Beta-naphthoflavone (DB06732) mediates estrogen receptor-positive breast cancer cell cycle arrest through AhR-dependent regulation of PI3K/AKT and MAPK/ERK signaling

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Beta-naphthoflavone (BNF, DB06732) is an agonist of aryl hydrocarbon receptor (AhR) and a putative chemotherapeutic agent that has antitumor activity against mammary carcinomas in vivo. However, the mechanism by which BNF exerts this antitumor effect remains unclear. Thus, we explored mechanisms of BNF’s antitumor effects in human breast cancer cells. This study showed that BNF suppressed cell proliferation and induced cell cycle arrest in the G_{2}/M phase with downregulation of cyclin D1/D3 and CDK4 and upregulation of p21^{Cip1/Waf1}, ultimately leading to a senescence-like phenotype in estrogen receptor (ER)-positive MCF-7 cells, but not in ER-negative MDA-MB-231 cells. In addition, BNF inhibited PI3K/AKT signaling, and the PI3K inhibitor, LY294,002, exhibited the same inhibitory effects on cyclinD1/D3, CDK4 and the cell cycle as BNF. Interestingly, BNF activated mitogen-activated protein kinase-extraacellular signal-regulated kinase (MAPK-ERK) signaling, and notably, MEK inhibitor PD98059 significantly blocked the BNF-induced cell cycle arrest and upregulation of p21^{Cip1/Waf1}. Furthermore, specific ER{α} and AhR siRNA studies indicate that ER{α} is required in BNF-induced p21^{Cip1/Waf1} expression, and BNF-mediated cell cycle arrest and modulation of AKT and ERK signaling is AhR-dependent. Taken together, AhR-dependent inhibition of the PI3K/AKT pathway, activation of MAPK/ERK and modulation of ER{α} is a novel mechanism underlying BNF-mediated anti-tumor effects in breast cancer, which may represent a promising strategy to be exploited in future clinical trials.

Introduction

Beta-naphthoflavone (BNF, DB06732), also called 5,6-benzoflavone, is a synthetically derived flavonoid and a known agonist of aryl hydrocarbon receptor (AhR), through which it induces cytochrome P450 IA (CYP1A) expression (1). The therapeutic potential of flavonoids has been touted for treatment of medical problems ranging from cardiovascular disease (2) to cancer (3). BNF also exhibits potent antitumor activity against mammary carcinomas in vivo (4–6); however, its mechanism of action remains unclear.

AhR is a ligand-activated transcription factor that mediates the effects of many environmental contaminants through inducing CYP1A (7). Ligand binding to AhR prompts nuclear translocation and subsequent heterodimerization with aryl hydrocarbon nuclear translocator (ARNT), as well as with transcriptional co-activators or co-repressors (7). The activated AhR complex binds to specific DNA sequences, termed dioxin-response elements and regulates transcription of genes. AhR has long been investigated for its role in mediating dioxin toxicity (8). However, recent studies have also explored other intriguing biological roles of AhR, including regulation of development, immunity, circadian rhythm and cancer biology (7,9,10).

The role of AhR in breast cancer biology has been extensively investigated, and notably increasing evidence indicates that the ultimate response of breast cancer to AhR is dependent upon estrogen receptor (ER) status, ligand presence and cell type (11–13). Deregulation of ER expression is crucial in the development of breast cancer, and estrogen-mediated ER alpha (ER{α}) activation promotes breast cancer growth (14). Under certain circumstances, agonist-activated AhR prompts ER{α} protein ubiquitination and degradation, and upregulation of enzymes that metabolize estrogen, which synergistically inhibits estrogen-induced ER-positive breast cancer cell proliferation (7,13). In contrast, other agonists activate AhR and subsequently inhibit ER-negative breast cancer cell proliferation or cell cycle progression independent of ER{α} (11,15). Clearly, AhR has effects on breast cancer cell proliferation that are both dependent and independent of its crosstalk with ER{α}. In addition, some AhR agonists can also directly bind to ER and regulate breast cancer cell proliferation independent of AhR (16). Taken together, these data suggest that mechanisms underlying the effects of AhR agonists on breast cancer are very complex.

In the present study, we explored the effects and molecular mechanisms of an AhR agonist, BNF, on ER-positive MCC-7 and ER-negative MDA-MB-231 breast cancer cells. Our results show that BNF inhibits the proliferation of MCC-7 cells, but not MDA-MB-231 cells through a novel mechanism in which BNF induces G_{2}/M phase arrest and senescence through AhR-mediated inhibition of PI3K/AKT signaling, consequently downregulation of cyclin D1/D3 and CDK4, as well as activation of the mitogen-activated protein kinase-extraacellular signal-regulated kinase (MAPK/ERK) causing ER{α}-dependent upregulation of p21^{Cip1/Waf1}.

