

Bexarotene-induced Cell Death in Ovarian Cancer Cells Through Caspase-4-mediated pyroptosis

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Abstract

Purpose: Bexarotene is selectively activates retinoid X receptor, which is a commonly used anticancer agent for cutaneous T-cell lymphoma. In this study, we aimed to investigate the anticancer effect of bexarotene and its underlying mechanism in ovarian cancer *in vitro*.

Methods: The ES2 and NIH:OVACAR3 ovarian cancer cell lines were treated with 0, 5, 10, or 20 μM bexarotene. After 24 hours, cell number measurement and lactate dehydrogenase (LDH) cytotoxicity assay were performed. The effect of bexarotene on CDKN1A expression, pyroptosis, and apoptosis were evaluated.

Results: Bexarotene reduced cell proliferation in all concentrations in both cells. At concentrations above 10 μM , it increased extracellular LDH activity with cell rupture. In both cells, 10 μM bexarotene treatment increased the *CDKN1A* mRNA levels and reduced cell cycle related protein expression. In ES2 cells, caspase-4 and GSDME were activated, whereas caspase-3 was not, indicating that bexarotene-induced cell death might be pyroptosis.

Conclusion: A clinical setting dose of bexarotene induced cell cycle arrest and cell death through caspase-4-mediated pyroptosis in ovarian cancer cell lines. Thus, bexarotene may serve as a novel therapeutic agent for ovarian cancer.

Introduction

Ovarian cancer is the fifth most common cause of cancer-related death and the most deadly gynecological cancer in western countries (Siegel et al. 2019). Although the initial clinical response is generally satisfactory, more than 70% of affected patients experience recurrences and ultimately die. Multimodality treatment with cytoreductive surgery and platinum-taxane-based chemotherapy combined with molecularly targeted drugs has shown prolonged survival. However, the overall cure rate of the disease has not considerably changed. Therefore, additional treatment strategies that can improve survival are urgently required.

Bexarotene (LGD1069, Targretin®) is a retinoid X receptor (RXR)-selective agonist that binds and activates all three RXR isoforms (RXR α , RXR β , and RXR γ) with equivalent affinity and potency (Boehm et al. 1994). Bexarotene is approved by the FDA and is a widely used drug for treating cutaneous manifestations in cutaneous T-cell lymphoma (CTCL) (Gniadecki et al. 2007). This drug has gained increasing attention in cancer treatment; hence, it has also been used as an off-label drug for non-small cell lung cancer (Dragnev et al. 2011), and breast cancer (Esteva et al. 2003). *In vitro* studies have shown that bexarotene can also prevent and overcome acquired drug resistance in advanced breast cancer (Yen et al. 2004a; Yen and Lamph 2005), non-small cell lung cancer (Yen et al. 2004b; Tooker et al. 2007), and even advanced prostate cancer (Yen and Lamph 2006). An *in vitro* analysis revealed that bexarotene could kill cancer cells via apoptosis, decrease TGF- α and EGFR expressions, induce cellular senescence

associated with increased p21 and p16 expressions, and promote G1 phase cell cycle arrest (Song et al. 2001). However, the precise mechanism of cell proliferation inhibition by bexarotene remains unclear.

Pyroptosis is one of the regulated cell deaths triggered by certain caspases that are mainly activated by inflammation, anticancer drugs, and endoplasmic reticulum (ER) stress. Inflammatory caspases (caspase-1/4/5 in humans) cleave gasdermin D (GSDMD), which is required and sufficient for pyroptosis (Shi et al. 2015; Yu et al. 2021). The polymerized GSDMD N-terminal forms pores in the cell membrane to increase permeability and osmosis, thereby causing cell rupture, which is a characteristic of pyroptosis. Caspase-3, a well-known apoptotic caspase, causes pyroptosis with gasdermin E (GSDME/DFNA5). Chemotherapy drugs such as cisplatin, etoposide, and doxorubicin induce pyroptosis in tumor cells with high GSDME levels caused by caspase-3 (Wang et al. 2017). Several anticancer drugs such as chemotherapy drugs and miRNA can reduce tumor viability and invasiveness by inducing tumor pyroptosis (Xia et al. 2019). Hence, pyroptosis induction might be a potent anticancer drug target (Tan et al. 2021).

In epithelial ovarian cancer, retinoic acid receptors are frequently and strongly expressed and may indicate an adverse prognosis (Kaiser et al. 2005). RXR-selective retinoids, such as bexarotene, might potentially treat ovarian cancer; however, the effect of bexarotene on ovarian cancer remains unclear. Hence, this study aimed to investigate whether bexarotene could suppress the proliferation of ovarian cancer cells in vitro and to better understand the antitumorigenic potential of bexarotene by exploring the precise mechanism of bexarotene on cell death, particularly pyroptosis.

Methods

Cell culture

ES2 cell lines and NIH:OVACAR-3 cell lines were purchased from ATCC (Manassas, VA, USA) and RIKEN BioResource Center (Tsukuba, Japan). These ovarian cancer cell lines were routinely cultured in RPMI 1640 (Nacalai Tesque, Kyoto, Japan) containing inactivated 2% fetal bovine serum (Equitech-Bio, Kerrville, TX, USA) and 1% penicillin–streptomycin (Nacalai Tesque) at 37°C with 5% CO₂ in room air.

Cell proliferation assay and lactate dehydrogenase (LDH) cytotoxicity assay

We seeded the cells in 12-well plates (100,000 cells/well) in the culture medium. At 80% confluence, the cells were treated with 0–20 μM of bexarotene (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) for 24 hours. The cells were then treated with trypsin–EDTA solution (Nacalai Tesque) and mixed with the total amount of trypan blue solution (Nacalai Tesque). The live cells were counted using a cell-counting chamber (WakenBtech Co., Ltd., Kyoto, Japan) under a stereomicroscope (Olympus, Tokyo, Japan).

