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RESEARCH PAPER

Honokiol sensitizes breast cancer cells to TNF-α induction of apoptosis by inhibiting Nur77 expression

Lei Xie[1,*], Fuquan Jiang[1,*], Xindao Zhang[1], Gulimiran Alitongbieke[1], Xinlei Shi[1], MinJun Meng[2], Yiming Xu[2], Anshi Ren[1], Jing Wang[1], Lijun Cai[1], Yunxia Zhou[1], Yang Xu[1], Ying Su[3], Jie Liu[1], Zhiping Zeng[1], Guanghui Wang[1], Hu Zhou[1], Quan Cheng Chen[1] and Xiao-kun Zhang[1,3]

[1]School of Pharmaceutical Sciences, Xiamen University, Xiamen 361102, China, [2]Zhongshan Hospital, Xiamen University, Xiamen 361102, China, and [3]Sanford Burnham Prebys Medical Discovery Institute Cancer Center, La Jolla, CA 92037, USA

Correspondence
Dr Xiao-kun Zhang, Sanford Burnham Prebys Medical Discovery Institute, Cancer Center, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA.
E-mail: xzhang@sanfordburnham.org
Dr Quan Cheng Chen, School of Pharmaceutical Sciences, Xiamen University, Xiang'an South Road, Xiamen, 361102, China.
E-mail: chenqc@xmu.edu.cn

*These authors contributed equally to the work.

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BACKGROUND AND PURPOSE

The orphan nuclear receptor Nur77 is implicated in the survival and apoptosis of cancer cells. The purpose of this study was to determine whether and how Nur77 serves to mediate the effect of the inflammatory cytokine TNF-α in cancer cells and to identify and characterize new agents targeting Nur77 for cancer therapy.

EXPERIMENTAL APPROACH

The effects of TNF-α on the expression and function of Nur77 were studied using *in vitro* and *in vivo* models. Nur77 expression was evaluated in tumour tissues from breast cancer patients. The anticancer effects of honokiol and its mechanism of action were assessed by *in vitro*, cell-based and animal studies.

KEY RESULTS

TNF-α rapidly and potently induced the expression of Nur77 in breast cancer cells through activation of IκB kinase and JNK. Knocking down Nur77 resulted in TNF-α-dependent apoptosis, while ectopic Nur77 expression in MCF-7 cells promoted their growth in animals. Levels of Nur77 were higher in tumour tissues than the corresponding tissues surrounding the tumour in about 50% breast cancer patients studied. Our *in vitro* and animal studies also identified honokiol as an effective sensitizer of TNF-α-induced apoptosis by inhibiting TNF-α-induced Nur77 mRNA expression, which could be attributed to its interference of TNFR1's interaction with receptor-interacting protein 1 (RIPK1).

CONCLUSIONS AND IMPLICATIONS

TNF-α-induced Nur77 serves as a survival factor to attenuate the death effect of TNF-α in cancer cells. With its proven human safety profile, honokiol represents a promising agent that warrants further clinical development.

Abbreviations

IKK, IκB kinase; TPA, 12-O-tetradecanoyl-13-phorbol acetate

Tables of Links

TARGETS	
Nuclear hormone receptors[a]	**Enzymes**[c]
Nur77 (NGF1B; NR4A1)	Caspase 3
RXRα	IKKβ
Catalytic receptors[b]	JNK
TNFR1	p38 MAPK
Other protein targets	RIPK1 (RIP1)
Bcl-2	TRAF2
TNF-α	

LIGANDS	
9-cis-Retinoic acid	IL-1β
Bay-11-7082	MG132
Cycloheximide	SB203580
Hsp60	SP600125
	TPA

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*,) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Nur77 (NR4A1), also known as nerve growth factor I-B (NGF1B) and TR3, is an orphan member of the nuclear receptor superfamily (Zhang, 2007; Pearen and Muscat, 2010; Lee *et al.*, 2011; McMorrow and Murphy, 2011; Deutsch *et al.*, 2012; Mohan *et al.*, 2012; To *et al.*, 2012; Kurakula *et al.*, 2014). It is also an immediate–early response gene whose expression can be induced by many stimuli including serum, inflammatory factors, growth factors and stress in different cell types (Pearen and Muscat, 2010; Lee *et al.*, 2011; McMorrow and Murphy, 2011; Deutsch *et al.*, 2012; Mohan *et al.*, 2012; To *et al.*, 2012; Kurakula *et al.*, 2014). Consistently, Nur77 exerts multiple biological functions, regulating cell proliferation, differentiation, apoptosis, development, metabolism and immunity. Nur77 has been shown to induce apoptosis in a number of cancer cell types, but can also paradoxically act as a death inhibitory factor, suggesting that it has both a tumour suppressive and pro-oncogenic effect in cancer development (Pearen and Muscat, 2010; Lee *et al.*, 2011; Mohan *et al.*, 2012; To *et al.*, 2012; Kurakula *et al.*, 2014). Nur77 exerts these physiological functions through expression regulation, post-translational modification and subcellular localization. While the cell survival effect of Nur77 correlates with its nuclear localization (Wu *et al.*, 1997b; Kolluri *et al.*, 2003), the death effect of Nur77 has been largely attributed to its migration to mitochondria where Nur77 binds Bcl-2 to activate the mitochondria-dependent apoptotic pathway (Li *et al.*, 2000; Lin *et al.*, 2004; Kolluri *et al.*, 2008).