Materials and methods

Reagents

BNF (DB06732), LY294,002, PD98059 and MG132 from Sigma–Aldrich (St Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) as stock solutions, diluted in culture medium and added to cells at a final DMSO concentration of 0.1%. The following primary antibodies were used for immunoblot: ER{α}, p53 and CYP1A1 from Santa Cruz Biotechnology (Santa Cruz, CA); AhR from Abcam; β-Actin from Sigma–Aldrich; poly(ADP-ribose) polymerase (PARP), p-ERK1/2, ERK1/2, p-AKT pathway antibody and cell cycle regulation sampler kit were purchased from Cell Signaling Technology (Beverly, MA). Other chemicals and biochemistry reagents were obtained from Sigma–Aldrich unless otherwise mentioned.

Cell culture and siRNA transfection

Two human breast cancer cell lines MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) (ATCC) were cultured and treated in DMEM Reduced Serum medium (HyClone) with 7.5% bovine serum growth (HyClone) and penicillin/streptomycin/amphotericin (PM Biobodmedical) at 37°C in a humidified, 5% CO_{2} atmosphere. Cultures were treated with BNF or an equal volume of the DMSO vehicle (0.1% of the total volume). Negative, AhR and ER{α} siRNAs (40nM, Life Technologies) were delivered into cells (2.5 x 10^{5}) in 6-well plates using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s recommendations. The sequences used were AhR siRNA sense 5′-GGC UGA UAG UUU UCC GGC UdTdT and AhR siRNA antisense 5′-AGG CCG AAA ACU AUC AUG C dTdT. ERs siRNA sense 5′-GUA GAA AGG UGG GUA GACG A dTdT and ER{α} siRNA antisense 5′-UGC UAU CCC ACC UUU CAU C dTdT′ (17).

Abbreviations: AhR, aryl hydrocarbon receptor; BNF, beta-naphthoflavone; CDK, cyclin-dependent kinases; DMSO, dimethyl sulfoxide; ER, estrogen receptor; MAPK-ERK, mitogen-activated protein kinase-extra cellular signal-regulated kinase; PARP, poly(ADP-ribose) polymerase; TBST, Tris-buffered saline containing 0.05% Tween 20.

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Immunoblot analysis

Cells were washed with cold phosphate-buffered saline and homogenized in lysis buffer (Roche); total protein (80–100 μg) was separated using 10 or 14% sodium dodecyl sulfate–polyacrylamide gel electrophoresis depending on protein size, followed by electrophoretic transfer to nitrocellulose membranes (Bio-Rad). The transblotted membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBST) containing 5% non-fat milk for 60 min at room temperature, and then washed three times with TBST. The membrane was incubated with a primary antibody at an appropriate dilution according to the manufacturer’s recommendations in TBST at 4°C overnight, and then washed three times with TBST. The membrane was probed with anti-mouse, anti-rabbit or anti-goat secondary antibody (LI-COR Bioscience) for 60 min at room temperature and washed three times with TBST. Images were taken by LI-COR imaging system (LI-COR Bioscience).

MITT colorimetric assay

Cells were seeded onto 96-well tissue culture plates at a density of 5 × 10^4 per well and incubated overnight. After 24, 48 and 72 h exposures with 10 or 100 μM BNF, MITT (Research Products International) was added to each well (final concentration, 2.5 mg/ml) and incubated for 3 h to allow metabolism of MITT by mitochondrial dehydrogenase to an insoluble formazan product. The medium was aspirated and formazan was solubilized by the addition of 100 μl of DMSO. Cell viability was determined by absorbance at 570 nm on a universal microplate reader.

Cell cycle analysis

The MCF-7 and MDA-MB-231 cell lines were plated in 60 mm dishes at a density of 5 × 10^4 cells. After treatment with 10 μM BNF for 48 h, the cells were trypsinized and washed once with cold phosphate-buffered saline. Cells were then fixed in 70% ethanol overnight at 4°C for cell cycle analysis. Before the assay, RNase A (final concentration, 0.2–0.5 mg/ml) digestion was performed at 37°C for 1 h. The cells were stained with propidium iodide (final concentration, 100 μg/ml) and kept in the dark at 4°C until analysis. Flow cytometry was performed in the Core Lab at South Illinois University School of Medicine.