Moreover, we collected the cell culture supernatant and measured the LDH activity using the LDH Cytotoxicity Assay Kit (Nacalai Tesque) according to the manufacturer's protocol. The LDH levels of the cell culture supernatant derived from the bexarotene-treated cell group were normalized relative to the cultured medium derived from the vehicle group. We repeated each experiment at least three times and compared the LDH activity with that of the vehicle group.

Protein extraction and western blot

We washed the cultured cells with PBS and extracted the total protein using a complete Lysis-M reagent (Roche, Basel, Switzerland) containing 1% Halt™ Phosphatase Inhibitor Cocktail (Thermo Fisher Science, Waltham, MA, USA) for 15 minutes at room temperature. We subsequently centrifuged the cell lysate at 15,000×g for 15 minutes at 4 °C, separated 7.5 µg of total protein in 10% or 4%–15% gradient SDS-PAGE (Bio-Rad, Hercules, CA, USA), and transferred it to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Burlington, MA, USA). Nonspecific binding to the PVDF membrane was blocked in Blocking one-P solution (Nacalai Tesque) at room temperature for 30 minutes. The first antibody was reacted at 4 °C for overnight. Then, we washed the PVDF membrane in PBS-T and enhanced the protein signal by anti-mouse or rabbit IgG antibody (1/10000 or 5000) at room temperature for 1 hour. We used ECL select or ECL Prime reagent (Roche) to detect protein signals.

RNA extraction and Real-time quantitative PCR

Total RNA from cultured cells was extracted using the RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Science, Waltham, MA, USA), we reverse-transcribed the extracted RNA into complementary DNA (cDNA). Real-time quantitative PCR (RT-qPCR) was conducted using Light Cycler DNA Master SYBR Green I Kit (F. Hoffmann–Roche Ltd., Basel, Switzerland) with Light Cycler Nano System (F. Hoffmann–Roche Ltd). Furthermore, we evaluated the *CDKN1A* and *GAPDH* (internal control) mRNA expression levels. The expression levels of measured genes were normalized relative to that of *GAPDH* mRNA. The $2^{-\Delta\Delta Cq}$ method was used to obtain the relative quantitative value (Schmittgen and Livak 2008). Supplementary Table S1 lists the gene-specific primers.

Small interfering RNA (siRNA) transfection

Using Lipofectamine RNAiMAX (Thermo Fisher Science, Waltham, MA, USA), we transfected ES2 cell lines with a siRNA. The si-protein kinase R-like endoplasmic reticulum kinase (PERK; SI02223718; Qiagen, Hilden, Germany) RNA, si-inositol-requiring enzyme 1-A (IRE1A; SI00605255; Qiagen), or a non-target siRNA (control) were used. To prepare the siRNA transfection solution for each tube, we mixed 5 pmol of siRNA with 50 µL of Opti-MEM reduced-serum medium (Thermo Fisher Science, Waltham, MA, USA). Concurrently, we mixed 1 µL of Lipofectamine RNAiMAX with 50 µL of Opti-MEM. Then, the two solutions

were mixed by gentle pipetting and incubated for 5 minutes at room temperature to allow siRNA/lipid complexes to form. The treated cells forming siRNA/lipid complexes were incubated for 48 h at 37°C, followed by bexarotene treatment.

Antibodies and other reagents

To detect the target protein, we used anti-caspase-4 antibody (1/1000; Cell Signaling Technology, Danvers, MA, USA), anti-GSDME antibody (1/1000; Proteintech Group, Rosemont, IL, USA), anti-cyclin D1 antibody (1/1000; Cell Signaling Technology), anti-Rb antibody (1/1000; Cell Signaling Technology), anti-phospho-Rb antibody, anti-CDK4 antibody (1/1000; Cell Signaling Technology), anti-CDK6 antibody (1/1000; Cell Signaling Technology), anti-PERK antibody (1/1000; Cell Signaling Technology), anti-Binding immunoglobulin protein (BiP) antibody (1/1000; Cell Signaling Technology), and anti- β actin antibody (1/5000; Cell Signaling Technology) as the first antibodies.

We used ZYVAD-FMK (R&D SYSTEMS, Minneapolis, MN, USA) for caspase-4 inhibition experiments.

Statistical analysis

The cell proliferation assay was statistically analyzed using an independent *t*-test. All comparisons were performed using a two-sided test. In addition, $P < 0.05$ was considered statistically significant. All statistical data were analyzed using the JMP statistical software (SAS Institute Inc., Cary, NC, USA).

Results

Bexarotene reduced cell viability and affected cell shape

After 24 hours of bexarotene treatment, cell viability was significantly reduced dose-dependently both in ES2 and NIH:OVACAR3 cells (Fig. 1a), and the morphology in cancer cells drastically changed, as observed microscopically (Fig. 1b). Bexarotene-treated cells were detached from the surface of the culture dish, and the plasma membrane clearly expanded. Moreover, some cells exhibited a post-cell burst appearance ($\geq 10 \mu\text{M}$ of bexarotene).

The effect of bexarotene on plasma membrane damage was evaluated by measuring the released LDH activity in the culture supernatant. In living cells, LDH is present in the cytoplasm, but it is released extracellularly due to cell membrane changes that occur during the cell death processes, such as in necroptosis and pyroptosis (Schneider et al. 2017). The levels of released LDH significantly increased at the 10 and 20 μM bexarotene-treated group compared with the vehicle group in ES2 cells (Fig. 1c, $P < 0.05$). Likewise, the LDH levels in NIH:OVACAR3 cells also significantly increased in the 10 and 20 μM bexarotene-treated group ($P < 0.05$). These results indicated that bexarotene induced cell death by causing plasma membrane damage in ovarian cancer cell lines.