Chronic inflammation plays a causal role in the development of cancer (Nakanishi and Toi, 2005; DiDonato *et al.*, 2012). NF-κB, the central mediator of the inflammatory process, is activated during inflammation or as a consequence of the formation of an inflammatory microenvironment during malignant progression (Nakanishi and Toi, 2005; DiDonato *et al.*, 2012). In unstimulated cells, NF-κB is sequestered in an inactive state in the cytoplasm by a family of inhibitors called IκBs. Canonically, pro-inflammatory stimuli such as TNF-α induce a sequential recruitment of various adaptors including TNF receptor-associated death domain protein (TRADD), receptor-interacting protein (RIP) and TNF receptor-associated factor 2 (TRAF2) to TNF receptor (TNFR), which is followed by the recruitment and activation of the IκB kinase (IKK) complex to phosphorylate IκBs, leading to their ubiquitination and degradation (Mocellin and Nitti, 2008; Balkwill, 2009). The NF-κB complex is then released from IκBs and translocated to the nucleus for the expression of tumour-promoting cytokines and survival genes. Nur77 has also emerged as an important regulator of inflammation (Zhang, 2007; Pearen and Muscat, 2010; Lee *et al.*, 2011; McMorrow and Murphy, 2011; Deutsch *et al.*, 2012; Mohan *et al.*, 2012; To *et al.*, 2012; Kurakula *et al.*, 2014). It is rapidly induced in macrophages by inflammatory stimuli such as TNF-α (Pei *et al.*, 2006), acting either to inhibit or to activate the NF-κB pathway (Pei *et al.*, 2006; Shao *et al.*, 2010). Although less characterized, Nur77 was shown to crosstalk with the NF-κB pathway in other cell types, serving as a survival factor in TNF-α signalling in mouse embryonic fibroblasts (Suzuki *et al.*, 2003). Whether and how Nur77 plays a role in mediating the biological effects of inflammatory stimuli in cancer cells remains largely unknown.

Honokiol is a phenolic compound initially isolated from the bark of *Magnolia obovata* (Fried and Arbiser, 2009; Arora *et al.*, 2012; Kumar *et al.*, 2013). It has been used to treat fever, headache, anxiety and nervous disturbance in Asia for decades. Recent studies indicate that this naturally occurring small molecule exerts anti-angiogenic, anti-invasive and anti-proliferative activities in a variety of cancer cells (Fried and Arbiser, 2009; Arora *et al.*, 2012; Kumar *et al.*, 2013). Honokiol induces growth inhibition and apoptosis in different cancer cell lines *in vitro* and represses tumour growth in animal xenograft models (Bai *et al.*, 2003; Hahm *et al.*, 2008; Leeman-Neill *et al.*, 2010). Honokiol also sensitizes conventional chemotherapy and radiotherapy in different models of human cancer (Liu *et al.*, 2008; Leeman-Neill *et al.*, 2010; Tian *et al.*, 2013; Wang *et al.*, 2014). Recent studies have shown that honokiol can modulate a number of signal transduction pathways, including the NF-κB pathway (Tse *et al.*, 2005; Zhang *et al.*, 2013; Fan *et al.*, 2014). It inhibits TNF-α-stimulated NF-κB activation through suppression of IKK activation in cancer cells, leading to a reduction of inflammatory gene

products (Tse *et al.*, 2005). The anti-inflammatory effect of honokiol has been subsequently confirmed in various models of inflammation, showing remarkable inhibition of various inflammatory responses (Tse *et al.*, 2005; Zhang *et al.*, 2013; Fan *et al.*, 2014). Thus, the anti-inflammatory effect of honokiol probably contributes to its antitumour activity.

In this study, we showed that TNF-α strongly and rapidly induces Nur77 mRNA and protein expression through its activation of IKK and JNK in MCF-7 breast cancer cells. TNF-α-induced Nur77 resided in the nucleus and acted as a survival factor to blunt the apoptotic effect of TNF-α. We also identified honokiol as an effective sensitizer of TNF-α-induced apoptosis through its inhibition of TNF-α-induced Nur77 expression due to its interference of TNFR1 recruitment of its adaptor protein RIPK1. These results suggest that Nur77 is an attractive drug target for therapeutic intervention in breast cancer and identify honokiol as a promising lead for breast cancer therapy.

Methods

Small interfering RNA

The siRNA sense sequences for Nur77 (5′-GGC UUG AGC UGC AGA AUG AdTdT-3′, 5′-CGC UUC AUG CCA GCA UUA UdTdT-3′, and 5′-CUC UGG AGG UCA UCC GCA AdTdT 3′), TNFR1 (5′ GUC UCC UGU AGU AAC UGU AdTdT-3′, 5′-GUG GAG AUC UCU UCU UGC AdTdT-3′, 5′-GAA CCU ACU UGU ACA AUG AdTdT-3′), p38 MAPK (5′-CUG ACA UAA UUC ACA GGG AdTdT-3′, 5′-GUC CAU CAU UCA UGC GAA AdTdT-3′, 5′-GGU CUA AAG UAU AUA CAU UdTdT-3′) and three respective pairs of control siRNA oligonucleotides were synthesized by Sigma-Aldrich Co. Ltd. They (20 mM) were transfected into MCF-7 cells using Lipofectamine 2000 according to the recommendations of the manufacturer. Specifically, cells were grown to 70–90% confluence at the time of transfection. Lipofectamine 2000 transfection reagent, 5 μL, was mixed with 5 μL of siRNA in Opti-MEM I Reduced Serum Medium. After 20 min of incubation at room temperature, the complexes were applied to cells and incubated for 24–36 h.

Cell culture

MCF-7, MDA-MB-231, HeLa and HepG2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). MCF-7, MDA-MB-231 and HepG2 cells were maintained in DMEM medium (Hyclone), while HeLa cells were maintained in Minimum Essential Medium (MEM) medium (Hyclone). Subconfluent cells with exponential growth were used throughout the experiments.

Construction of plasmids

Nur77 cDNA generated by PCR using forward primer (5′-CGG AAT TCC GAT GCC CTG TAT CCA AGC CCA-3′) and reverse primer (5′-GCG TCG ACT CAG AAG GGC AGC GTG TCC ATG-3′) were cloned into EcoR I and Sal I sites of the pIRES2-EGFP vector (Clontech, Mountain View, CA, USA). The resulting pIRES2-EGFP-Nur77 expression vector was verified by sequencing. For transfection, 1 μg of pIRES2-EGFP-Nur77 expression vector or the pIRES2-EGFP control vector was transfected into MCF-7 cells with 2.5 μL of Lipofectamine 2000 transfection reagent. A pool of stable clones was selected with 500 $\mu g \cdot mL^{-1}$ G418 for 12 days and used for experiments.

Animals and xenograft tumour mouse model

Nude mice (BALB/c, 18–20 g, 4 to 5 weeks-old) were housed at 28°C in a laminar flow under sterilized conditions. Mice were injected s.c. with 10^6 cells in 100 μL PBS. For compound treatment, mice (n = 6) were treated with honokiol or vehicle (Tween 80) once a day at a dose of 20 $mg \cdot kg^{-1}$ using gavage, 2 days after the transplantation of cells. Body weight and tumour size were measured every 2 days. Mice were killed at the end of the experiment, and the tumours were harvested for further assessments. All animal care and experimental procedures were approved by the Animal Care and Use Committee of Xiamen University.