RNA isolation, reverse transcription and real-time quantitative PCR

MCF-7 and MDA-MB-231 cells were plated in a 6-well plate at a density of 2.5 × 10^5 cells, grown overnight and transfected with siRNA and/or treated with 10 μM BNF or DMSO for 36–48 h. Total RNA was extracted using TRIzol (Life Technologies) according to the manufacturer’s recommendations. Following cDNA synthesis (Promega protocol), 5 μl cDNA (1:5 dilution) was used for quantitative polymerase chain reaction (qPCR) using SYBR green (Quanta Biosciences) in a Smart Cycler rapid thermal cycler (Cepheid). Each assay included a no reverse transcriptase negative control. β-Actin was used for normalization. The primers used were 5′ CGT GAG ACT CTC AGG GTG AAA TGT 3′ for P21 mRNA (18), 5′ CCG ACA TCT CTT CGT 3′ and 5′ GGT TGA GCC ACT GGT TT3 for CYP1A1, and 5′ ATG TCG CAC ACC TTC TAC 3′ and 5′ GTA CAT GOG TGG GTG GAT GGA G3′ for β-Actin (19). Relative standard curve was created to evaluate primer’s amplification efficiency. Relative expression was assessed by the comparative CT method and normalized by β-Actin. ‘PCR product specificity from each primer pair was confirmed using melting curve analysis and subsequent agarose gel electrophoresis.’

Senescence-associated β-galactosidase activity

Cells growing on 6-well tissue culture plates were fixed with 2% formaldehyde and 0.2% glutaraldehyde. Senescence-associated β-galactosidase (SAβ-gal) activity was detected following the Senescence β-galactosidase Staining Kit (Cell Signaling Technology). Cells were photographed and scored under an inverted microscope. SAβ-gal-positive rate (SAβ-gal+/total cells) (%) was calculated as follows: number of SAβ-gal-positive cells/number of total cells) × 100.

Apoptosis detection

Apoptosis was evaluated by PARP cleavage and DNA fragmentation because both of them are key features of apoptosis. PARP cleavage was detected by western blot as described above; DNA fragmentation was examined as addressed previously (20). In brief, cells growing on 60 mm cell culture dishes were harvested and lysed in a lysis buffer containing 0.5% Triton X-100, 5 mM Tris-HCl (pH 7.4) and 5 mM EDTA for 20 min on ice. Supernatants were collected by centrifugation at 10 000 g for 15 min, and incubated with 100 μg/ml of proteinase K (Applied Biosystems) and 0.5% sodium dodecyl sulfate (final concentration) for 2 h at 56°C. DNA was extracted with phenol-chloroform and precipitated with ethanol. DNA pellets were dissolved in 20 mM Tris-HCl (pH 7.4) with 50 μg/ml of RNase A and incubated at 37°C for 1 h. DNA products were then separated on 2% agarose gel.

Statistics

Statistical analysis was performed using Student’s t-test or one-way ANOVA with Tukey’s post hoc comparison. The P values were considered statistically significant at P < 0.05. All data are means ± SEM for at least three separate experiments.

Results

BNF induces CYP1A1 differently in MCF-7 versus MDA-MB-231 cells

To study the effect of BNF on breast cancer cells, we used two typical human breast cancer cell lines, MCF-7 and MDA-MB-231 cells. Both cell lines express AhR protein (Supplementary Figure S1A, available at Carcinogenesis Online), and the AhR level is higher in MDA-MB-231 cells than in MCF-7 cells. We evaluated the AhR target gene CYP1A1 after BNF treatment in these cell lines; in MCF-7 cells both CYP1A1 protein and mRNA were increased after 10 μM BNF treatment (Supplementary Figure S1B and C, available at Carcinogenesis Online). In MDA-MD-231 cells, however, CYP1A1 mRNA was only weakly upregulated and CYP1A1 protein was almost unchanged (Supplementary Figure S1B and C, available at Carcinogenesis Online), consistent with a previous report (21).

BNF inhibits cell proliferation, induces cell cycle arrest and senescence and regulates cell cycle proteins in MCF-7 cells

We examined the effects of BNF on cell proliferation in both MCF-7 and MDA-MB-231 cell lines. BNF (10 μM) inhibited MCF-7 proliferation at 72 h, but had no effect on MDA-MB-231 cells (Figure 1). Increasing the BNF dose to 100 μM nearly completely abolished MCF-7 cell proliferation, but still had no effect on MDA-MB-231 cells (Supplementary Figure S2, available at Carcinogenesis Online).

