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Rexinoid inhibits Nrf2-mediated transcription through retinoid X receptor alpha

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ABSTRACT

NF-E2 P45-related factor 2 (Nrf2) is a key transcription factor that controls genes encoding cytoprotective and detoxifying enzymes through antioxidant response elements (AREs) in their regulatory regions. We reported recently that retinoid X receptor alpha (RXRα) inhibits Nrf2 function by direct interaction with the Neh7 domain of Nrf2 in a ligand-independent manner. Here, we provide evidence that an RXRα-specific ligand, bexarotene, dose-dependently inhibits the mRNA expression of ARE-driven genes. Knock-down of RXRα by siRNA abolished the inhibitory effect of bexarotene. Conversely, the over-expression of RXRα enhanced the inhibition by bexarotene, indicating that the effect is mediated by RXRα. The inhibition by bexarotene was also found in the non-small-cell lung cancer cell line A549, which carries a dysfunctional somatic mutation of Kelch-like ECH-associated protein 1 (KEAP1), suggesting that KEAP1 is not involved. Our results demonstrate that rexinoid is able to inhibit the transcriptional activity of Nrf2, and that RXRα can repress the cytoprotection pathway in a ligand-dependent manner.

1. Introduction

NF-E2 p45-related factor 2 (Nrf2) is a cap'n'collar transcription factor which plays a key role in protecting cells from pro-oxidants and electrophiles. Nrf2 regulates the basal and inducible expression of genes that contain antioxidant response element (ARE) sequences in their promoter regions. Nrf2 target genes include those encoding antioxidant and detoxification enzymes such as aldo-keto reductase, heme oxygenase 1 (HO-1), glutathione S-transferase (GST), glutamate-cysteine ligase, and NADP(H): quinone oxidoreductase-1 (NQO1) [1–3].

It has been established that the ubiquitin ligase substrate adaptor Kelch-like ECH-associated protein 1 (KEAP1) is a major repressor of Nrf2. Under normal conditions, Nrf2 is constantly degraded via the ubiquitin–proteasome pathway in a KEAP1-dependent manner. Under stressful conditions, reactive oxygen species or electrophiles modify cysteine residues in KEAP1 causing loss of its adaptor activity, and in turn its failure to ubiquitinate Nrf2. Upon inactivation of KEAP1, Nrf2 accumulates in the nucleus where it heterodimerizes with small Maf proteins and activates ARE-driven genes [4,5]. At least 10 chemical classes of monofunctional phase 2 gene inducers have been identified [6]. Nrf2 activators react with sulfhydryl groups of Keap1, resulting in disruption of the Keap1–Nrf2 complex [4]. The activation of Nrf2 is regarded as an effective strategy for the chemoprevention of cancer and many other diseases [7].

On the other hand, Nrf2 is up-regulated in numerous types of cancer, including those of the head and neck [8], lung [9–11], skin [12], gallbladder [10], breast [13], prostate [14], and endometrium [15]. Nrf2 likely confers a growth advantage on cancer cells by enhancing cytoprotection and anabolism [16]. Interruption of Nrf2 signaling has been recognized as a new strategy for the sensitization of chemoresistant tumors to therapeutic and cytotoxic agents. Therefore, small-molecule inhibitors of Nrf2 might have therapeutic utility.

Recently, we reported that retinoid X receptor alpha (RXRα) inhibits the transcriptional activity of Nrf2 through a physical interaction between the two factors in the absence of ligand [17]. The Neh7 domain of Nrf2 and the DNA-binding domain of RXRα

Abbreviations: AKR1C, aldo-keto reductase 1C; ATRA, all-*trans* retinoic acid; ARE, antioxidant response element; CTCL, cutaneous T-cell lymphoma; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HO-1, heme oxygenase 1; KEAP1, Kelch-like ECH-associated protein 1; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF-E2 p45-related factor 2; RT-PCR, real-time quantitative PCR; NSCLC, non-small-cell lung cancer; RAR, retinoic acid receptor; RXRα, retinoid X receptor alpha; siRNA, small interfering RNA; tBHQ,

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are required for this interaction [17]. To further understand the antagonistic effect of RXRα on the Nrf2 signaling pathway, we investigated whether RXRα also modulates Nrf2 activity in a ligand-dependent manner. Bexarotene, 4-[1-(3,5,5,8,8,-pentamethyl-5,6,7,8-tetrahydro-naphthalen-2-yl)-vinyl]-benzoic acid, is a synthetic RXR agonist [18]. In the present study, we found that bexarotene inhibited Nrf2 transcriptional activity via RXRα, indicating that RXRα antagonizes Nrf2 in a ligand-dependent manner.