Human tissues and evaluation

Breast tumour tissues and their surrounding tissues were obtained by surgical resection from cancer patients. Histologically normal specimens, which were at least 3–5 cm distant from the tumour nodule, were obtained from the corresponding patients. The study was approved by the Institute for Biomedical Research Ethics Committee at Xiamen University, and all patients were given informed consent. Tissues from patients (n = 18) were collected for studying the expression of Nur77 by Western blotting.

Subcellular fractionation

Cytosolic and nuclear fractions were prepared as described previously (Li *et al.*, 2000; Lin *et al.*, 2004; Kolluri *et al.*, 2008). Briefly, cells were lysed with 0.4 mL cold buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT) containing 0.05% NP-40 and proteinase cocktail inhibitors for 10 min. Cell lysates were then centrifuged at 14000 g for 30 s, and the cytoplasmic fraction was collected. The nuclei pellets were washed twice with cold PBS, resuspended in cold high-salt buffer C (20 $mmol \cdot L^{-1}$ HEPES-KOH, pH 7.9, 25% glycerol, 420 $mmol \cdot L^{-1}$ NaCl, 1.5 $mmol \cdot L^{-1}$ $MgCl_2$, 0.2 $mmol \cdot L^{-1}$ EDTA and 0.5 $mmol \cdot L^{-1}$ DTT) containing proteinase cocktail inhibitors on ice for 30 min. Cellular debris was removed by centrifugation at 14000 g at 4°C for 15 min, and the nuclear fraction was then collected.

Reverse transcription-PCR (RT-PCR)

First-strand cDNA was generated with the RevertAid First Strand cDNA Synthesis Kits (Fermentas, Waltham, MA, USA). Primers (forward primer, 5′-TCA TGG ACG GCT ACA CAG-3′; reverse primer, 5′-GTA GGC ATG GAA TAG CTC-3′) and (forward primer, 5′-CTG GAG AAG AGC TAC GAG-3′; reverse primer, 5′-TGATGG AGT TGA AGG TAG-3′) were used to amplify Nur77 and β-catenin respectively.

Western blotting, co-immunoprecipitation and immunofluorescence assays

Western blotting, immunoprecipitation and immunofluorescence assays were carried out as described previously (Li *et al.*, 2000; Lin *et al.*, 2004; Kolluri *et al.*, 2008). For Western blotting assay, cellular proteins (20–50 μg) separated by sodium

dodecyl sulfate–polyacrylamide gel electrophoresis were transferred onto PVDF membranes. The membranes were blocked with 5% skim milk in TBST buffer (150 mM NaCl, 0.1% Tween 20 and 50 mM Tris–HCl, pH 7.4), incubated with primary antibody and detected with secondary antibody. The final immunoreactive products were detected by using enhanced chemiluminescence system.

For co-immunoprecipitation assay, cells were lysed in NETN (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10 mM EDTA and 0.2% NP-40) buffer containing protease inhibitors. Lysate was incubated with primary antibody at 4°C for 12 h, then with 40 μL of protein A/G-sepharose (Santa Cruz Biotechnology) for another 4 h. After extensive washing with NETN buffer, beads were boiled in 100 μL loading buffer and analysed by Western blotting.

For the immunofluorescence assay, cells were plated on glass slides for 24 h, treated in accordance with corresponding experimental conditions, permeabilized with PBS containing 0.05% Triton X-100 and 0.1 M glycine for 15 min on ice and blocked with 1% BSA in PBS for 30 min at room temperature. To immunostain Nur77, cells were incubated with rabbit anti-Nur77 antibody (1:200) and mouse anti-Hsp60 antibody (1:200) for 12 h at 4°C and detected by anti-rabbit IgG conjugated with Cy3 (1:500) and anti-mouse IgG conjugated with FITC respectively. The images were acquired using the LSM-510 confocal laser scanning microscope system (Carl Zeiss, Oberkochen, Germany).

Luciferase reporter assay

pGL6-NF-κB-luc (100 ng; Beyotime, Haimen, China) and 50 ng of β-galactosidase were transiently transfected into MCF-7 cells for 24 h. NF-κB luciferase activities were assayed after cells were treated with or without 10 ng·mL^{-1} TNF-α in the presence or absence of 10 μM honokiol for an additional 12 h. Each transfection included β-galactosidase for normalization.

Statistical analysis

Data are expressed as mean ± SEM. Each assay was repeated in triplicate in three independent experiments. Statistical significance of differences between groups was analysed by using Student's *t*-test or ANOVA. *$P < 0.05$ was considered significant.

Materials and Reagents

Honokiol was isolated from the stem bark of *M. officinalis* subsp. *biloba* (Rehd. et Wils.) Cheng et Law. Its purity was determined to be a minimum of 99% by HPLC (Supporting Information Fig. S1). Lipofectamin 2000 from Invitrogen (Carlsbad, CA, USA); BAY11-7082 from Santa Cruz Biotechnology (Dallas, TX, USA); SB203580, MG132, SP600125, DAPI, IL-1β (IL-1β) and 12-O-tetradecanoyl-13-phorbol acetate (TPA) from Sigma-Aldrich (St Louis, MO, USA); actinomycin D from MP Biomedicals (Santa Ana, CA, USA); TNF-α from PeproTech Inc. (Rocky Hill, NJ, USA); antibodies against Nur77, p-IKKβ, JNK, anti-cleaved caspase 3, p38 MAPK and TRAF2 from Cell Signaling (Danvers, MA, USA); antibodies against RXRα (D20), PARP, GAPDH, α-tublin, Hsp60 and TNFR1 from Santa Cruz Biotechnology; anti-β-actin antibody from Sigma-Aldrich; anti-RIPK1 antibody from BD Biosciences (Franklin Lakes, NJ, USA); anti-IκBα antibody from Abcam (Cambridge, UK) and anti-rabbit and anti-mouse secondary antibodies conjugated to HRP from Thermo Fisher Scientific (Waltham, MA, USA) were used in the study.

