NJK14013, a novel synthetic estrogen receptor-α agonist, exhibits estrogen receptor-independent, tumor cell-specific cytotoxicity

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Abstract. Estrogens act through interactions with estrogen receptors (ERs) to play diverse roles in various pathophysiological conditions. A number of synthetic selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, have been developed and used to treat ER-related diseases, including breast cancer and osteoporosis. Here, we identified a novel compound, bis(4-hydroxyphenyl) methanone-*O*-isopentyl oxime, designated NJK14013, as an ER agonist. NJK14013 activated ER-dependent transcription in a concentration-dependent manner, while suppressing androgen receptor-dependent transcriptional activity. It induced the activation-related phosphorylation of ER and enhanced the transcription of growth regulation by estrogen in breast cancer 1 (*GREB1*), further supporting its ER-stimulating activity. NJK14013 exerted anti-proliferative effects on various cancer cell lines, including an ER-negative breast cancer cell line, suggesting that it is capable of suppressing the growth of cancer cells independent of its ER-modulating activity. In addition, NJK14013 treatment resulted in significant apoptotic death of MCF7 and Ishikawa cancer cells, but did not induce apoptosis in non-cancer human umbilical vein endothelial cells. Collectively, our findings demonstrate that NJK14013 is a novel SERM that can activate ER-mediated transcription in MCF7 cells and suppress the proliferation of various cancer cells, including breast cancer cells and endometrial cancer cells. These results suggest that NJK14013 has potential as a novel SERM for anticancer or hormone-replacement therapy with reduced risk of carcinogenesis.

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Introduction

Estrogens serve various physiological functions and are critical for the development and maintenance of diverse tissues, including reproductive tissues, adipose tissues, and skeletal tissues though their interactions with estrogen receptors (ERs). In turn, estrogens and their receptors have been implicated in various pathophysiologic conditions, including cancers, cognitive diseases, and postmenopausal syndrome. Accordingly, there have been numerous efforts to develop compounds capable of modulating ER-mediated signaling pathways, and several selective estrogen receptor modulators (SERMs) and aromatase inhibitors have been approved. To date, the major clinical applications of SERMs are in the treatment of ER-positive breast cancer and the prevention and treatment of postmenopausal symptoms caused by the low levels of estrogens that accompany osteoporosis (1-3). In addition, recent studies suggest the possible use of SERMs as neuroprotective agents (4,5).

Anti-estrogenic ER-modulating agents such as tamoxifen and toremifene, which directly interact with ERs and suppress their function, have been commonly used to treat patients with ER-positive breast cancer (6-8). However, a majority of patients treated with tamoxifen eventually develop resistance and experience metastases (9,10). Moreover, tamoxifen is known to increase the risk of thromboembolism and endometrial changes, including endometrial cancer (11-13).

On the other hand, estrogen and estrogenic compounds have been used to treat estrogen deficiency-related postmenopausal symptoms. It has been reported that ~75% of perimenopausal and postmenopausal women experience various symptoms owing to estrogen withdrawal, including vasomotor menopausal syndrome (VMS) and hip, spine or wrist fracture due to osteoporosis (1,14,15). Hormone replacement therapy (HRP) was once widely used to ameliorate and prevent postmenopausal symptoms, including VMS and osteoporosis. However, its use has drastically declined since a report by the Women's Health Initiative (WHI) showed that HRP increases the risk of strokes, breast cancer, and pulmonary embolism (16-19). To

overcome these limitations, several next generation SERMs have been developed or are under development (3). For example, raloxifene, a SERM that exerts estrogenic effects on bones and anti-estrogenic effects on the uterus and breast, has been used clinically to prevent osteoporosis in postmenopausal women (2,20). Recently, another estrogenic SERM, ospemifene, was approved by the US Food and Drug Administration for dyspareunia associated with vulvar and vaginal atrophy and menopause (21). Considering the increased risks and adverse effects of using SERMs, there are still unmet needs for alternative, safer SERMs with an improved risk-benefit ratio. In an effort to identify new SERMs, we generated a chemical library composed of synthetic compounds structurally related to bisphenol A, which is well known to exert hormone-like actions. In the present study, we identified a synthetic compound that acts as an ER-signaling agonist and characterized its anticancer effects.