2. Materials and methods

2.1. Chemicals and cell cultures

Unless otherwise stated, all chemicals were from Sigma–Aldrich Co., Ltd. (Shanghai, China), and all antibodies were from Santa Cruz Biotechnology (Shanghai, China). Bexarotene (Targretin) was from Ligand Pharmaceuticals Inc. (San Diego, CA). Antibody against actin was from Sigma (China). MCF7 (human breast carcinoma), Caco2 (human colon cancer) and A549 (non-small-cell lung cancer, NSCLC) cell lines were from ATCC (China). All cells were cultured at 37 °C, in 95% air and 5% CO_2, and passaged every 3–4 days. All media supplements for cell cultures were from Invitrogen (China).

2.2. Analysis of luciferase reporter gene activity

The ARE-luciferase reporter plasmid pGL-*GSTA2*.41 bp-ARE was used for transient transfections with Lipofectamine 2000 (Invitrogen) as described previously [19,20]. Briefly, 24 h after seeding, cells were transfected with the ARE-luciferase reporter plasmid. The plasmid pRL-TK encoding *Renilla* luciferase was used as a control for transfection efficiency. Following transfection, the culture medium was replaced 24 h later and cells were left for 6–24 h to respond to xenobiotics before being harvested. For control experiments, vehicle alone (0.1% v/v DMSO) was added to the growth medium. Firefly and *Renilla* luciferase activities in cell lysates were measured using a 96-microplate luminometer (GloMAX, Promega) following the addition of Luciferase Assay Reagent II (Promega). The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of *Renilla* luciferase.

2.3. siRNA and transient plasmid transfection

The siRNAs against human RXRα (RXRα siRNA) or non-targeting negative control siRNA (scrambled siRNA) synthesized by TaKaRa Biotechnology (Dalian, China) were described previously [17]. The plasmid pEGFP-mRXRα, encoding GFP-tagged mRXRα, was as described previously [17]. Cells were transfected with siRNA or plasmid using Lipofectamine 2000 according to the manufacturer's protocol.

2.4. Real-time quantitative PCR (RT-PCR)

The isolation of total RNA and RT-PCR were performed as described previously [17]. The primers and probes were synthesized by TaKaRa Biotechnology. Each assay was performed in triplicate. The results were analyzed with 7500 Real Time PCR System software (ABI, Shanghai, China). The level of 18S rRNA was used as an internal standard. The sequences of the primers and probes for measuring cDNAs corresponding to human AKR1C1 mRNAs have been described previously [21,22].

2.5. Western blot analysis

Whole-cell extracts were prepared as described previously [22]. Protein samples were separated on SDS–PAGE gels, and immunoblots were carried out using the standard protocol. Immunoblotting with antibody against actin was performed to confirm equal loading of whole-cell extracts.

2.6. Statistical analysis

Statistical comparisons were performed using the unpaired Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Bexarotene inhibits antioxidant response element-driven gene expression