Results

Induction of Nur77 expression by TNF-α in MCF-7 breast cancer cells

It has been shown that different inflammatory stimuli can induce the expression of Nur77 in macrophages (Pei *et al.*, 2006). To study whether Nur77 plays a role in mediating inflammatory signalling in cancer cells, we examined the effect of TNF-α on the expression of Nur77 in MCF-7 cells. Western blotting showed that TNF-α could rapidly and strongly induce the expression of Nur77 protein in a time-dependent manner (Figure 1A). The induction of Nur77 protein by TNF-α was very fast, occurring as early as 1 h after exposure to TNF-α, with a maximal induction observed after 3 h of treatment, which then gradually decreased. TNF-α also induced Nur77 protein expression in MDA-MB231 breast cancer cells. RT-PCR revealed that Nur77 mRNA expression in MCF-7 cells was also significantly up-regulated upon TNF-α treatment for 1.5 h in MCF-7 cells (Figure 1B), similar to the effect of TPA known to induce Nur77 mRNA expression in many different types of cells (Li *et al.*, 2000; Kolluri *et al.*, 2003). These results suggested that TNF-α-induced Nur77 expression was mainly regulated at transcriptional level. Consistently, the protein synthesis inhibitor cycloheximide and the transcriptional inhibitor actinomycin D suppressed the effect of TNF-α on inducing Nur77 expression (Supporting Information Fig. S2).

TNF-α-induced Nur77 acts as a survival factor to attenuate its killing effect

A unique property of Nur77 is that depending on the cellular environment it can exert opposing biological effects, such as survival and death, even in the same cell line (Kolluri *et al.*, 2003). We showed previously that the cytoplasmic Nur77 is apoptotic, while the nuclear Nur77 is a survival factor in lung cancer cells (Kolluri *et al.*, 2003). As the first step to determine the biological function of TNF-α-induced Nur77, we examined its subcellular localization. Our cellular fractionation data showed that Nur77 was mainly found in the nuclear fraction of MCF-7 cells treated with or without TNF-α (Figure 1C). This was confirmed by immunostaining analysis showing that TNF-α-induced Nur77 protein was stained mainly in the nucleus (Figure 1D). TNF-α is a multifunctional cytokine implicated in the regulation of diverse events such as cell survival and cell death (Mocellin and Nitti, 2008; Balkwill, 2009). However, the death effect of TNF-α is often antagonized by its own survival function in cancer cells, such as the activation of the NF-κB pathway (Mocellin and Nitti, 2008; Balkwill, 2009). Our observation that TNF-α-induced Nur77 resided in the nucleus raised the possibility that it might act to mediate the survival function of TNF-α. To test the hypothesis, we used siRNA approach to knock down Nur77 and then examined its effect on TNF-α activity. Transfection of Nur77 siRNA effectively inhibited Nur77 expression in MCF-7 cells treated with or without TNF-α (Figure 2A). When the apoptotic effect of TNF-α was examined, we found that it could significantly induce PARP cleavage in cells transfected with

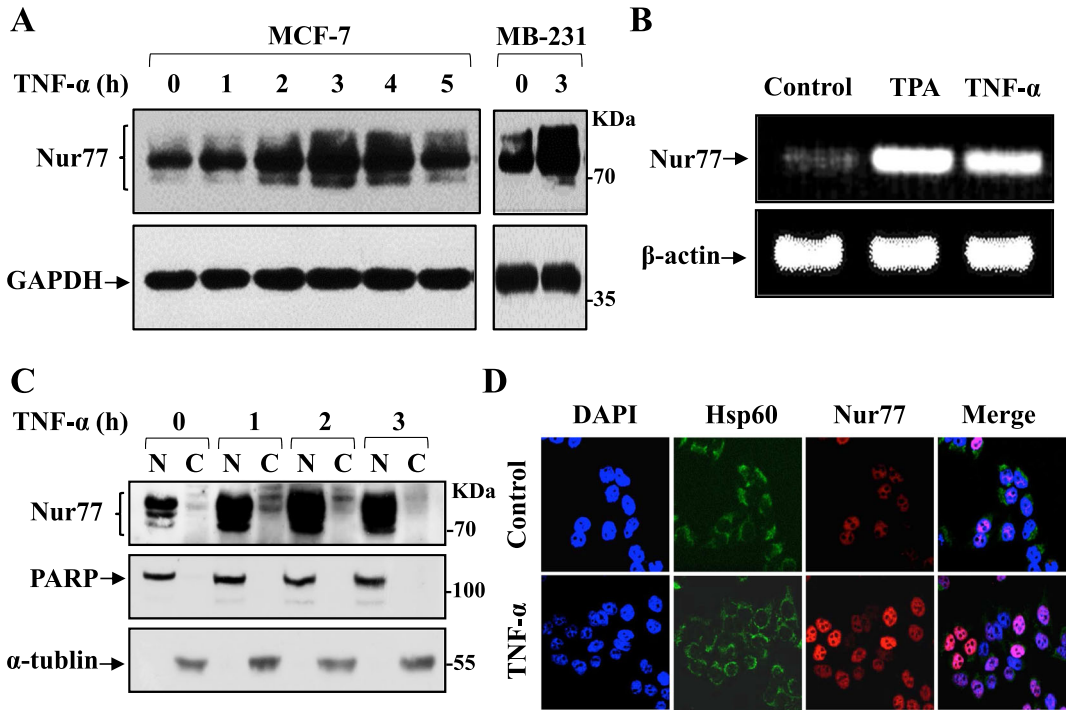

Figure 1

Induction of Nur77 expression by TNF-α. (A) Time course analysis. The indicated breast cancer cell lines treated with TNF-α (10 ng·mL^{-1}) for the indicated time were analysed by Western blotting. (B) The induction of Nur77 mRNA expression. MCF-7 cells treated with TNF-α (10 ng·mL^{-1}) or TPA (100 ng·mL^{-1}) for 1.5 h were analysed by RT-PCR. (C) TNF-α-induced Nur77 protein mainly resides in the nucleus. Nuclear and cytoplasmic fractions were prepared from MCF-7 cells treated with TNF-α (10 ng·mL^{-1}) for the indicated time and analysed for Nur77 expression by Western blotting. (D) Immunostaining of TNF-α-induced Nur77 protein. MCF-7 cells treated with or without TNF-α for 3 h were stained with anti-Nur77 antibody followed by Cy3-conjugated secondary antibody to detect Nur77, or with antibody against Hsp60, a mitochondria-specific protein, followed by FITC-conjugated secondary antibody to detect mitochondria. Nuclei were detected by DAPI staining. Immunostaining was visualized by confocal microscopy, and the images merged. Medium containing 0.1% DMSO was used as vehicle control.