Materials and methods

Synthesis of NJK13054 and NJK14013

NJK13054 [(E)-1-(4-hydroxyphenyl)ethanone-O-butyl oxime]. To a solution of 4-hydroxyacetophenone (30 mg, 0.22 mmol) in ethanol (1 ml) was added *O*-butylhydroxylamine hydrochloride (33 mg, 0.26 mmol). The reaction mixture was stirred at ambient temperature for 4 h, quenched with H_2O, and diluted with ethyl acetate (EtOAc). The combined organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated *in vacuo*. Purification of the residue via flash column chromatography on silica (EtOAc:*n*-hexane = 1:6-1:5) yielded 36 mg of NJK13054 (79%); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.46 (2H, d, *J*=8.7 Hz), 6.71 (2H, d, *J*=8.7 Hz), 6.53 (1H, s), 4.17 (2H, t, *J*=6.6 Hz), 2.21 (3H, s), 1.71-1.64 (2H, m), 1.44-1.38 (2H, m), 0.94 (3H, t, *J*=7.4 Hz).

NJK14013 [bis(4-hydroxyphenyl)methanone-O-isopentyl oxime]. To a solution of 4,4'-dihydroxybenzophenone (50 mg, 0.23 mmol) in ethanol (2 ml) was added *O*-isopentylhydroxylamine hydrochloride (33 mg, 0.32 mmol). The reaction mixture was stirred at 70˚C for 6 h, quenched with H_2O, and diluted with EtOAc. The combined organic layer was washed with H_2O, dried over $MgSO_4$, and concentrated *in vacuo*. Purification of the residue via flash column chromatography on silica (EtOAc:*n*-hexane = 1:6-1:5) yielded 36 mg of NJK14013 (53%); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.31 (2H, d, *J*=8.6 Hz), 7.25 (2H, d, *J*=8.6 Hz), 6.80 (2H, d, *J*=8.6 Hz), 6.70 (2H, d, *J*=8.6 Hz), 6.00 (1H, s), 4.19 (2H, t, *J*=6.9 Hz), 1.65 (1H, m, *J*=6.8 Hz), 1.60-1.55 (2H, m), 1.26 (1H, m), 0.89 (6H, d, *J*=6.5 Hz).

Cells. MCF7 and MDA-MB-231 human breast cancer cell lines and the LNCaP (CRL-1740) human prostate cancer cell line were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml). The Ishikawa human endometrial adenocarcinoma cell line and A549 human lung adenocarcinoma epithelial cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and penicillin/streptomycin (100 U/ml). Human umbilical vein endothelial cells (HUVECs; ATCC CRL-1730) were propagated in endothelial cell growth media (Lonza). For luciferase assays and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) assays using 17β-estradiol (E2) or test compounds, MCF7 cells were maintained in phenol red-free RPMI containing 10% charcoal-stripped FBS for 1 day before treatment.

Luciferase assays. Stimulation of ER transcriptional activity by test compounds was assessed by dual-luciferase assays as previously described, with slight modifications (22). Briefly, MCF7 cells were co-transfected with estrogen response element (ERE)-Luc and thymidine kinase-Renilla (pRL-TK) expression plasmids and maintained in phenol-red-free RPMI containing 10% charcoal-stripped (CS) FBS for 1 day. Cells were treated with the indicated compounds for 24 h and analyzed using dual-luciferase assays (Promega, Madison, WI, USA) according to the manufacturer's instructions. Activation of the androgen receptor (AR) was assessed in a similar manner by transfecting LNCaP cells with a prostate-specific antigen (PSA)-enhancer/promoter-luciferase reporter construct or mouse mammary tumor virus (MMTV) enhancer/promoter-luciferase reporter plasmid together with a pRL-TK expression plasmid.

Immunoblotting. Effects of NJK14013 on the phosphorylation status of ERα were analyzed by immunoblotting. MCF7 cells were treated with different concentrations of NJK14013 for 24 h. After lysing cells with RIPA buffer containing phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA), proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted using anti-phospho-ER (Cell Signaling Technology, #8644, Beverly, MA, USA), anti-ERα (Cell Signaling Technology, #8644), and anti-β-actin (Santa Cruz, sc47778, Dallas, TX, USA) antibodies. Cell proliferation was assessed by treating cells with E2 or NJK14013 for 24 h, then lysing cells and analyzing cell lysates by immunoblotting using an anti-PCNA antibody (Santa Cruz, sc7907).

Molecular modeling of interactions between ER and NJK14013. Molecular docking studies were performed based on the human-ERα crystal structure (PDB code: 2QXS) using Autodock 4.2 (Molecular Graphic Laboratory) (23). Results of docking studies were visualized using Chimera 1.10 software (24).