To assess the mechanism responsible for the growth-inhibitory activity of BNF, cell apoptosis detection and flow cytometric analysis of the cell cycle were performed in both MCF-7 and MDA-MB-231 cells. BNF (10 μM for 48 h) did not induce cell apoptosis by DNA ladder analysis and cleaved PARP detection (data not shown). However, BNF treatment (10 μM for 48 h) inhibited a cell cycle progression in MCF-7 cells, but not in MDA-MB-231 cells, which was reflected by a distinct increase of the percentage of cells in the G1/S phase and a significant reduction in the S and Go/M phase (Figure 1B).

Cell cycle progression through G1/S phase is tightly regulated by the interplay between Cyclin D, CDK4/6 and their inhibitors, p21Cip/Waf1, p16INK4A and p15INK4B. Thus, we examined the effects of BNF on expression of these cell cycle proteins. Consistent with its effects on cell cycle arrest, BNF (10 μM) reduced cyclin D1 and CDK4, slightly reduced cyclin D3 and distinctly increased p21Cip/Waf1 protein and mRNA levels in MCF-7 cells (Figure 1C and D) in a time- and dose-dependent manner (Figure 1E and F). BNF induced more p21Cip/Waf1 mRNA than protein in 48 h of treatment (Figure 1C and D) possibly because mRNA was not fully translated into protein in short time, and more p21Cip/Waf1 mRNA was translated into protein in 72 h (Figure 1F). The upstream regulator of p21Cip/Waf1, the p53 tumor suppressor (22), however, was not changed in BNF-treated MCF-7 cells with functional p53 or MDA-MB-231 cells with p53 mutation (Figure 1C), suggesting that BNF-induced p21Cip/Waf1 increase may be p53-independent.

p21Cip/Waf1-mediated cell cycle arrest is accompanied by phenotypic features of senescence (23,24). Cell senescence assay detected a large flat morphology and SAβ-gal activity (Figure 1G), an indicator of cell senescence (25), in MCF-7 cells exposed to BNF for 3 days.

BNF regulates the PI3K/AKT pathway and MAPK signaling

The phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) is critical in breast cancer development and therapy due to a frequent mutation in this pathway that causes hyperactivation and promotes a cell cycle progression (26–28). Although the MAPK-ERK pathway is generally associated with cell proliferation (29), increasing evidence indicates that prolonged hyperactivation of ERK leads to p21Cip/Waf1 upregulation, cell cycle arrest and cell senescence (23,30).
Fig. 1. BNF inhibits cell proliferation, induces cell cycle arrest and cell senescence, and regulates the expression of cell cycle proteins MCF-7 cells. (A) $5 \times 10^5$ MCF-7 and MDA-MB-231 cells were seeded in 96-well plates. After an overnight culture, cells were treated with 10 $\mu$M BNF for 0, 1, 2 or 3 days. Cell proliferation was assessed by MTT. (B) Distribution of cell cycle in MCF-7 and MDA-MB-231 cells following treatment with 10 $\mu$M BNF for 48h. Upper panel, representative cell cycle distribution; Lower panel, quantitation of cell cycle distribution. (C) Expression levels of cell cycle proteins were detected by western blot in 10 $\mu$M BNF-treated MCF-7 and MDA-MB-231 cells for 48h. (D) The transcription level of p21$^{Cip1/Waf1}$ was tested by qPCR in 10 $\mu$M BNF-treated MCF-7 and MDA-MB-231 cells. (E and F) BNF regulates the expression of cell cycle proteins by dose dependence (E) and time dependence (F) in MCF-7 cells. (G) Cell senescence was examined by SA$\beta$-gal activity analysis. Right panel, representative senescent morphology; Left panel, quantitation of SA$\beta$-gal activity, and SA$\beta$-gal-positive rate (SA$\beta$-gal+) (%) is calculated as follows: (number of SA$\beta$-gal staining-positive cells/number of total cells) $\times$ 100. The data represent the mean $\pm$ SEM from three to five separate experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, versus DMSO-treated cells.
Considering the importance of PI3K/AKT pathway and MAPK signaling in cell cycle regulation, we examined the effects of BNF on PI3K/AKT and ERK signaling. MCF-7 and MDA-MB-231 cells were exposed to 10 µM BNF for 48 h. BNF significantly reduced phosphorylated AKT protein (Ser\(^{473}\) and Thr\(^{308}\)) and increased phosphorylated ERK protein (Figure 2A) in MCF-7 cells. Accordingly, phosphorylation of PI3K/AKT pathway proteins, p-PDK1 (Ser\(^{241}\)) and p-GSK-3β (ser\(^{9}\)), and p-c-Raf (Ser\(^{259}\)) were also significantly reduced (Figure 2A). The effects of BNF on PI3K/AKT and ERK in MCF-7 cells were dose- and time-dependent (Figure 2B and C).