Bexarotene increased *p21* mRNA expression and induced cell growth inhibition

First, we measured the mRNA expression levels of *RXR-α*, *RXR-β*, and *RXR-γ* in ES2 and NIH:OVACAR-3 cells using RT-PCR to confirm the RXR expression. The *RXR-α* and *RXR-β* mRNA were expressed, but *RXR-γ* mRNA was not in both cells (data not shown). Tanaka et al. reported that a ligand-activated RXR homodimer upregulates cyclin-dependent kinase inhibitor p21 (CDKN1A) expression by directly binding to its promoter region and regulating cell proliferation (Kehn et al. 2004). To determine whether bexarotene acted as an RXR agonist in ovarian cancer cell lines, we measured the *CDKN1A* mRNA expression levels after 24 hours of bexarotene treatment. Bexarotene significantly increased the *CDKN1A* mRNA expression levels dose-dependently in both cell lines (Fig. 2a, b).

Next, the levels of cell cycle-related proteins, specifically the downstream of p21 protein, CDK4, CDK6, Cyclin D1, and phospho-RB, were examined using western blot in 10 μM bexarotene-treated cells (Kehn et al. 2004). After 48 hours of bexarotene treatment, the levels of CDK4, CDK6, Cyclin D1, and phospho-RB decreased in both ovarian cancer cell lines (Fig. 2c, d). These results indicated that bexarotene acted as an RXR activator in ovarian cancer cell lines.

Bexarotene-induced pyroptosis via caspase-4 and GSDME-related pathway

As above, bexarotene-treated ovarian cancer cell lines exhibited drastic morphological changes with the release of intracellular LDH. The swelling structure formation and membrane rupture during cell death is related to pyroptosis (Shi et al. 2015; Zhang et al. 2018). Thus, the bexarotene-induced cell death might be pyroptosis. We used ES2 cells, which were highly sensitive to bexarotene, to determine whether bexarotene induces pyroptosis in ovarian cancer cells. To determine whether the expression and activation of caspase-1 and -4 are caused by bexarotene stimulation in molecular levels, we performed western blot using an antibody specific to caspase-1 and -4. In ES2 cells, caspase-4 was expressed and activated after 8 and 24 hours from 10 μM bexarotene treatment (Fig. 3a). Meanwhile, caspase-1 mRNA and protein were not detected in ES2 cell line (data not shown). Bexarotene stimulation could not activate caspase-3, the apoptosis-related caspase (Fig. 3a). During pyroptosis, the activated caspase cleaves GSDM and the N-terminal domain dimer of GSDM induces cell membrane perforation (Feng et al. 2018). Therefore, we also evaluated GSDME and GSDMD cleavage using western blot. From 8 hours after the start of bexarotene treatment, we detected the cleaved GSDME (Fig. 3a). Meanwhile, bexarotene treatment did not cleave GSDMD (Fig. 3a).

Then, we evaluated whether 20 μM ZYVAD-FMK (caspase-4 inhibitor) pretreatment can mitigate bexarotene-induced cell viability reduction and LDH release into the culture media. As shown in Fig. 3b, pretreatment with ZYVAD-FMK could not restore bexarotene-induced cell viability reduction ($P = 0.7742$, bexarotene-treated group vs. bexarotene with ZYVAD-FMK-treated group). Meanwhile, pretreatment with

ZYVAD-FMK suppressed the increase of extracellular LDH levels induced by bexarotene (Fig. 3c; bexarotene-treated group vs. bexarotene with ZYVAD-FMK-treated group; $P < 0.05$). In addition, the pretreatment of ZYVAD-FMK with bexarotene attenuated morphological changes in ES2 cells (Fig. 3d). These results indicate that bexarotene has two functions on ovarian cancer cells: cell proliferation suppression and caspase4-GSDME related-pyroptosis, and these are independent actions.

Bexarotene-induced ER stress, which is not associated with pyroptosis

The ER stress might cause pyroptotic cell death in several cells *in vitro* (Lebeauin et al. 2015; Ke et al. 2020). Thus, we examined whether bexarotene-induced ER stress in ES2 cells. We also evaluated whether bexarotene activated the unfold protein response (UPR) signaling pathways, which are common ER stress markers, in ES2 cells. We used PERK phosphorylation, BiP protein accumulation, and X-binding protein 1 (*XBP1*) mRNA splicing as ER stress response markers in this study.

After 2 hours of bexarotene treatment, the molecular weight of PERK was rapidly shifted to a higher molecular form (Fig. 4a), indicating that PERK protein was activated via phosphorylation. After 24 hours of bexarotene treatment, BiP significantly increased (Fig. 4a). Spliced *XBP1* worked as a transcriptional factor for upregulating or downregulating the UPR-related gene under ER stress conditions. From 2 hours after bexarotene treatment, *XBP1* mRNA was spliced in an increased time-dependent manner (Fig. 4b). These results indicated that bexarotene rapidly induced ER stress in ES2 cells. Next, we examined whether the knockdown of ER stress sensor proteins attenuated cell death caused by bexarotene in ES2 cells. We found that PERK and IRE1 knockdown could not attenuate the release of LDH (Fig. 4c) and restore cell morphological changes (Fig. 4d) caused by bexarotene treatment.

Discussion

This study revealed that bexarotene induced cell death in ovarian several cancer cell lines under a clinical setting dose. Furthermore, this study clarified the following points: 1) bexarotene induced cell death might be pyroptosis, which was mediated by caspase-4 activation and GSDME cleavage; 2) bexarotene rapidly induced ER stress but might not be related to pyroptosis.