RNA preparation and RT-qPCR. The transcriptional activity of ER was analyzed by determining transcript levels of the ER target, growth regulation by estrogen in breast cancer 1 (*GREB1*), by RT-qPCR, as described previously (22). MCF7 cells were seeded and maintained in phenol-red-free RPMI containing 10% CS FBS for 1 day prior to treatment with E2 or NJK14013. After incubating cells for an additional 24 h, RNA was extracted and cDNA, used as a template for quantitative PCR, was synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo$_{20}$(dT) primers. β-actin was used as a reference gene for normalization. The following primer pairs were used for qPCR: GREB1, 5'-gtggtagccgagtggacaat-3' (sense) and 5'-aaac ccgtctgtggtacagc-3' (antisense); and β-actin, 5'-gggaaatcgt gcgtgacatt-3' (sense) and 5'-ggagttgaaggtagttt cgt-3' (antisense).

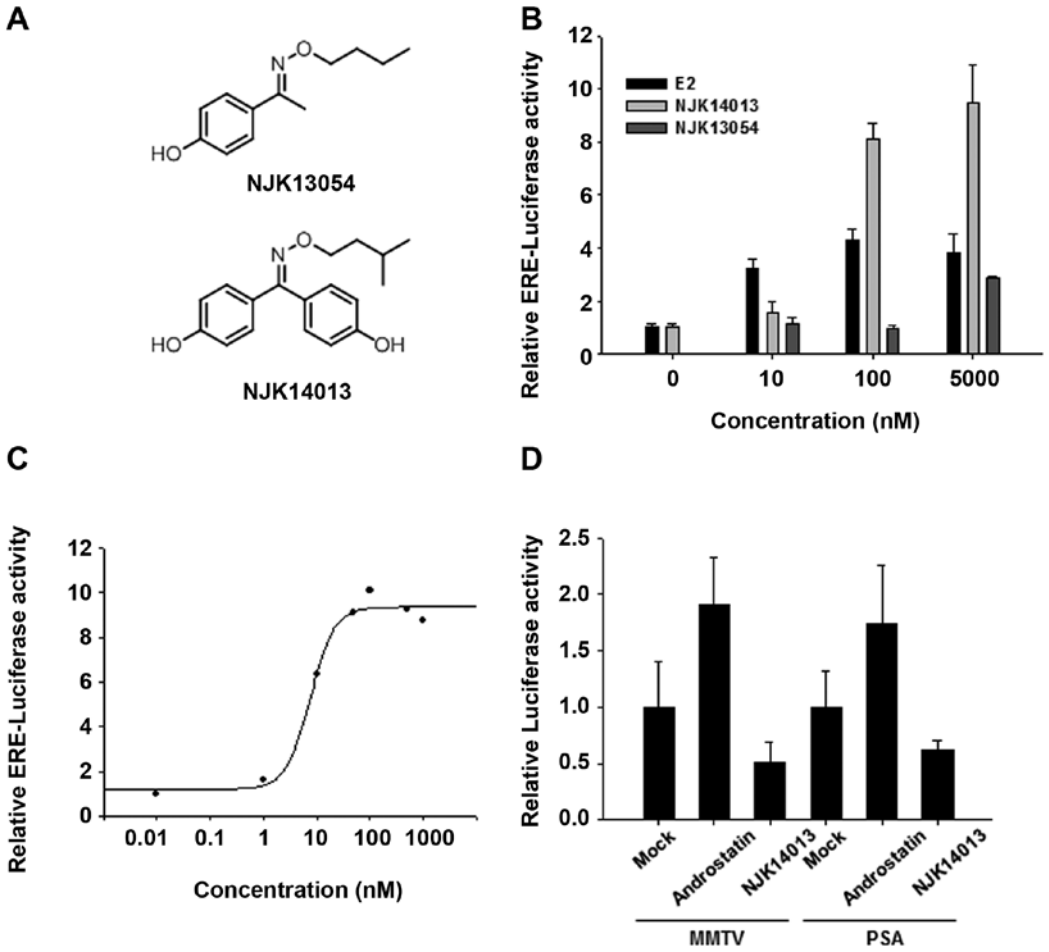

Figure 1. Identification of NJK14013 as a novel agonist of estrogen signaling. (A) Chemical structures of NJK13054 and NJK14013. (B) Agonistic activity of NJK14013 and NJK13054 on ERE-luciferase promoter activity. MCF7 cells transfected with an ERE-luciferase reporter and TK-Renilla luciferase reporter plasmids were treated with the indicated concentrations of NJK13054, NJK14013 or E2, and then subjected to dual-luciferase assays. (C) Concentration-dependent stimulation of ERE-promoter activity by NJK14013. MCF7 cells were treated with increasing concentrations of NJK14013 as in (B). EC_{50} value was determined from triplicate samples. (D) Effects of NJK14013 on MMTV and PSA promoter activity. LNCaP cells were transfected with a PSA-enhancer/promoter- or an MMTV enhancer/promoter-luciferase construct together with a TK-Renilla reporter plasmid. Cells were treated with androstain (1 μM) or NJK14013 (10 μM) for 24 h and assayed for luciferase activity. Data are presented as means ± SD.

Cell proliferation and cell cytotoxicity assay. The effects of compounds on tumor cell growth were determined by seeding MCF7, Ishikawa, MDA-MB-232, and A549 cells ($1x10^4$ cells/well) onto 96-well tissue culture plates and treating with different concentrations of E2 or NJK14013 for 24, 48 or 72 h. Cell proliferation was determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. After incubation with the compound, the culture medium was replaced with fresh medium