Nur77 siRNAwhen compared with cells transfected with control siRNA in a dose- (Figure 2A) and time-dependent (Figure 2B) manner. To further study the survival function of Nur77, we stably transfected Nur77 in MCF-7 cells. A pool of Nur77 stable clones and the corresponding control stable clones (Figure 2C) were inoculated s.c. into the right and left hind-side flanks, respectively, in the mammary fat pad of the same mouse. Our results showed that the growth of MCF-7 cells stably expressing Nur77 in mice (Figure 2D) was 1.3-fold faster than the control cells (Figure 2E). Thus, the expression of Nur77 confers MCF-7 cells their growth advantage *in vivo*. We also studied the clinical relevance of Nur77 by examining its expression in breast tumour tissues. Immunoblotting showed that the levels of Nur77 were elevated in breast tumour tissues as compared with the corresponding surrounding tissues from nine out of 18 breast cancer patients studied (Figure 2F and Supporting Information Fig. S3). Together, these results suggest that TNF-α-induced Nur77 acts as a survival factor to promote the growth of breast cancer cells.

Honokiol sensitizes MCF-7 breast cancer cells to the killing effect of TNF-α by inhibiting Nur77 expression

In our effort to screen compounds for inhibiting the survival activity of TNF-α-induced Nur77 in cancer cells, we found that honokiol (Figure 3A), a potent anti-inflammatory agent (Tse *et al.*, 2005; Vaid *et al.*, 2010; Li *et al.*, 2011; Tian *et al.*, 2013), could effectively inhibit TNF-α induction of Nur77 expression and induce apoptosis in cancer cells. Treatment of MCF-7 cells with 10 μM honokiol alone for 6 h did not show any effect on apoptosis as revealed by a lack of PARP cleavage (Figure 3B). Interestingly, its combination with either 15 or 30 ng·mL^{-1} TNF-α resulted in significant PARP cleavage, while TNF-α alone had no effect. Thus, honokiol and TNF-α could synergistically induce apoptosis of MCF-7 cells. The antitumour effect of honokiol was also evaluated in a xenograft mouse model of MCF-7 breast cancer. The administration of honokiol to MCF-7 tumour-bearing mice effectively inhibited the growth of the MCF-7 tumour, with more than 50% inhibition after a 2 week treatment (Figure 3C).

The effect of honokiol on sensitizing MCF-7 cells to TNF-α induction of apoptosis was similar to that of Nur77 siRNA transfection. We therefore examined whether honokiol inhibited the ability of TNF-α to induce Nur77 expression. Indeed, our results showed that TNF-α-induced Nur77 expression was inhibited by honokiol in a dose-dependent manner (Figure 3D). Honokiol also inhibited TNF-α induction of Nur77 expression in MDA-MB231 breast cancer cells (Supporting Information Fig. S4). The inhibitory effect of honokiol appeared to be TNF-α specific as IL-1β-induced Nur77 expression was not affected by honokiol (Supporting Information Fig. S5). Interestingly, honokiol