To investigate whether rexinoids affect the Nrf2/ARE pathway, we started by testing the effect of the RXRα-specific agonist bexarotene on Nrf2 transcriptional activity. The ARE-reporter plasmid pGL-*GSTA2*.41 bp-ARE was transiently transfected into Caco2 cells. The transfected cells were treated with the Nrf2 activator *tert*-butylhydroquinone (tBHQ; 20 μM) for 6 h in the presence of different doses of bexarotene. We found that bexarotene dose-dependently inhibited the induction of ARE-luciferase activity with an estimated IC_{50} of 50 nM (Fig. 1A). RT-PCR analysis of the mRNA levels of endogenous Nrf2-regulated genes revealed that the tBHQ-induced *AKR1C1*, *HO-1*, *NQO1*, *GCLC*, and *MRP3* mRNAs were similarly inhibited by bexarotene (Fig. 1B). Over 60% reduction of tBHQ-induced *AKR1C1* mRNA occurred in the presence of 100 nM bexarotene, while *KEAP1* and *Nrf2* mRNA expression was not affected (Fig. 1B). We also carried out similar experiments in MCF7 cells, and western immunoblotting revealed that the induction of HO-1 protein by tBHQ (20 μM) was reduced from 3-fold to 2-fold by bexarotene (300 nM) (Fig. 1C). Previous studies have shown that upon tBHQ exposure, Nrf2 translocates to the nucleus within 15 min [23], and ARE-mediated transcription is detectable within 6 h [19]. The fact that the inhibition occurred 6–8 h after the addition of bexarotene suggests that the rexinoid might act on Nrf2 through a ligation with RXRα.

3.2. Knockdown of retinoid X receptor alpha abolishes the inhibitory effect of bexarotene

To determine whether RXRα is required for the inhibition by bexarotene, siRNA specific to RXRα was transfected into MCF7 cells. When the cells were co-transfected with the ARE-reporter plasmid pGL-*GSTA2*.41 bp-ARE and treated with tBHQ (20 μM), the induced ARE-luciferase activity was reduced 52% by bexarotene (300 nM) in the cells transfected with scrambled siRNA. In contrast, the cells transfected with hRXRα-siRNA showed no response to bexarotene (300 nM) (Fig. 2A). We carried out similar experiments in Caco2 cells and, consistent with the results in MCF7 cells, the tBHQ-induced *AKR1C1* and *HO-1* mRNA expression was reduced >20% by bexarotene (300 nM). Again, when RXRα expression was knocked down by hRXRα-siRNA, *AKR1C1* and *HO-1* mRNA levels were not changed by bexarotene (Fig. 2B). These data indicated that RXRα is essential for the anti-Nrf2 effect of bexarotene.

3.3. Over-expression of retinoid X receptor alpha enhances the inhibitory effect of bexarotene

We next over-expressed exogenous GFP-mRXRα in Caco2 cells after transient transfection with the pEGFP-mRXRα expression vector. In agreement with our recent report that mRXRα inhibits