containing 20 μl of MTT (5 mg/ml), and cells were incubated for an additional 4 h. Thereafter, the medium was removed, and cells were lysed with dimethyl sulfoxide. The absorbance of lysates was determined at 570 nm using a microplate reader. The cytotoxicity of compounds toward tumor cells was analyzed by seeding Ishikawa cells and HUVEC cells ($4x10^4$ cells/well) onto 96-well plates and treating as indicated in the text. Cell viability was determined by MTT assays in a similar manner.

Apoptosis assay. Apoptotic cell death was assessed by Annexin V/7-AAD staining. For Annexin V assays, cells were treated with vehicle or NJK14013 (10 μM) as indicated. Cells were stained with Annexin V-PE and 7-AAD using a Guava Nexin kit (Millipore, Billerica, MA, USA) and analyzed with a Guava easyCyte flow cytometer according to the manufacturer's instructions.

Results

NJK14013 is a novel synthetic ER agonist that stimulates ER transcriptional activity. With the aim of developing new ER-modulating agents, we screened an in-house synthetic library containing bisphenol-A-inspired compounds. These screens identified two compounds, NJK14013 and NJK13054, that upregulated the activity of an estrogen response element (ERE)-containing luciferase reporter construct (ERE-luciferase) in MCF7 cells (Fig. 1A and B). Notably, NJK14013 showed higher activity than E2, the endogenous ligand, at concentrations of 100 nM and 1 μM, but displayed weaker activity at 10 nM (Fig. 1B). Since the agonistic effect of NJK14013 was greater than that of NJK13054, NJK14013 was used for subsequent studies. NJK14013 induced concentration-dependent stimulation of ERE-mediated transcriptional

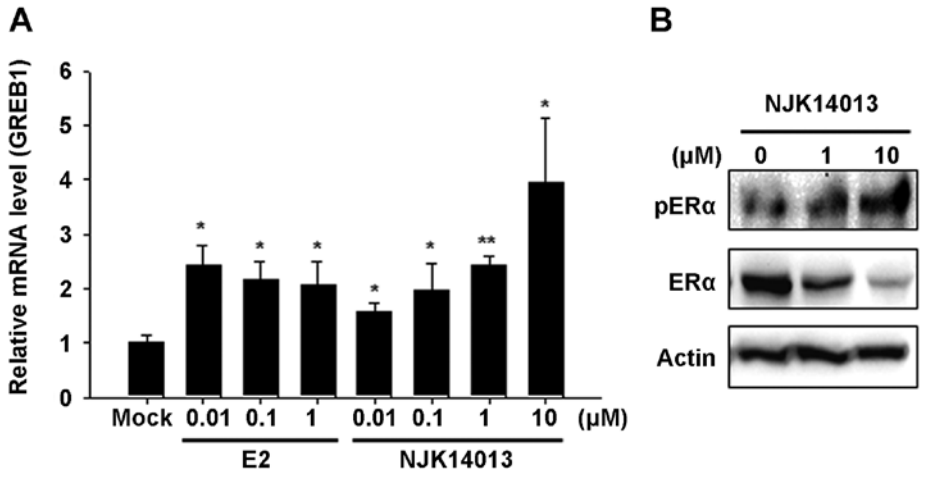

Figure 2. Stimulation of ER transcriptional activity by NJK14013. (A) Assessment of GREB1 mRNA levels by RT-qPCR. Total RNA was prepared from MCF7 cells treated with the indicated concentrations of E2 or NJK14013, and GREB1 mRNA levels were determined by RT-qPCR as described in Materials and methods. Data are presented as means ± SD ($^{*}p<0.05$ and $^{**}p<0.01$ versus mock-treated). (B) Induction of ER phosphorylation by NJK14013. MCF7 cells were treated with the indicated concentrations of NJK14013 for 24 h. After lysis, samples were analyzed by immunoblotting using anti-phospho-ERα (Ser104/106), anti-ERα and anti-β-actin antibodies.