BNF inhibits cyclinD1/D3 and CDK4 and induces p21\(^{Cip1/Waf1}\) by modulating PI3K/AKT and MAPK signaling respectively

To determine if the inhibition of PI3K/AKT pathway and/or the activation of MAPK signaling are responsible for the change of cell cycle regulating proteins, we used two chemical inhibitors of the PI3K and MAPK kinase, LY294,002 and PD98059, respectively. Increasing concentrations of LY294002 (24 h) resulted in dose-dependent down-regulation of p-AKT, cyclinD1/D3 and CDK4, but did not change p21\(^{Cip1/Waf1}\) expression, ERK activation or the basal CYP1A1 protein level (Figure 3A). These data demonstrate that the PI3K/AKT pathway positively regulates the expression of cyclinD1/D3 and CDK4, but is not related to the increasing of p21\(^{Cip1/Waf1}\) expression or ERK activation in BNF-treated MCF-7 cells. Similar to BNF, LY294002 treatment also resulted in a cell cycle arrest in G\(_1\) (Figure 3B).

Activation of MAPK signaling can induce p21\(^{Cip1/Waf1}\) expression and cell cycle arrest/cell senescence (30). To determine if the MEK/ERK pathway is involved in BNF-induced p21\(^{Cip1/Waf1}\) transcriptional upregulation, we exposed MCF-7 cells to the MEK inhibitor PD98059 (10 µM). Preincubation with PD98059 significantly reduced the expression of p21\(^{Cip1/Waf1}\) protein and mRNA induced by BNF (Figure 3C and D). Furthermore, PD98059 pre-incubation significantly reversed BNF-induced cell cycle arrest (Figure 3E), but did not affect AKT phosphorylation, CYP1A1 induction or expression of cyclinD1/D3 and CDK4 regulated by BNF (Figure 3C).

ER\(_{\alpha}\) is required for BNF-induced p21\(^{Cip1/Waf1}\) expression

BNF had different effects on p21\(^{Cip1/Waf1}\) induction in ER-positive MCF-7 and ER-negative MDA-MB-231 cells. Previous studies have shown that ER\(_{\alpha}\) increases p21\(^{Cip1/Waf1}\) protein and mRNA expression through protein-protein interactions and transcriptional functions of the receptor in MCF-7 cells (31,32). To investigate whether ER\(_{\alpha}\) is important for BNF-induced p21\(^{Cip1/Waf1}\) expression in MCF-7 cells, we used specific siRNA to knockdown ER\(_{\alpha}\) expression. RNAi-mediated
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knockdown of endogenous ERα significantly inhibited the basal levels of p21Cip1/Waf1, and BNF still induced a little p21Cip1/Waf1 (Figure 4A and B). To allow better comparison of the induction effect of BNF on p21Cip1/Waf1 between negative and ERα siRNA groups, we calculated the fold increase of p21Cip1/Waf1 mRNA relative to DMSO treatment. BNF-induced p21Cip1/Waf1 expression is significantly inhibited after ERα knockdown (Figure 4A and C). Concomitantly, BNF significantly reduced ERα protein (Figure 4A). Studies show that agonist-dependent activation of AhR initiates proteasomal degradation of ERα protein (33). To determine if the BNF-mediated reduction of ERα protein is due to protein degradation, we used the proteasome inhibitor, MG132. MG132 blocked the decrease of ERα protein mediated by BNF (Figure 4D). ERα knockdown did not affect the phosphorylation of ERK1/2 induced by BNF (Figure 4A), implying that ERα may be directly involved in BNF-induced p21Cip1/Waf1 expression.

BNF induces cell cycle arrest through AhR

To determine if the effect of BNF on MCF-7 cells is dependent on AhR, we used specific AhR siRNA to knockdown AhR protein. RNAi-mediated knockdown of endogenous AhR significantly inhibited BNF-induced CYP1A1 protein and mRNA (Figure 5A and B). Furthermore, AhR knockdown significantly inhibited BNF-induced phosphorylation of ERK and p21Cip1/Waf1 increase (Figure 5A and B). AhR knockdown also blocked the effect of BNF on inhibition of AKT phosphorylation and ERα degradation (Figure 5A). Although AhR knockdown itself slightly resulted in a cell cycle arrest in G0/G1 phase (Figure 5C), consistent with other studies that suggest the AhR endogenously facilitate cell cycle progression through G1 in the absence of an exogenous ligand (15,34), BNF did not further induce cell cycle arrest in G0/G1 phase when AhR was knocked down (Figure 5C).