Our study confirmed that bexarotene (5–20 μM) induced cell death and inhibited cell proliferation in ovarian cancer cell lines. These experimental doses of bexarotene have almost the same or rather lower concentrations than those of the clinical dose (C_{max} : 10.4 μM , Targretin capsules 75 mg, interview form, Minophagen Pharmaceutical Co., Ltd., Zama, Japan). Conversely, the dose of all-trans retinoic acid (ATRA), which is a well-investigated retinoic acid against ovarian cancer *in vitro*, has supraphysiologic concentrations. The ATRA concentration (sometimes as high as 10 μM) in *in vitro* studies was 5–50 times higher than that in the clinical setting (C_{max} : 0.18 μM , VESANOID Capsule 10mg, interview form, Fuji Pharma Co., Tokyo, Japan). ATRA increases apoptosis and decreases cell proliferation, thereby

proving effective against several ovarian cancer cell lines (Krupitza et al. 1995; Wu et al. 1997; Karabulut et al. 2010; Lokman et al. 2019). In the study by Lockman et al. (Lokman et al. 2019), the cell survival of two of five serous ovarian cancer cell lines (OVCAR-3 and OV-90) was inhibited by more than 20%. However, several ovarian cell lines, such as COV362, COV318, SKOV-3, and CP70, are resistant to ATRA. Contrary to ATRA, bexarotene had the potential of clinical application in ovarian cancer.

We confirmed that bexarotene-induced cell death is pyroptosis mediated by caspase-4 activation and GSDME cleavage. As far as we know, this is the first report of bexarotene-induced death of pyroptotic cells in an ovarian cancer cell line. Apoptosis is induced by bexarotene in CTCL cells, according to research (Zhang et al. 2002). Apoptosis is characterized by many distinct morphological features, including cell shrinkage and fragmentation into membrane-bound apoptotic bodies (Saraste and Pulkki 2000), and mediated by caspase-3 and -8 activation (Shi 2002). However, the characteristic morphology of apoptosis was not observed in bexarotene-treated ovarian cancer cell lines in ES2 cells and NIH: OVACAR3 cells. In addition, the extracellular activity of LDH, which is initially present intracellularly, is not elevated on apoptosis. This type of cell death featured cell proliferation like the morphological changes during pyroptosis (de Vasconcelos et al. 2019). Pyroptosis is a proinflammatory programmed cell death typically induced by a viral infection, toxin, and chemotherapy drugs (Yu et al. 2021). Three main pathways of inducing pyroptosis have been reported. The first is the canonical pathway of pyroptosis, which is regulated by inflammation-activated caspase-1. The second is the non-canonical pyroptotic pathway regulated by caspase-4/5 in humans and caspase-11 in the mouse. GSDMD is a common substrate for caspase-1/4/5/11 for both canonical and non-canonical pyroptosis (Feng et al. 2018). The last pathway is mediated by caspase-3 and GSDME, which can be activated by chemotherapy drugs (Wang et al. 2017). The study showed that bexarotene induced pyroptotic cell death through the caspase-4–GSDME-dependent signaling pathway in an ovarian cancer cell line. It is the first report demonstrating the induction of pyroptosis by caspase-4 activation and GSDME cleavage.

In this study, we also showed that bexarotene activated ER stress in ovarian cancer cells was consistent with the findings of a previous report wherein the human neuroblastoma cell line was induced by a high concentration of bexarotene (Dheer et al. 2018). Several studies have reported that ER stress can lead to pyroptosis (Lebeaupin et al. 2015; Ke et al. 2020; Cheng et al. 2019). Palmitic acid and the ER stressor tunicamycin induce ER stress, leading to pyroptosis. An ER stress inhibitor can inhibit pyroptosis caused by palmitic acid or tunicamycin-induced ER stress. In this study, we have also demonstrated that the attenuation of ER stress by si-PERK and si-ERE1a could not restore bexarotene-induced pyroptosis, suggesting that bexarotene-induced pyroptosis is not mediated by ER stress in ovarian cancer cells. Hence, future research should investigate how bexarotene induces pyroptosis in ovarian cancer cells.

The anticancer effect of bexarotene was due to its genomic function as an RXR agonist. In this study, bexarotene upregulated CDKN1A, which codes a cyclin-dependent kinase inhibitor p21, and downregulated cell cycle-related proteins downstream of p21. The ligand-activated RXR homodimer binds two RXR consensus domains within the promoter region of *CDKN1A* genes and upregulates the *CDKN1A* mRNA expression (Tanaka et al. 2007). Therefore, bexarotene also acts as an RXR agonist in ovarian

cancer cells. Several studies indicated that the accumulation of p21 could induce growth inhibition and cell cycle arrest but could not induce cell death (Almond and Cohen 2002). Thus, the induction of CDKN1A alone could not explain bexarotene-induced cell death. However, in ovarian cancer cell lines, bexarotene induced cell death with characteristic morphological changes through caspase-4 and GSDME. In addition, the knockdown of RXR α or RXR β by siRNA could not attenuate the LDH release induced by bexarotene (Supplementary Figure S1). Therefore, we speculated that bexarotene has both genomic and nongenomic functions (Fig. 5).

Conclusion

Our study has shown that the dose of bexarotene has the effect of inhibiting cell proliferation by inducing p21, which is still commonly known and can also cause cell death, possibly pyroptosis, in ovarian cancer cells lines. Therefore, bexarotene is a potential anticancer drug for ovarian cancer. This study, however, was unable to elucidate the mechanisms of bexarotene-induced pyroptosis. To better understand the pharmacological action of bexarotene, future research should investigate further into mechanisms of bexarotene-induced pyroptosis in ovarian cancer cells.

Declarations

Data availability

All data analyzed in this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Authors' contributions

TK, AM, KN, and MS conceived, designed the experiment while TK, PH, and MS performed the experiments. TK, KN, and AM wrote the article and conducted the analyses. All authors have read and

approved the final manuscript.

Competing interests:

The authors declare no competing interests.