activity with a median effective concentration (EC_{50}) value of 46.3±12 nM (Fig. 1C). To determine whether this activity was specific for ER-mediated transcription, we analyzed the effect of NJK14013 on AR-mediated transcription using MMTV promoter- and PSA promoter-driven luciferase reporters. Unlike ER-driven transcription, AR-driven PSA promoter- and MMTV enhancer/promoter-reporter activity in LNCaP cells were not activated by NJK14013 (Fig. 1D). These results suggest that NJK14013 differentially interacts with and regulates the ER and AR, although the molecular basis for these differential effects is not clear.

To further confirm the agonistic activity of NJK14013 on ER transcriptional activity, we analyzed the effects of NJK14013 on the transcription of *GREB1,* an ER-responsive gene. As depicted in Fig. 2A, treatment with E2 or NJK14013 induced a significant increase in GREB1 mRNA. Since interaction with estrogen causes the ER to undergo phosphorylation, which results in ER dimerization and nuclear translocation, we examined whether NJK14013 affected the phosphorylation status of the ER. As shown in Fig. 2B, cells treated with NJK14013 showed increased levels of phosphorylated ER (Ser104/106) and decreased levels of total ER protein.

These results suggest that NJK14013 may interact with ER and modulate its activity. To investigate the binding modes of NJK14013 in the active site of the ER ligand-binding domain(LBD) active site, we performed molecular docking studies based on the human ERα crystal structure (PDB code: 2QXS). As expected, the compound fit well into the active site, with an estimated free energy of binding of -8.08 kcal/mol. One of the phenols in NJK14013 interacted with Glu353 of the protein, whereas the other phenol formed a hydrogen bond with His524. In addition, an isopentyl tether occupied the hydrophobic region of the pocket. The key hydrogen bonding interaction and overall binding mode with ERα were similar to those of raloxifene (Fig. 3).

NJK14013 suppresses proliferation of tumor cells in an ER-independent manner. Next, we tested the effect of

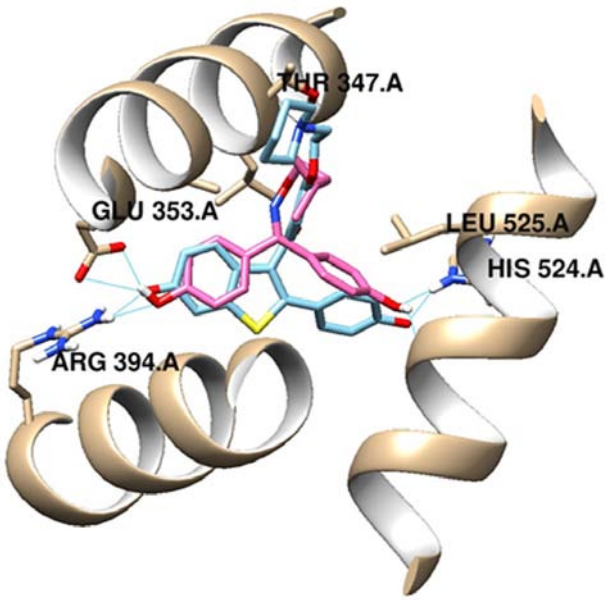

Figure 3. Molecular modeling of NJK14013 interaction with ERα. Docking of NJK14013 (pink) and raloxifene (blue) into ERα (PDB code: 2QXS) was performed using Autodock 4.2 and the results were visualized using Chimera 1.10.

NJK14013 on the proliferation of various cancer cell lines. To our surprise, treatment with 10 μM NJK14013 clearly suppressed the proliferation of MCF7 cells; in contrast, the same concentration of E2 did not significantly affect MCF7 cell proliferation (Fig. 4A). NJK14013 also suppressed the proliferation of Ishikawa endometrial cancer cells, MDA-MB-231 breast cancer cells, and A549 lung adenocarcinoma cells, indicating that it is capable of suppressing diverse types of tumor cells (Fig. 4B-D). Consistent with MTT assay results, the protein level of proliferating cell nuclear antigen (PCNA), a well-known proliferation marker, was significantly decreased by NJK14013 treatment in MCF7 cells (Fig. 4E).