Discussion

In the present study, we demonstrated a strong antitumor effect of BNF on human ER-positive breast carcinoma cells (MCF-7) through inducing cell cycle arrest and cell senescence (Figure 1). BNF inhibited PI3K/AKT activation, leading to decreased levels of the cell cycle promoters, cyclinD1/D3 and CDK4, and activated MAPK/ERK signaling. Furthermore, BNF increased the cell cycle inhibitor, p21Cip1/Waf1, an effect dependent upon ERK activation and ERα. Collectively, these events resulted in cell cycle arrest and cell senescence. Importantly, the effects of BNF on MCF-7 cells are dependent upon expression of the AhR (Figure 6). The modulation of cell cycle proteins by AhR-mediated PI3K/AKT, MAPK/ERK and ERα signaling is a possible molecular mechanism underlying the effects of BNF on MCF-7 breast cancer cells (Figure 6).

Two key classes of regulatory molecules control cell cycle progression, i.e., positive regulators, cyclins and cyclin-dependent kinases.
Fig. 3. BNF inhibits the expression of cyclinD1/D3 and CDK4 and induces p21<sup>Cip1/Waf1</sup> by modulating PI3K/AKT and MAPK signaling respectively. For A and B, MCF-7 cells were treated with PI3K inhibitor LY294, 002 for 48 h. (A) Western blot analysis of the cell cycle proteins and the PI3K/AKT and ERK signaling proteins. (B) Flow cytometric analysis of cell cycle arrest. Upper panel, representative cell cycle distribution; Lower panel, quantitation of cell cycle distribution. For C, D and E, MCF-7 cells were preincubated with MEK inhibitor PD98059 (10 µM) for 1 h and then treated with 10 µM BNF for 48 h. (C) Western blot analysis of the cell cycle proteins, AKT and ERK phosphorylation and (D) qPCR analysis of p21Cip1/Waf1 mRNA level. (E) Flow cytometric analysis of cell cycle arrest. Left panel, representative cell cycle distribution; Right panel, quantitation of cell cycle distribution. The data represent the mean ± SEM from four separate experiments. *<i>P</i> < 0.05, **<i>P</i> < 0.01 and ***<i>P</i> < 0.001, versus DMSO-treated cells; $<i>P</i> < 0.05 and $$<i>P</i> < 0.01, versus PD98059-treated cells; #, <i>P</i> < 0.05, ##, <i>P</i> < 0.01 and ###, <i>P</i> < 0.001, versus BNF-treated cells.
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(CDKs) prompt cells to progress through the cell cycle; negative regulators, cyclin-dependent kinase inhibitors (p21\textsuperscript{Cip1/Waf1}, p27\textsuperscript{Kip1}, and so on) cause cell cycle arrest. The balance between the activation of cyclin D/CDK4 complex and the binding of p21\textsuperscript{Cip1/Waf1} and p27\textsuperscript{Kip1} to cyclin/CDK complexes determines the progression from G\textsubscript{1} to S phase (35). Many studies have shown that AhR affects cell cycle progression through multiple mechanisms that are ligand and cell-context dependent (36). AhR can affect the cell cycle through regulating the expression of p21\textsuperscript{Cip1/Waf1}, p27\textsuperscript{Kip1} and cyclin G\textsubscript{2} (37, 38) or interacting with retinoblastoma protein (pRb), E2F and CDK4 (15, 39). Our results provide a novel mechanism for AhR-mediated cell cycle arrest in BNF-treated MCF-7 cells by negatively regulating the PI3K/AKT pathway and simultaneous activation of ERK signaling, which may lead to a decrease in cyclinD1/D3 and CDK4 proteins and increases p21\textsuperscript{Cip1/Waf1} expression, respectively. 