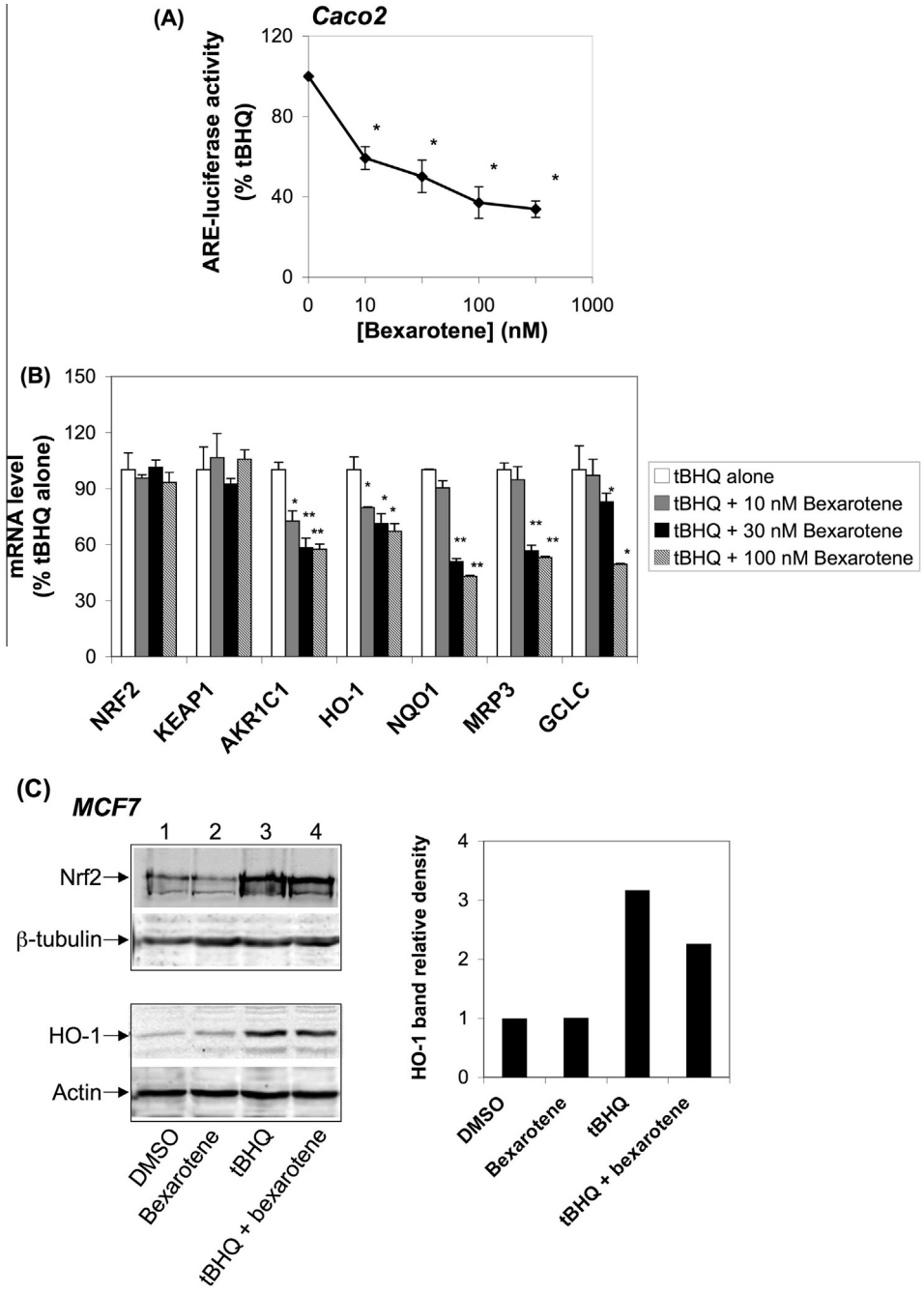

Fig. 1. Bexarotene dose-dependently inhibits ARE-driven gene expression. (A) Bexarotene inhibited the induction of ARE-driven luciferase activity by tBHQ in Caco2 cells. Caco2 cells were transiently transfected with the ARE reporter plasmid pGL-*GSTA2*.41 bp-ARE and the plasmid pRL-TK. Twenty-four hours later the cells were treated with 20 μM tBHQ in the presence 0–300 nM bexarotene for 6 h, then the dual luciferase activity was determined. The relative activity was calculated by normalizing firefly luciferase activity to that of *Renilla* luciferase. The value for cells treated with tBHQ alone was set at 100%. (B) Bexarotene inhibited the induction of endogenous ARE-driven transcription by tBHQ in Caco2 cells. Caco2 cells were treated with 20 μM tBHQ in the presence 10, 30, or 100 nM bexarotene for 24 h. *NRF2*, *KEAP1*, *AKR1C1*, *HO-1*, *NQO1*, *GCLC*, and *MRP3* mRNA levels were measured by RT PCR. The value of the same RNA from cells treated with tBHQ alone was set at 100%. (C) Bexarotene inhibited the induction of HO-1 in MCF7 cells. MCF7 cells were treated with 20 μM tBHQ in the presence of 300 nM bexarotene for 8 h. The nuclear and cytoplasmic fractions were prepared and analyzed by western immunoblotting with specific antibodies against Nrf2 and HO-1. β-Tubulin and actin were used as loading controls for the nuclear and cytoplasmic fractions, respectively. Values are mean ± SD. Results are from three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.005$.

ARE-driven gene expression in a ligand-independent manner [17], the over-expression of mRXRα reduced tBHQ-induced *AKR1C1* mRNA by 80%. Strikingly, the combination of over-expression of mRXRα and treatment with bexarotene (300 nM) reduced the induced *AKR1C1* mRNA level by 98% (Fig. 3). Similar inhibition of induced *HO-1* mRNA also occurred, whereas the over-expression of mRXRα resulted in 47% inhibition, which dropped further to nearly 80% with bexarotene (Fig. 3). These data further indicated that RXRα is able to inhibit the ARE gene battery ligand-dependently.