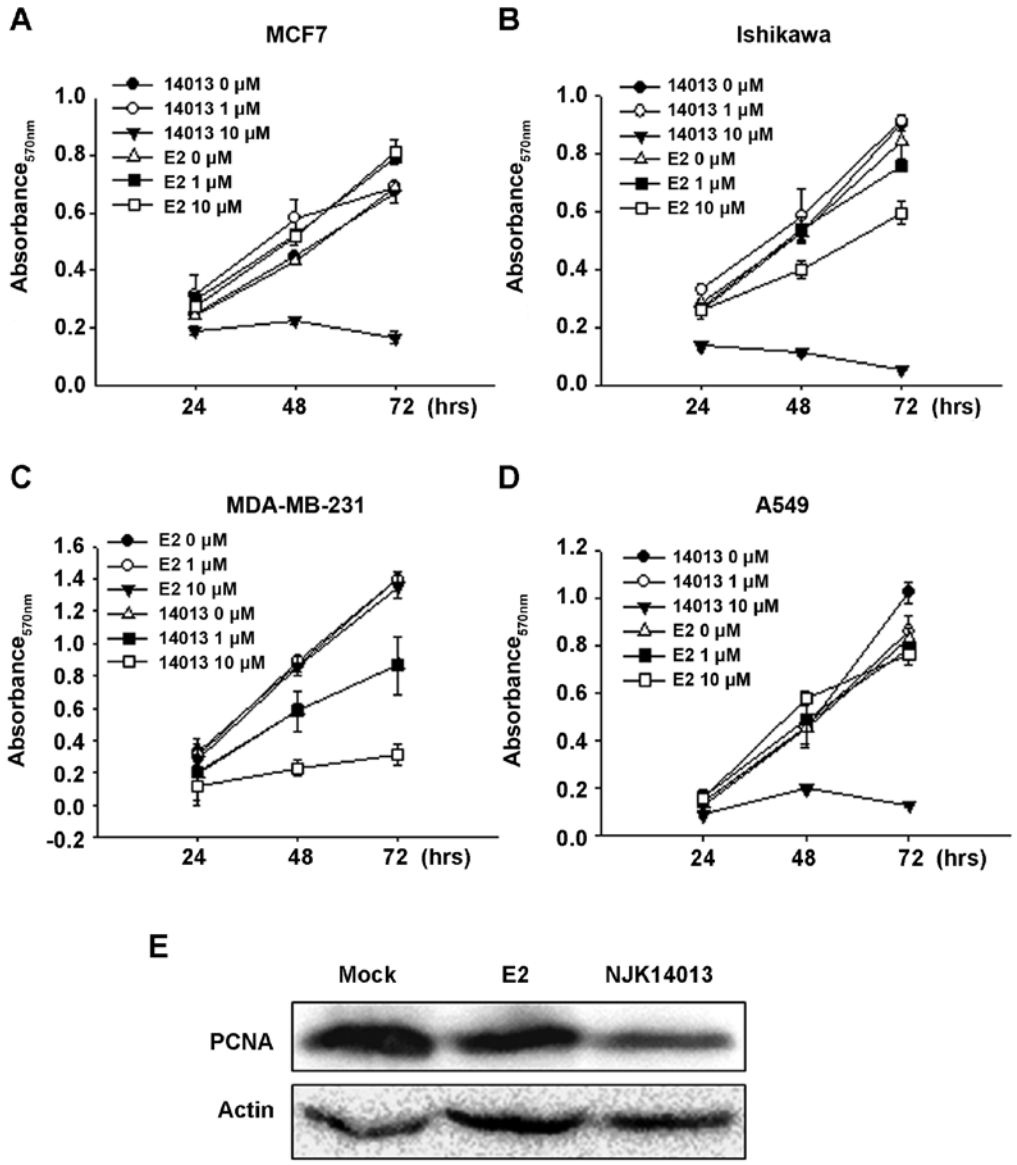

Figure 4. ER-independent suppression of tumor-cell proliferation by NJK14013. (A-D) MCF7 (A), Ishikawa (B), MDA-MB-231 (C) and A549 (D) cells were seeded onto 96-well tissue culture plates (1x10^4 cells/well). After incubating for 18 h, cells were treated with increasing concentrations of E2 or NJK14013 (14013) for 24, 48 or 72 h. Proliferation of cells was determined by MTT assays. Data are presented as means ± SD. (E) MCF7 cells were treated with 10 μM E2 or NJK14013 for 24 h and then analyzed by immunoblotting using anti-PCNA and anti-β-actin antibodies.