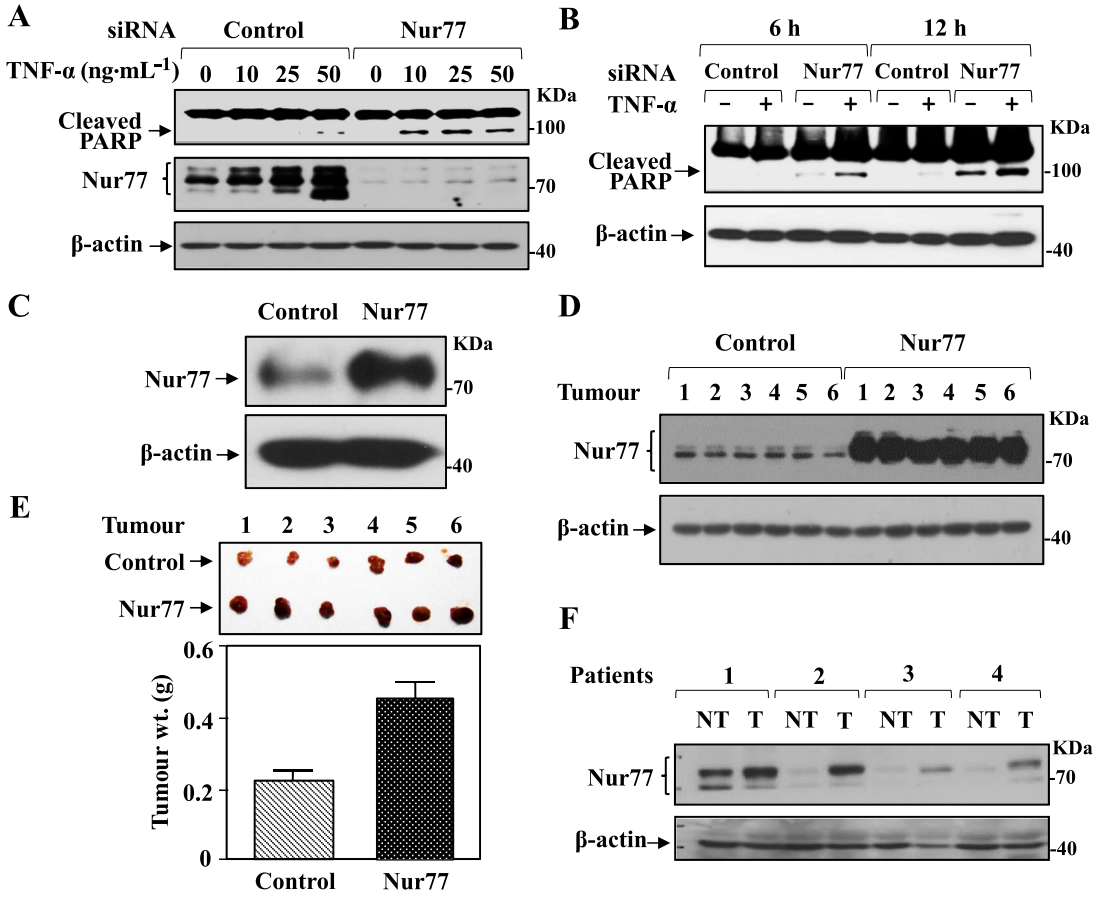

Figure 2

TNF-α-induced Nur77 acts as a survival factor. (A and B) Knocking down Nur77 promotes TNF-α-induced apoptosis. MCF-7 cells transfected with Nur77 siRNA or control siRNA for 36 h were treated with or without the indicated concentration of TNF-α for 9 h (A) or at the indicated time (B). PARP cleavage and Nur77 expression were analysed by Western blotting. Medium containing 0.1% DMSO was used as vehicle control. (C) The expression of Nur77 in a pool of MCF-7 clones stably transfected with either the control vector or Nur77 expression vector analysed by Western blotting. (D) The expression of Nur77 in tumour tissues prepared from mice inoculated with MCF-7 stable clones transfected with control vector or Nur77 expression vector. (E) The ectopic expression of Nur77 promotes the growth of MCF-7 tumour in animals. MCF-7 stable clones transfected with Nur77 expression vector or control vector were inoculated into the left and right forelimb armpits of same BALB/c nude mice respectively. Two weeks later, tumours were harvested and weighed. Tumours excised from each animal are shown (n = 6). F, overexpression of Nur77 in breast tumour tissues. Expression of Nur77 in tumour tissues (T) and the corresponding surrounding tissues (NT) from four patients were analysed by Western blotting.

inhibition of Nur77 expression correlated with its induction of PARP cleavage. At doses (10 and 20 μM) that effectively inhibited Nur77 expression, honokiol potently induced the cleavage of PARP when used together with TNF-α. Thus, honokiol could sensitize MCF-7 cells to TNF-α-induced cell death by inhibiting Nur77 expression. Honokiol-induced PARP cleavage also correlated with its inhibition of Nur77 expression in HepG2 hepatocellular carcinoma cells (Supporting Information Fig. S6), suggesting that the inhibitory effect of honokiol on Nur77 expression is not limited to MCF-7 cells. To further illustrate the role of Nur77 in mediating the killing activity of the honokiol/TNF-α combination, we examined caspase 3 activation in MCF-7 cells stably expressing Nur77. Immunostaining of cells by anti-cleaved caspase 3 antibody showed that overexpression of Nur77 suppressed the activation of caspase 3 by TNF-α combination with honokiol (Figure 4A). Ectopic expression of Nur77 also attenuated the effect of the honokiol/TNF-α combination at inducing PARP cleavage (Figure 4B). Moreover, the potent inhibitory effect of honokiol on the growth of the MCF-7 tumour in nude mice was compromised by Nur77 overexpression (Figure 4C and 4D).

Honokiol inhibits TNF-α induction of Nur77 expression by suppressing IKK and JNK

To study how honokiol inhibited the TNF-α induction of Nur77 expression, we examined the effect of different chemical inhibitors to determine the pathway involved. Treatment of cells with the IKK inhibitor Bay-11-7082 almost completely attenuated the effect of TNF-α at inducing Nur77 expression (Figure 5A), demonstrating that TNF-α activation of the NF-κB pathway is critical for its induction of Nur77

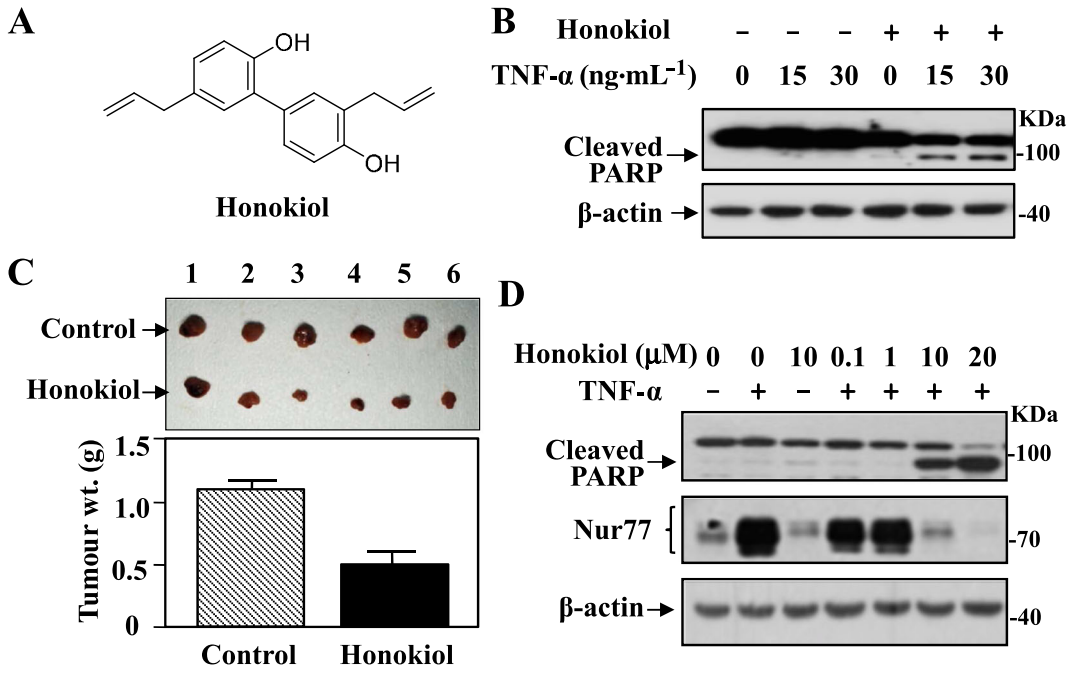

Figure 3

Honokiol sensitizes MCF-7 cells to TNF-α-induced apoptosis. A, chemical structure of honokiol. B, honokiol sensitizes cancer cells to TNF-α induction of apoptosis. MCF-7 cells treated with TNF-α (15 or 30 ng·mL^{-1}), honokiol (10 μM) or their combination for 6 h were analysed by Western blotting. Medium containing 0.1% dimethyl sulfoxide (DMSO) was used as vehicle control. C, honokiol inhibits tumour growth in animals. MCF-7 cells (10^7 cells) were inoculated into the left and right forelimb armpits of BALB/c nude mice. Mice were then randomly divided into two groups, which were fed with vehicle control or honokiol at a dose of 20 mg·kg^{-1} per day for 2 weeks. Tumours were harvested and weighed. Tumours excised from each animal are shown. The results were presented as mean ± SEM (n = 6), statistical significance versus control group, *$P < 0.01$. D, honokiol induction of apoptosis and inhibition of TNF-α-induced Nur77 expression. MCF-7 cells pretreated with the indicated concentration of honokiol for 2 h were exposed to TNF-α (10 ng·mL^{-1}) for 3 h. PARP cleavage and Nur77 expression were analysed by Western blotting. Medium containing 0.1% DMSO was used as vehicle control.