The relevance of the PI3K/AKT pathway in breast cancer is becoming increasingly recognized; hyperactivation and aberrations of this pathway are major causes of breast cancer metastasis, poor survival rates and therapeutic resistance (28); in addition, drugs targeting PI3K/AKT pathway are in clinical development for breast cancer treatment (26). AKT regulates the cell cycle by preventing GSK-3\textbeta-mediated phosphorylation and degradation of cyclin D protein (27) and by negatively regulating the CDK inhibitors p21\textsuperscript{Cip1/Waf1} (23). To confirm PI3K/AKT-dependent regulation of cell cycle proteins in this study, we evaluated the effect of LY294,002, a pharmacologic PI3K inhibitor on MCF-7 cells. Increasing concentrations of LY294,002 inhibited AKT signaling and resulted in downregulation of cyclinD1/D3 and CDK4, and cell cycle arrest (Figure 3A and B). However, unlike BNF, LY294,002 had no effect on ERK1/2 phosphorylation or p21\textsuperscript{Cip1/Waf1} expression (Figure 3A), implying that BNF-mediated PI3K/AKT inhibition may only be responsible for decreasing of cyclinD1/D3 and CDK4, but not responsible for increasing of p21\textsuperscript{Cip1/Waf1} expression. Although one study shows that LY294,002 is an AhR antagonist (40), LY294,002 did not affect the basal CYP1A1 protein expression (Figure 3A) in this study.

Activation of the MAPK pathway is generally associated with increased cell proliferation (29), but increasing studies have shown that the prolonged MAPK/ERK activation can induce p21\textsuperscript{Cip1/Waf1} expression, leading to cell cycle arrest and cell senescence (23, 30). AhR-MAPK crosstalk has been reported; those studies indicate that TCDD activates ERK via an AhR-dependent or AhR-independent pathway, but TCDD-stimulated ERK appears only to affect AhR activity, with no effects on the ERK-mediated downstream transcriptional activity (41). In contrast, our study shows that BNF, induced in a dose- and time-dependent fashion, ERK phosphorylation that is dependent on AhR (Figures 2 and 5). Furthermore, BNF induced p21\textsuperscript{waf1} expression and cell cycle arrest by activating ERK (Figure 3C–E). Although one study has indicated that PD98059 may be a potent AhR antagonist (42), our data clearly indicate that PD98059 does not inhibit BNF-induced CYP1A1 expression (Figure 3C), thereby excluding PD98059 as an AhR ligand.
Fig. 5. The effect of BNF on MCF-7 cell cycle depends on AhR. For A, B and C, MCF-7 cells were transfected with 40 nM negative or AhR siRNA for 36h, then treated with 10 µM BNF for 36h. (A) Western blot analysis of the phosphorylation of AKT and ERK1/2, p21\(^{C_{eta}p/W_{af1}}\), CYP1A1 and ER\(\alpha\) protein. (B) qPCR analysis of p21\(^{C_{eta}p/W_{af1}}\) and CYP1A1 mRNA. (C) Flow cytometric analysis of cell cycle arrest. *\(P < 0.05\), **\(P < 0.01\) and ***\(P < 0.001\), versus DMSO-treated cells with negative siRNA; $$$, \(P < 0.001\), versus DMSO-treated cells with AhR siRNA #, \(P < 0.05\) and ###, \(P < 0.001\), versus BNF-treated cells with negative siRNA.
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Fig. 6. Hypothetical model for BNF-induced cell cycle arrest and senescence. BNF induces MCF-7 cells accumulation in G1/G0 phase of the cell cycle and cell senescence by activating AhR. BNF reduces cyclinD1/D4 and CDK4 through inhibiting PI3K/AKT pathway, and increases p21(Cip1/Waf1) through activating MAPK/ERK signaling in the presence of ERα. Meanwhile, BNF also causes ERα degradation through ubiquitination-proteasome pathway.

By using AhR siRNA, we demonstrated that BNF-mediated regulation of PI3K/AKT and ERK signaling is dependent on AhR (Figure 5). Furthermore, our data showed that BNF induced very high expression of CYP1A1 mRNA and protein in MCF-7 cells, but only very weak CYP1A1 mRNA induction in MDA-MB-231 cells (Supplementary Figure S1, available at Carcinogenesis Online), which is consistent with previous studies that demonstrate responsiveness of MCF-7 but not MDA-MB-231 cells to TCDD (21), and that the biological activity of AhR in MDA-MB-231 cells is altered due to expression of a variant ARNT protein (43). In addition, MDA-MB-231 cells showed inherently different characteristics in both pathways; Akt activation is very weak and ERK is hyperactivated compared to MCF-7 cells (Figure 2A). Our data, together with the findings reported by others provide good explanations for why BNF effects differ in MCF-7 compared to MDA-MB-231 cells.