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Figures

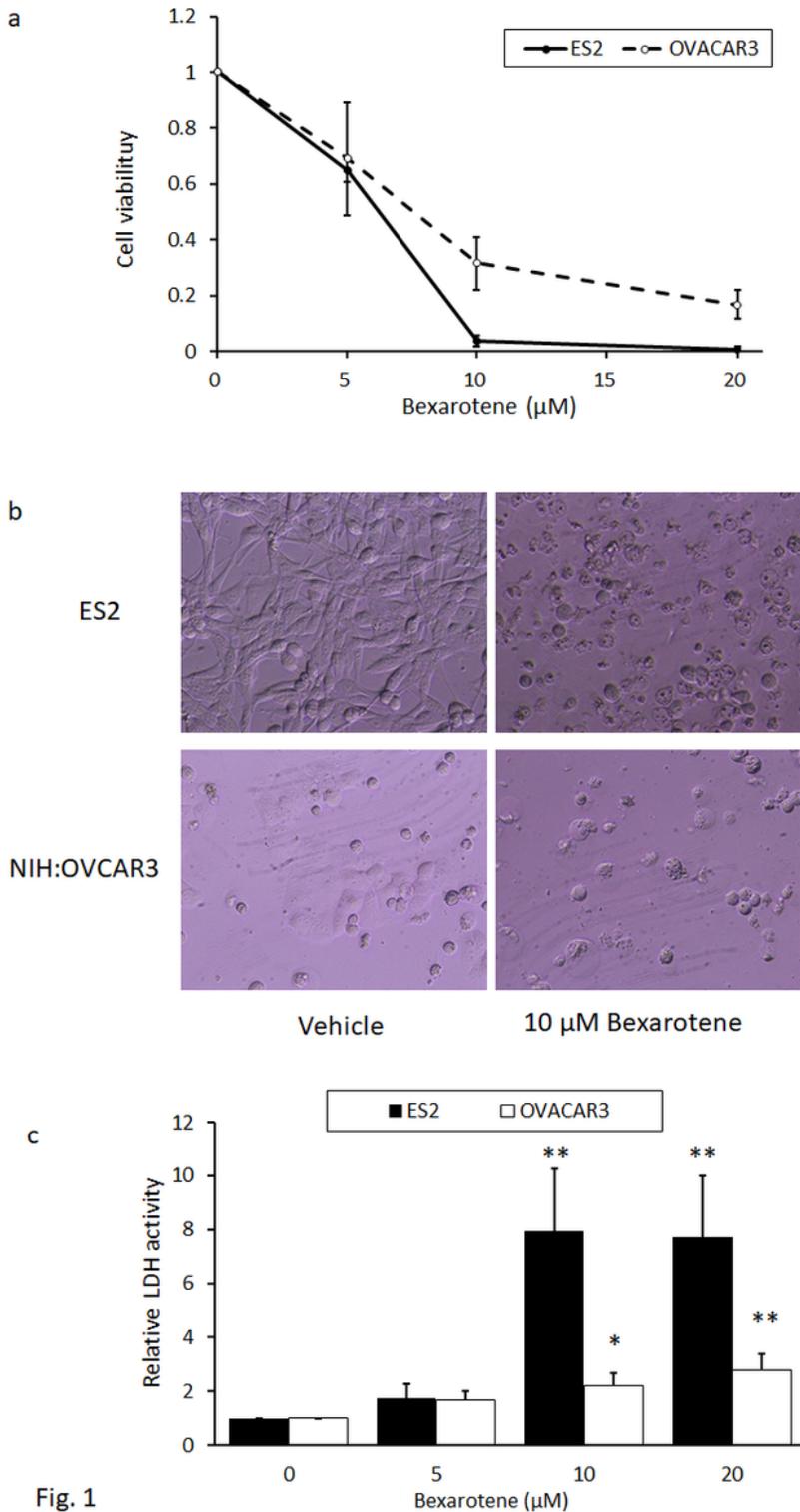


Fig. 1

Figure 1

Bexarotene treatment reduced cell viability and induced cell death by LDH release and plasma membrane bursting (a) In ES2 and NIH: OVACAR3 cells, counted manually were cell numbers after 24 hours of bexarotene treatment. Measured was the cell viability against the bexarotene nontreated group. The results presented were the mean, \pm standard deviation for at least three independent experiments. (b) Morphological analysis of ES2 cells (upper two panels) and NIH: OVACAR3 (lower two panels). The

plasma membrane expanded, and the broken cells had a swollen structure. (c) bexarotene treatment was extracellular LDH activity. The results presented were the mean, \pm standard deviation for at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with bexarotene-free control LDH, lactate dehydrogenase.