expression. Consistently, the proteasome inhibitor MG132 also inhibited TNF-α induction of Nur77 expression, probably due to its stabilization of IκBα, which in turn inhibits the NF-κB pathway. Interestingly, MG132 treatment also enhanced the basal Nur77 expression, suggesting that Nur77 expression might also be regulated through a proteasome-dependent pathway. TNF-α can also activate JNK, known to induce the expression of Nur77 (Li *et al.*, 1998; Kolluri *et al.*, 2003). Thus, treatment of MCF-7 cells with the JNK inhibitor SP600125, which inhibited JNK activation (Supporting Information Fig. S7), also inhibited TNF-α induction of Nur77 expression (Figure 5B). In contrast, the p38 MAPK inhibitor SB203580 had no effect. This was confirmed by data showing that a down-regulation of p38 MAPK expression by transfection of p38 MAPK siRNA had no effect on TNF-α induction of Nur77 expression (Figure 5C). These results demonstrated that TNF-α activation of NF-κB and JNK but not p38 MAPK mediates its induction of Nur77 expression. We next studied whether honokiol could interfere with the effect of TNF-α on activating the NF-κB and JNK pathways in MCF-7 cells. Treatment of MCF-7 cells with 10 ng·mL^{-1} TNF-α for 30 min resulted in strong activation of IKKβ and JNK and a reduction of IκBα expression (Figure 5D). When cells were pretreated with 10 μM honokiol for 1 h, the effects of TNF-α were completely suppressed. In addition, TNF-α induction of NF-κB transactivation measured by the NF-κB luciferase reporter assay was also inhibited by honokiol (Figure 5E). Thus, the potent inhibitory effect of honokiol on TNF-α induction of Nur77 mRNA expression in MCF-7 cells is largely attributed to its negative regulation of the NF-κB and JNK pathways.

Honokiol inhibits TNF-α signalling by interfering with TNFR1,s recruitment of RIPK1

TNF-α exerts its function through two distinct receptors, TNFR1 and TNFR2 (Mocellin and Nitti, 2008; Balkwill, 2009). TNFR1 appears to be the key mediator of TNF-α signalling in cancer cells (Mocellin and Nitti, 2008; Balkwill, 2009). To determine the role of TNFR1 in mediating TNF-α induction of Nur77 expression, we examine the effect of transfection of TNFR1 siRNA on the induction of Nur77 by TNF-α. Transfection of TNFR1 siRNA, which effectively inhibited the expression of TNFR1, completely impaired the inducing effect of TNF-α on Nur77 protein expression (Figure 6A), demonstrating that the expression of TNFR1 is necessary for the inducing effect of TNF-α.

TNF-α binding to TNFR1 recruits the adaptor protein TRADD, which serves as an assembly platform for the recruitment of other molecules, such as TRAF2 and RIPK1, for the activation of NF-κB and JNK (Mocellin and Nitti, 2008; Balkwill, 2009). We next studied whether the inhibitory effect