3.4. Inhibition of antioxidant response element-driven gene expression by bexarotene is independent of Kelch-like ECH-associated protein 1

Keap1 is the main repressor of Nrf2 [24]. To investigate whether Keap1 is involved in the inhibitory effect of bexarotene on Nrf2, we

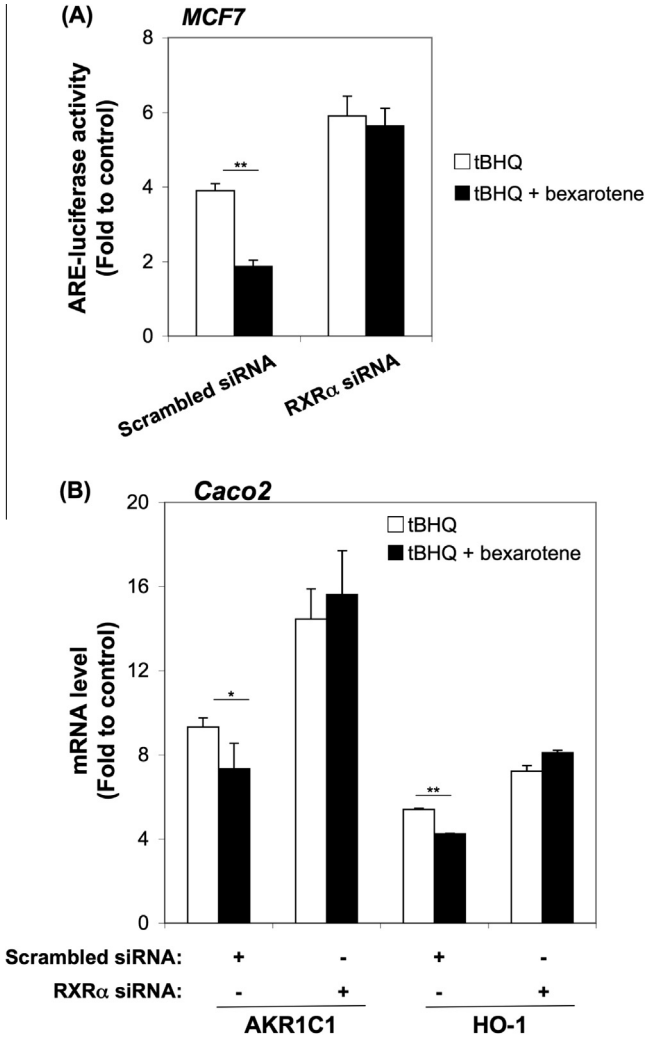

Fig. 2. RXRα is required for the inhibition of ARE-driven gene expression by bexarotene. (A) Bexarotene failed to inhibit tBHQ-induced ARE-driven luciferase activity in MCF7 cells when RXRα was knocked down. MCF7 cells were transiently transfected with siRNA specific to hRXRα, the ARE reporter plasmid pGL-*GSTA2*.41 bp-ARE, and the plasmid pRL-TK. Forty-eight hours later, the cells were treated with 20 μM tBHQ in the presence or absence of 300 nM bexarotene. After 6 h of incubation, the cells were harvested and the relative luciferase activity was determined. The value for cells transfected with scrambled siRNA and treated with DMSO was used as control and set at 1. (B) Knockdown of RXRα abolished bexarotene-mediated inhibition of the induction of *AKR1C1* and *HO-1* mRNAs in Caco2 cells. Caco2 cells were transfected with hRXRα-siRNA. Forty-eight hours later, the cells were treated with 10 μM tBHQ in the presence or absence of 300 nM bexarotene. After 6 h of incubation, the cells were harvested and the mRNA levels of *AKR1C1* and *HO-1* were determined by RT-PCR. The level of 18S rRNA was used as internal control. The value for cells transfected with scrambled siRNA and treated with DMSO was used as control and set at 1. Values are mean ± SD. Results are from three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.005$.

carried out similar studies in NSCLC A549 cells, which carry a somatic KEAP1 mutation that causes constitutively high expression and activity of Nrf2. In these cells, bexarotene dose-dependently inhibited *AKR1C1* mRNA expression, although the IC_{50} of 600 nM was much higher than that in Caco2 cells (Fig. 4A). After A549 cells were transfected with the ARE-reporter plasmid pGL-*GSTA2*.41 bp-ARE and treated with bexarotene (300 nM), the ARE-luciferase activity dropped by 40%, indicating that bexarotene inhibited the ARE-driven gene expression in these cells as well (Fig. 4B). Knock-down of RXRα expression by RXRα siRNA abolished the inhibition by bexarotene (Fig. 4C). Taken together, our results indicated that Keap1 is unlikely to be involved in the anti-Nrf2 effect of bexarotene.