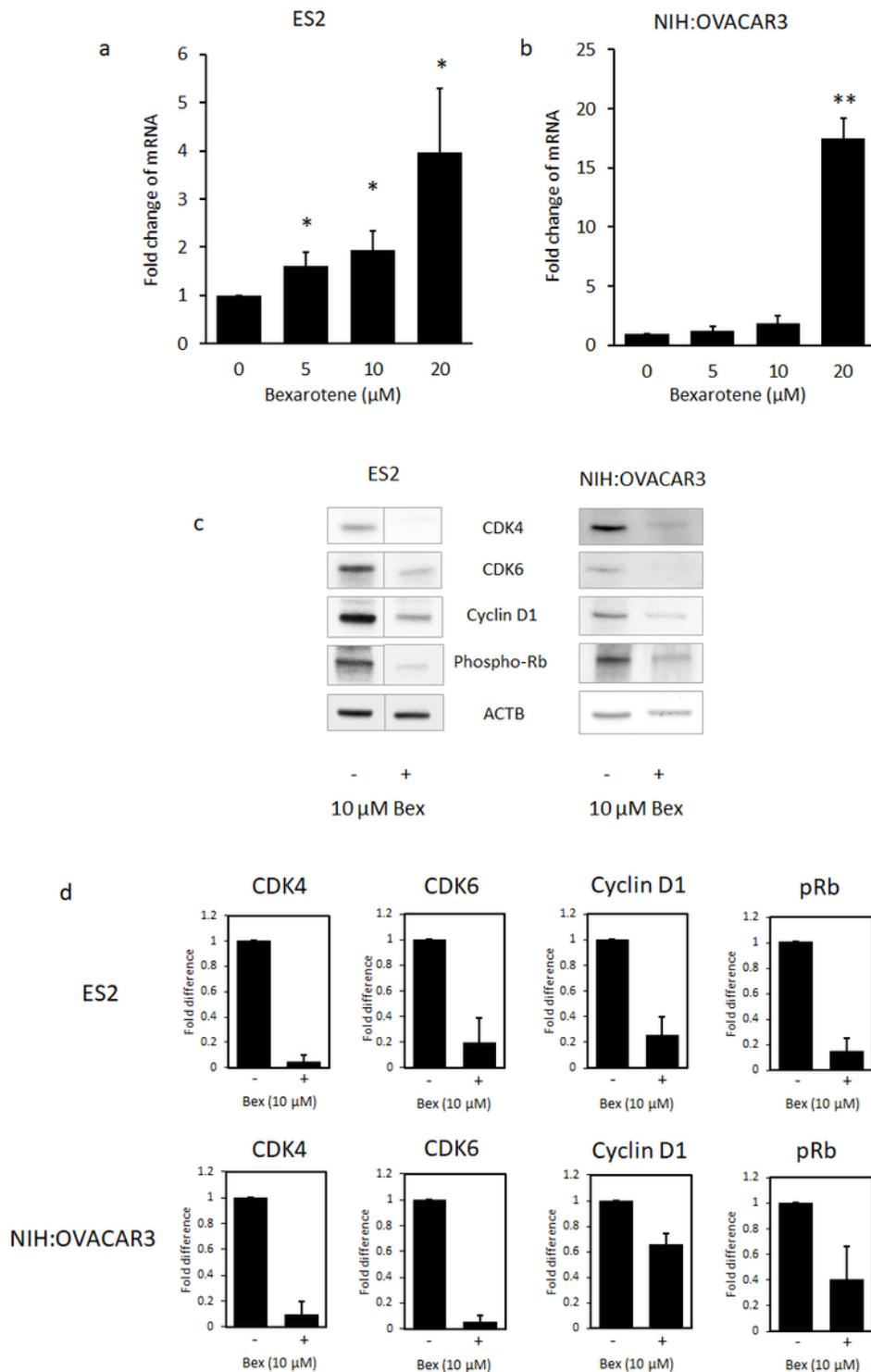


Fig. 2

Figure 2

Bexarotene treatment increased CDKN1A mRNA expression and reduced cell cycle-related proteins. Levels of CDKN1A mRNA were measured and compared between bexarotene-treated cells and nontreated cells 24 hours after bexarotene treatment in (a) ES2 and (b) NIH: OVACAR3 cells. CDKN1A expression was assessed using the $2^{-\Delta\Delta CT}$ quantitation method and normalized to housekeeping gene GAPDH using a no-treatment control as a calibrator. The results presented were mean, \pm standard deviation for at least three independent experiments. (c) Western blot analysis was performed to evaluate the effect of bexarotene on cell cycle-related proteins (CDK4, CDK6, cyclin D1, and phospho-Rb) in ES2 and NIH: OVACAR3 cells. Used as an internal control was β -actin. (d) Columns and bar graphs represent the mean \pm standard deviation for at least three independent experiments. Quantification of protein levels was performed by densitometry and normalized to β -actin. * $P < 0.05$, ** $P < 0.01$, compared with bexarotene-free control. ACTB, β -actin.

