in one report, β-phenylethyl isothiocyanate was shown to selectively kill oncogenically transformed cells through a ROS-dependent mechanism (46).

Rac plays crucial roles in migration and invasion, which are important steps in the metastasis process of cancer cells to near or
distant tissues and organs (35). Interestingly, Ferraro and colleagues (47) have shown that endogenous ROS levels are higher in the B16-F10 metastatic cells compared with the B16-F murine melanoma cells due to increased Rac1 activation. Nonetheless, our findings show that the activation of a protein involved in cancer promotion, such as Rac, may still lead to cell death in conjunction with 4HPR treatment because the metastatic cells showed an augmented activation of Rac by 4HPR, which resulted in the generation of higher ROS levels and enhanced apoptosis.

In conclusion, we have shown that Rac activation by 4HPR precedes ROS generation and is required for cell death induction by the proapoptotic 4HPR and that metastatic cancer cells exhibit increased sensitivity to 4HPR because of higher Rac activation in these cells compared with their primary isogenic cancer cell counterparts. Moreover, our findings support the use of the ROS-generating and promising anticancer agent 4HPR against metastatic cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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