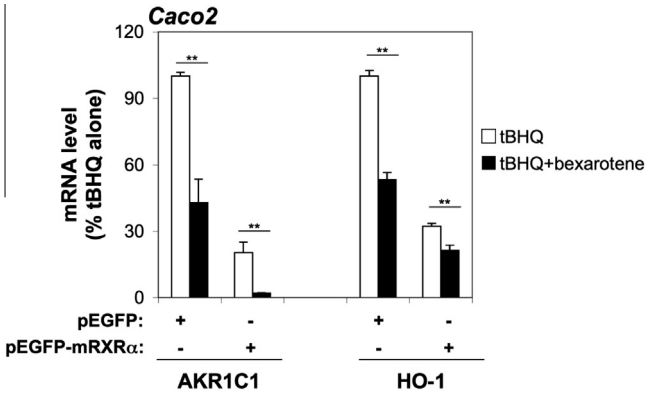

Fig. 3. Overexpression of RXRα enhances the inhibition of inducible ARE-driven gene expression by bexarotene. Caco2 cells were transiently transfected with pEGF-mRXRα. After 24 h recovery, the cells were treated with 20 μM tBHQ in the presence or absence of 300 nM bexarotene for 6 h and the mRNA levels of *AKR1C1* and *HO-1* were measured by RT-PCR. The level of 18S rRNA was used as internal control. The value for cells transfected with pEGFP and treated with tBHQ was used as control and set at 100%. Values are mean ± SD. Results are from three separate experiments. $^{**}p < 0.005$.

4. Discussion

Bexarotene is a novel, third-generation, RXR-selective retinoid ("rexinoid") that is FDA-approved for the treatment of cutaneous T-cell lymphoma (CTCL) [25]. When used as a single agent in advanced refractory CTCL, oral bexarotene has a total response rate of 45–71% with a duration of response from 3 to 17 months [25,26].

Bexarotene is known to induce apoptosis and affect cellular proliferation, differentiation, and cytokine production [27–31]. Atlucci et al. [32] showed that elevating the levels of cyclic AMP confers on rexinoids the ability to induce terminal granulocyte differentiation and the apoptosis of all-*trans* retinoic acid (ATRA)-resistant and insensitive acute myeloid leukemia cells and blasts from patients. Wang et al. [33] showed that bexarotene inhibits lung tumor progression in mutant A/J mouse models with genetic alterations in p53 or K-ras. Yen and Lamph [34] showed that when exposing human prostate cancer cells (PC3) to the therapeutic agents paclitaxel, doxorubicin, or cisplatin, combination treatment with bexarotene decreases the rate of spontaneous development of drug resistance, suggesting a role of bexarotene in preventing and overcoming drug resistance in advanced prostate cancer. Although the precise molecular mechanisms of action of bexarotene remain unknown, its multiple effects are also likely attributable to the RXRs, a family of nuclear hormone receptors that not only participate in retinoid signaling pathways as co-regulators of retinoic acid receptors (RARs), but also form heterodimers with at least 20 other nuclear receptors. In a previous study, we demonstrated that RXRα represses Nrf2 activity by forming a heterodimer with the transcription factor in a ligand-independent manner. Here, we showed that an RXRα ligand is capable of inhibiting Nrf2, indicating that RXRα can also inhibit the Nrf2/ARE system in a ligand-dependent manner.