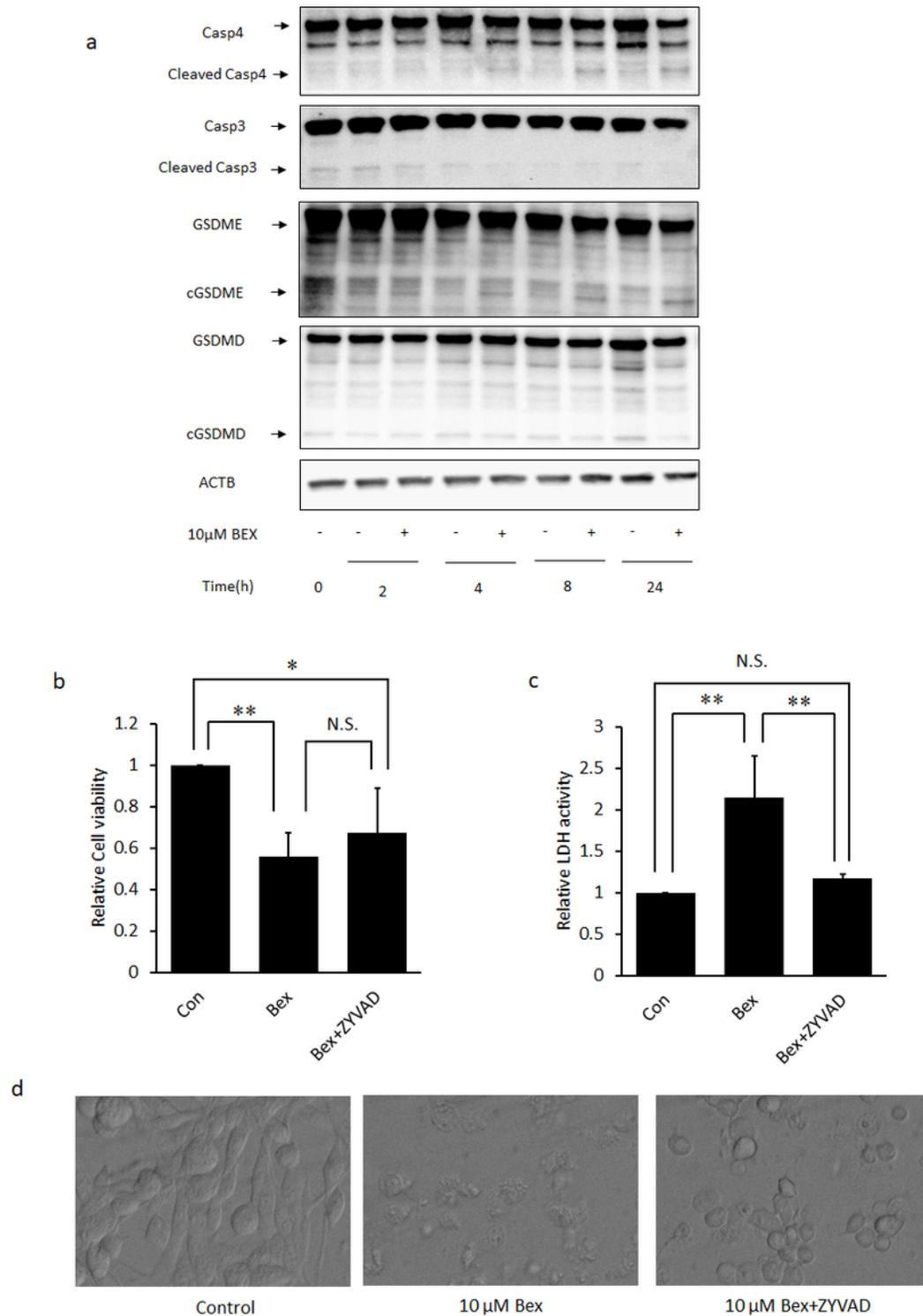


Fig. 3

Figure 3

Bexarotene induces pyroptosis in ES2 cells (a) Time-dependent changes on pyroptosis-(Casp4, GSDME, and GSDMD)- or apoptosis-(Casp3)-related proteins following exposure to 10 μ M bexarotene for 2–24 hours in ES2 cells. (b–d) ES2 cells were pretreated with or without 20 μ M ZYVAD-FMK (caspase-4 inhibitor) 1 hour before 10 μ M bexarotene treatment. (b) Cell numbers were manually counted 24 hours after bexarotene treatment, and cell viability against the bexarotene nontreated group was calculated.

Pretreatment with ZYVAD-FMK could not suppress bexarotene-induced cell viability reduction. (c) The extracellular LDH levels increased by bexarotene were reduced by pretreatment with ZYVAD-FMK. (d) Bexarotene-treated cells showed pyroptotic-like morphological changes (center panel), whereas pretreatment with ZYVAD-FMK attenuated bexarotene-induced cell shape alteration (right panel). β -actin was used as an internal control. Columns and bar graphs represent the mean \pm standard deviation for at least three independent experiments. * $P < 0.05$. ACTB, β -actin; cGSDME, cleaved GSDME; cGSDMD, cleaved GSDMD; ZYVAD-FMK, ZYVAD; NS, not significant.