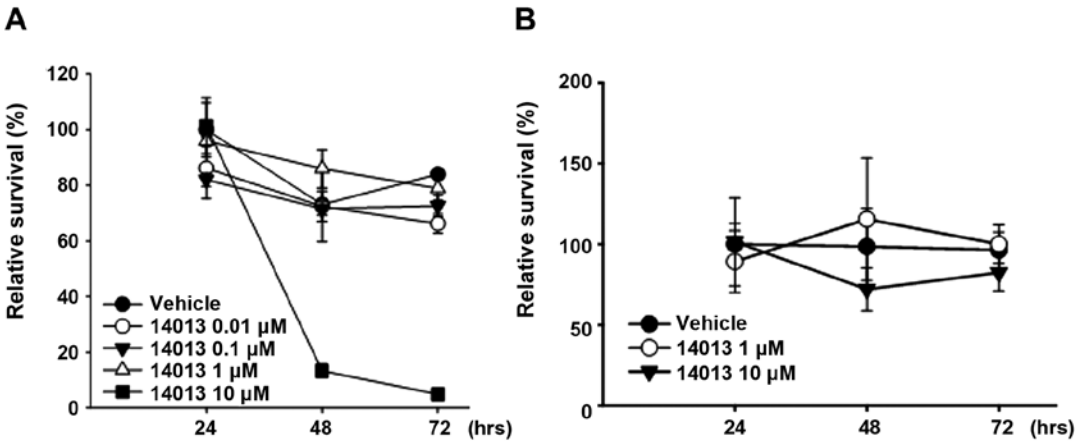

Figure 5. Induction of apoptotic death of tumor cells by NJK14013. (A and B) Induction of cell death by NJK14013 (14013) in Ishikawa cells. Ishikawa (A) and HUVEC (B) cells were seeded onto 96-well plates (4x10^4 cells/well). After incubation for 24 h, cells were treated with vehicle or the indicated concentrations of NJK14013 for 24, 48 or 72 h. Cell viability was determined by MTT assay as described in Materials and methods.