In spite of the fact that a number of Nrf2 inducers have been developed and tested in clinical trials [35,36], only a few reports are available on the inhibition of Nrf2 activity by small molecules. ATRA and other RARα ligands have been found to inhibit Nrf2 activity [37]. The flavonoids luteolin and apigenin also inhibit Nrf2 activity [22,38–40]. Procyanidin tetramers and pentamers are the active components in extracts from *Cortex Cinnamomi* that suppress Nrf2 activity [41]. Brusatol has also been identified as a unique inhibitor of the Nrf2 pathway [42]. Brusatol selectively

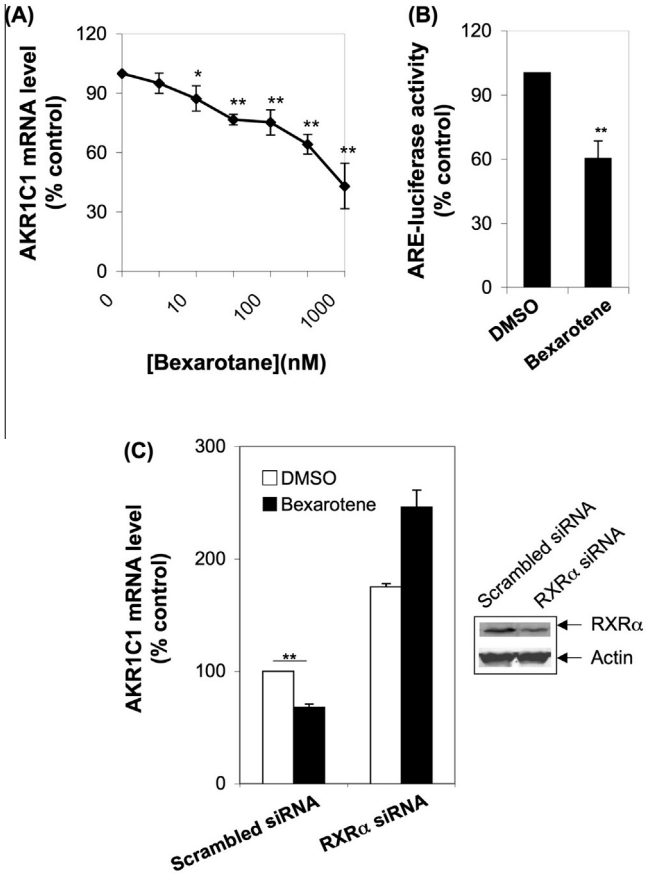

Fig. 4. Bexarotene inhibits ARE-driven gene expression in A549 cells. (A) Bexarotene dose-dependently inhibited *AKR1C1* mRNA expression in A549 cells. A549 cells were exposed to 0–1000 nM bexarotene for 24 h. *AKR1C1* mRNA levels were determined by RT-PCR. The level of 18S rRNA was used as internal control. The value for cells treated with DMSO was set at 100%. (B) Bexarotene inhibited ARE-luciferase activity in A549 cells. A549 cells were transiently transfected with the ARE reporter plasmid pGL-*GSTA2*.41 bp-ARE and the plasmid pRL-TK. Twenty-four hours later, the cells were treated with 300 nM bexarotene. After 6 h of incubation, the cells were harvested and the relative luciferase activity was calculated. The value for cells treated with DMSO alone was set at 100%. (C) Knockdown of RXRα abolished bexarotene-mediated inhibition of *AKR1C1* mRNA in A549 cells. Cells were transfected with hRXRα-siRNA. Forty-eight hours later, the cells were treated with 300 nM bexarotene. After 6 h of incubation, the cells were harvested and the mRNA levels of *AKR1C1* were determined by RT-PCR. The value for cells transfected with scrambled siRNA and treated with DMSO was used as control and set at 100%. Values are mean ± SD. Results are from three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.005$.

reduces the protein level of Nrf2 by enhancing its ubiquitination and degradation [42]. However, further studies are required to develop this compound into a chemotherapeutic drug. In the present study, we showed that, similar to other vitamin A derivatives such as ATRA, bexarotene is an inhibitor of Nrf2 transcriptional activity. Bexarotene is an established single agent for the treatment of CTCL [25]. Recently, bexarotene in combination with other anticancer drugs has also been used in the clinical trials for the treatment of other types of cancers such as NSCLC [43]. Our study highlights a novel mechanism by which bexarotene may control cancer progression.

Acknowledgments

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