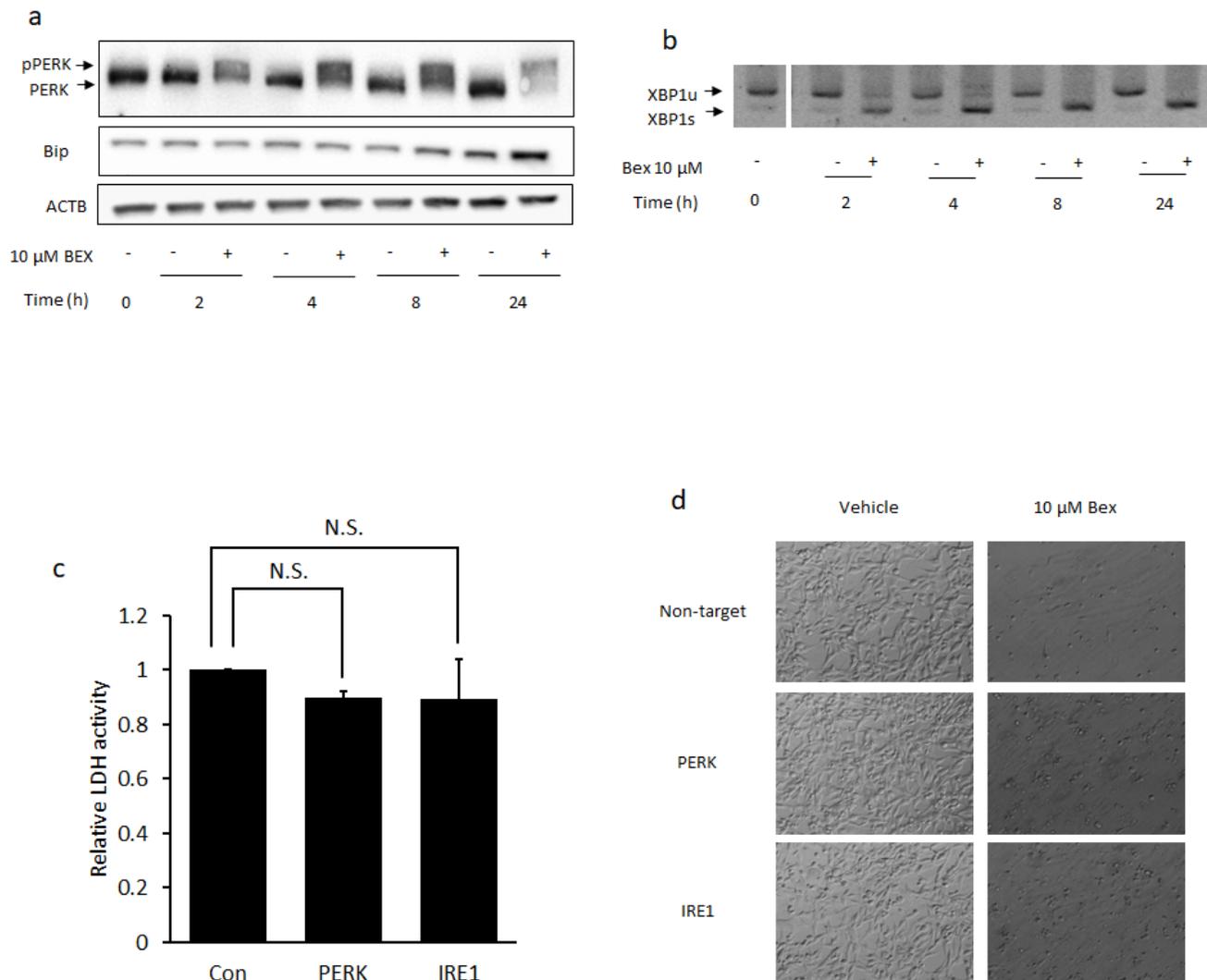


Fig. 4

Figure 4

Bexarotene-induced ER stress, which is not associated with pyroptosis Time-dependent changes in (a) PERK and BiP protein levels and (b) XBP1 mRNA following exposure to 10 μ M bexarotene for 2–24 hours. (a) BiP was increased as the molecular weight of PERK was rapidly altered to a higher molecular

form (active form). (b) Upregulated was the XBP1 mRNA splicing in a time-dependent manner. (c, d) Control, PERK, or IRE1 siRNA-transfected cells were incubated for 24 hours with 10 μ M bexarotene. (c) After bexarotene treatment, extracellular LDH was measured and compared between the control group and each siRNA-transfer group. Significant differences in LDH activity were not found between each siRNA transfer group and the control group. (d) Morphological analysis of control, PERK, or IRE1 siRNA-transfer ES2 cell line. β -actin was used as an internal control. Columns and bar graphs represent the mean \pm standard deviation for at least three independent experiments. NS, not significant.

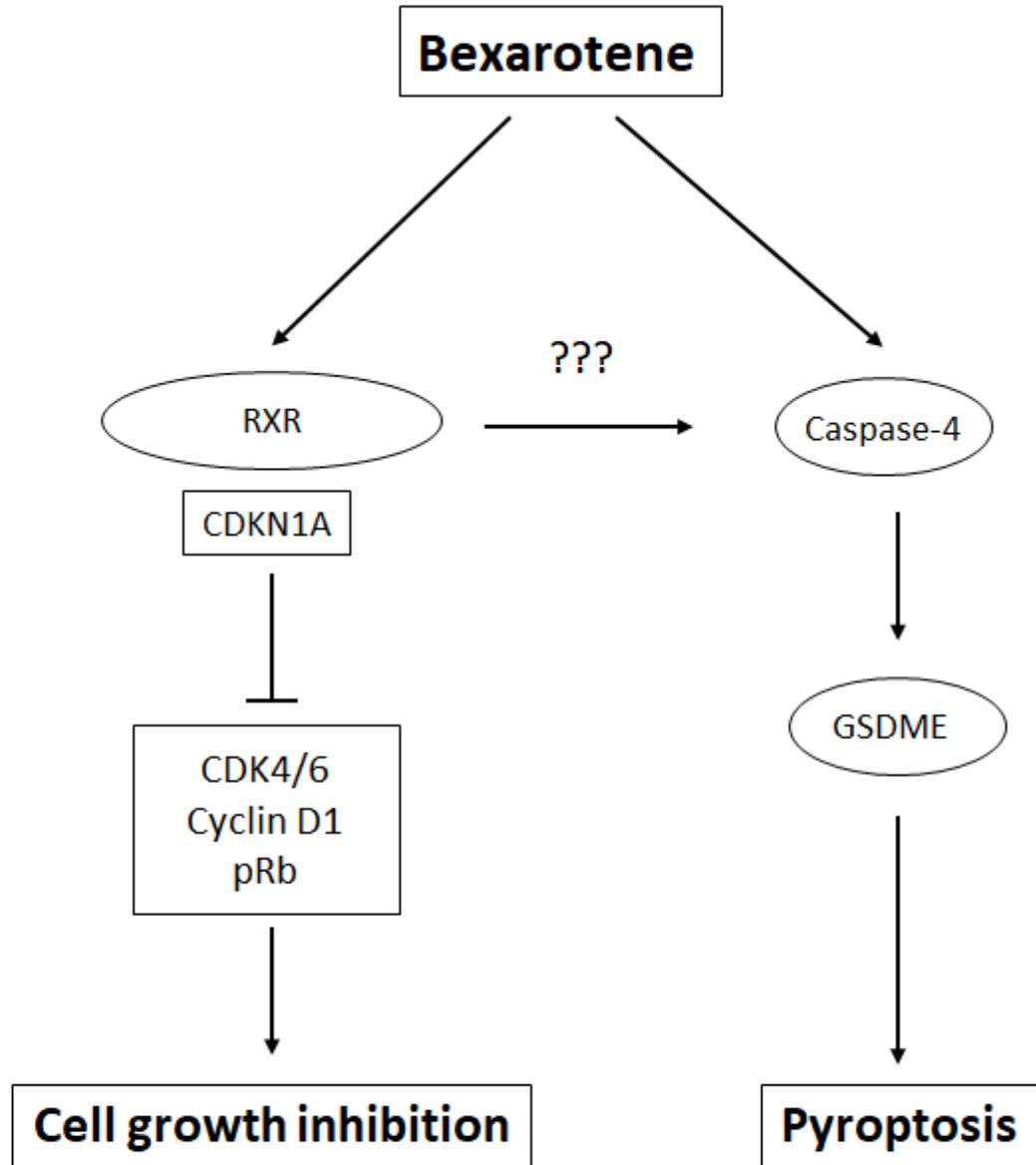


Fig. 5

Figure 5

Multiple effects of bexarotene on ovarian cancer cells Bexarotene inhibited cell cycle arrest and induced pyroptosis in ovarian cancer cell lines.

Supplementary Files

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- [SupplementaryFigurev2.pptx](#)
- [SupplementaryTableS1.xlsx](#)