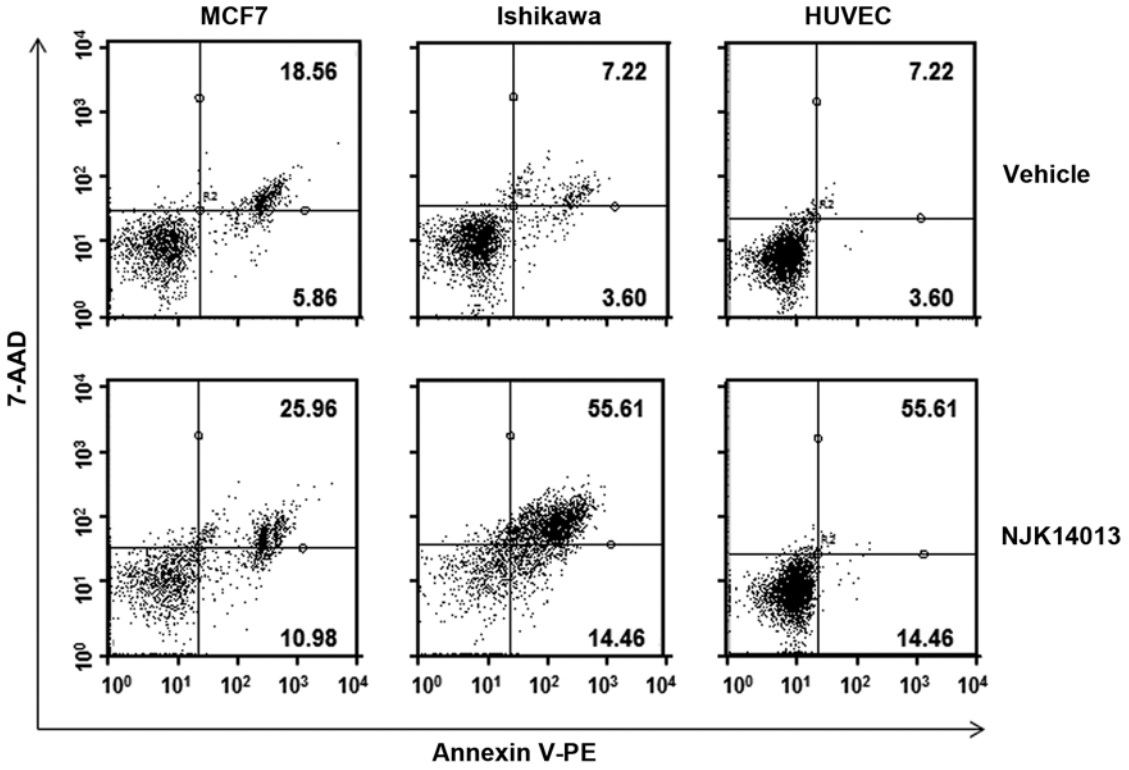

Figure 6. Induction of apoptotic death in cancer cells by NJK14013. MCF7, Ishikawa and HUVEC cells were treated with vehicle or NJK14013 (10 μM) for 48 h. Apoptotic cell death was assessed by Annexin V/7-AAD staining and analyzed by flow cytometry.

Considering that MDA-MB-231 is an ER-negative breast cancer cell line, these results imply that the tumor-suppressive effect of NJK14013 is not ER-dependent.

NJK14013 induces apoptotic death of tumor cells. Finally, we tested whether NJK14013 induced tumor cell death in addition to suppressing tumor cell proliferation using Ishikawa cells. Unlike E2, treatment with NJK14013 (10 μM) resulted in 87 and 95% cell death after 48 and 73 h, respectively (Fig. 5A). In stark contrast, the same concentrations caused no significant change in the viability of HUVECs within 72 h after treatment (Fig. 5B).

To examine whether NJK14013 is able to cause apoptotic death of cancer cells, we analyzed MCF7, Ishikawa, and HUVEC cells by Annexin V/7-AAD assays after treatment with NJK14013 for 48 h. As shown in Fig. 6, treatment with NJK14013 caused apoptotic cell death in 25.96 and 55.61% of MCF7 and Ishikawa cells, respectively. In contrast, NJK14013 treatment did not significantly increase apoptosis of HUVECs, further supporting cell viability data (Fig. 6).

Discussion

Estrogens and SERMs exert various effects on different types of tumor cells. For instance, tamoxifen displays an ER antagonistic effect in MCF7 cells and suppresses breast cancer cell proliferation, whereas it increases the risk of endometrial cancer. Hormone replacement therapy using estrogen to treat postmenopausal syndrome and osteoporosis has been associated with an increased risk of breast cancer. In this study, we sought to identify biologically active compounds that are capable of stimulating ER activity while suppressing proliferation of various tumor cells. We identified NJK14013 as a novel compound which enhances the transcriptional activity of ERα. The agonistic effect of NJK14013 on the ER suggests its possible use for estrogen-deficiency-related conditions, such as osteoporosis. However, the ER agonistic effect in MCF7 also raises concerns that NJK14013 might increase the risk of breast cancer or facilitate the proliferation of cancer cells.

Our results demonstrate that NJK14013 is a novel SERM capable of inducing apoptotic death in various cancer cell types. An ER-agonistic role of NJK14013 in MCF7 cells indicates that NJK14013 may act through a different mechanism than tamoxifen and raloxifene, which exert ER-antagonistic activity in MCF7 cells. The SERM actions of NJK14013 in various other tissues, including bone and endometrium, remain to be determined. Although the mechanism underlying the anticancer effect of NJK14013 is not clear, it does not appear to be dependent on its ER-modulating activity since NJK14013 showed similar effects on ER-negative cancer cells. Interestingly, a recent study identified ospemifene derivatives that were cytotoxic to both MCF7 and MDA-MB-231, despite the fact that ospemifene itself is selective for ER-positive MCF7; moreover, these compounds were not toxic to normal mouse embryonic fibroblasts (25). Thus, it is conceivable that some SERMs may be able to acquire ER-independent, cancer cell-specific cytotoxicity while retaining ER-modulating activity. Elucidating the molecular target and underlying cytotoxicity mechanism of NJK14013 will require further studies.

Collectively, our results suggest the potential use of NJK14013 as a novel SERM for hormone-replacement therapy with reduced risk of carcinogenesis or tumor progression.

In addition, its tumor-suppressive effects on various cancer cell types at concentrations that do not affect the viability of non-cancer cells (HUVECs) indicate its potential use as an anticancer therapeutic.

Acknowledgements

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