PI3K/AKT and Raf-MAPK-ERK mediate conflicting cellular responses including proliferation, apoptosis, cell cycle arrest and senescence; these pathways functionally synergize or antagonize each other depending on the extent and duration of the external stimulus and on cell type (23,44). Given this and our data, BNF-activated AhR may switch the balance toward MAPK/ERK activation, inducing p21(Cip1/Waf1), whereas PI3K/AKT–mediated cell cycle progression signal are suppressed, synergistically leading to cell cycle arrest and cell senescence. This model is completely different from previous reports that PI3K pathway inhibition results in compensatory ERK activation (45) because our study showed that PI3K inhibitor, LY294,002 had no effect on basic ERK phosphorylation (Figure 3A). The exact mechanism of BNF-activated, AhR-mediated modulation of both pathways remains unclear. Currently, there are almost no clear reports about the regulation of AhR on PI3K/AKT and MAPK/ERK pathways. Numerous studies have shown that the effect of AhR on cell cycle progression is ligand and cell-context dependent, implying that AhR activation is necessary but not sufficient for ligand-mediated cell cycle regulation. Many studies have shown the importance of CYP1 in cancer prevention (46,47). For example, some antitumor regents selectively inhibit breast cancer cell proliferation through AhR/CYP1A1-mediated drug metabolism (48,49). AhR ligands have an array of different chemical structures that may produce different metabolic products after induction of P450 metabolizing enzymes. Reactive metabolites may then act through different mechanisms downstream and/or independent of activating AhR. In this context, it is conceivable that BNF activates AhR to induce CYP enzymes that feed back to metabolize BNF into active compounds that may regulate PI3K/AKT and MAPK-ERK signal. Different breast cancer cell lines may have different biological characteristics, like PI3K/AKT mutations and Ras/Raf/ERK mutations, which provide incentive for examining cell types in cancer patients and providing individualized treatment in the clinic. Considering the endogenous biological differences in breast cancer cell lines, future studies are designed to thoroughly investigate and compare the effects of BNF and other AhR agonists on breast cancer in vitro with multiple cell lines and in vivo with tumor models. Our objective is to gain insight into crosstalk among the AhR response, ERα expression, agonists and cell types in breast cancer. p21(Cip1/Waf1) is usually activated by p53 triggered by DNA damage. Here, BNF did not change p53 protein, implying that p21(Cip1/Waf1) induction may be independent of p53, which agrees with another study that demonstrated induction of p21(Cip1/Waf1) expression by TCDD independent of p53 (37). Furthermore, our results showed that BNF-induced p21(Cip1/Waf1) expression is ERα dependent (Figure 4), consistent with previous studies that ERα is a known transcriptional regulator of p21(Cip1/Waf1) in MCF-7 cells (31,32). Like other AhR agonists (33), BNF treatment also resulted in ERα protein degradation through activating AhR. ERα knockdown did not impact the phosphorylation of ERK1/2 induced by BNF (Figure 4A), and different ERK1/2-activated gene expression also may directly regulate p21(Cip1/Waf1) expression (40,50). Implicating that ERK1/2 signaling and ERα act together to regulate p21(Cip1/Waf1). Although BNF-induced p21(Cip1/Waf1) expression is AhR-dependent (Figure 5), and AhR knockdown also blocked BNF-mediated ERK1/2 phosphorylation, implying that BNF-activated AhR increases p21(Cip1/Waf1) expression through ERK1/2 phosphorylation, we cannot exclude the possibility that AhR also may directly regulate p21(Cip1/Waf1) expression considering TCDD has been shown to regulate p21(Cip1/Waf1) expression through a direct transcriptional effect by activated AhR (37). Thus, transcriptional regulation of BNF-induced p21(Cip1/Waf1) is very complex. Additional experiments are warranted to investigate how AhR, ERα and ERK-activated transcription factors synergistically regulate BNF-mediated p21(Cip1/Waf1) expression.

In summary, we have firstly and clearly demonstrated that BNF exerts an antitumor effect through a completely different mechanism by activating AhR and PI3K/AKT pathway.
from other AhR agonists and flavonoids, i.e. AhR-mediated PI3K/AKT pathway inhibition, MAPK/ERK signaling activation and ERα signaling work together to prompt a cell cycle arrest. This novel mechanism represents a promising strategy to be exploited in future clinical trials of breast cancer treatment. It is important to further clarify the detailed molecular mechanisms and the exact nature of PI3K/AKT pathway inhibition and MAPK/ERK activation mediated by BNF in our future studies.

Supplementary material
Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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