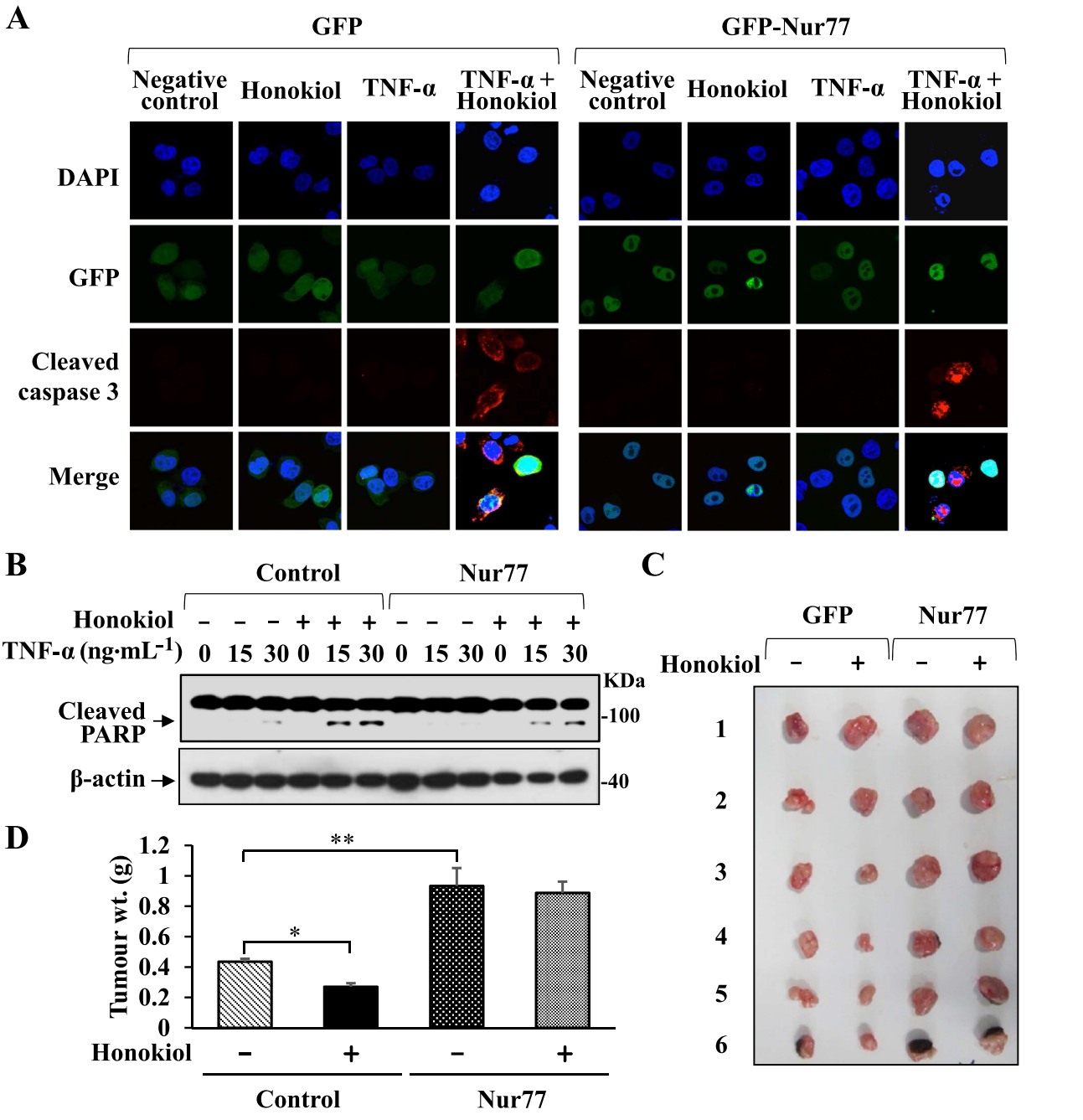

Figure 4

Transfection of Nur77 inhibits the effects of honokiol on inducing cancer cell apoptosis and growth. (A) Transfection of Nur77 inhibits caspase 3 activation by TNF-α/honokiol combination. MCF-7 stable clones expressing GFP-Nur77 or control GFP vector were treated with TNF-α, honokiol or their combination for 6 h. Caspase 3 activation was analysed by immunostaining with anti-cleaved caspase 3 antibody. Medium containing 0.1% DMSO was used as vehicle control. (B) Transfection of Nur77 inhibits PARP cleavage by TNF-α/honokiol combination. MCF-7 stable clones expressing Nur77 or control vector were treated with the indicated concentration of TNF-α in the presence or absence of honokiol (15 μM) for 12 h. PARP cleavage was analysed by Western blotting. Medium containing 0.1% DMSO was used as vehicle control. (C) Nur77 overexpression inhibits the antitumour effect of honokiol in animals. MCF-7 stable clones expressing Nur77 or control vector were inoculated into the left and right forelimb armpits of BALB/c nude mice. Mice were then randomly divided into two groups, which were fed with vehicle control or honokiol at a dose of 20 mg·kg^{-1} per day for 2 weeks. Tumours were harvested and weighed. The results are presented as mean ± SEM (n = 5 or 6), statistical significance versus control group, *$P < 0.01$. Tumours excised from each animal are shown (n = 6).

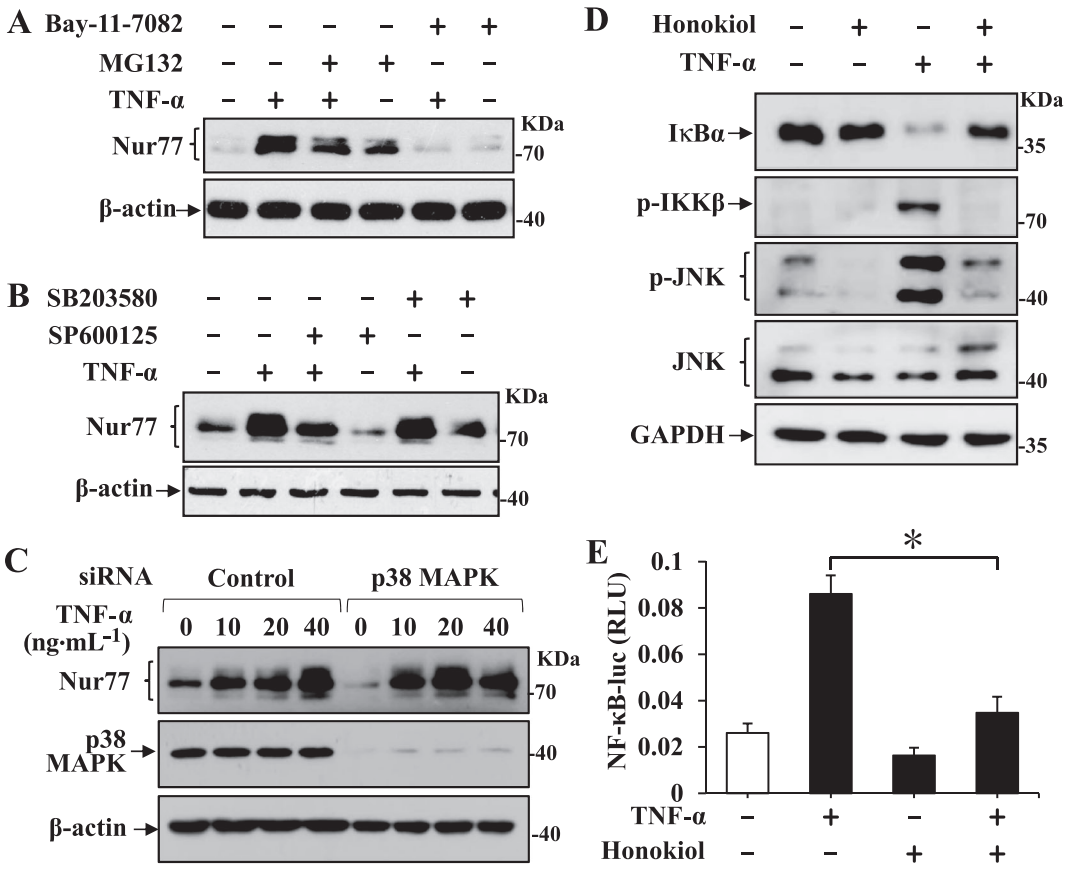

Figure 5

Honokiol inhibits TNF-α-induced Nur77 expression by suppressing its activation of IKK and JNK. (A and B) The effect of specific signalling pathway inhibitors. MCF-7 cells pretreated with vehicle control, MG132 (10 μM), Bay11-7082 (10 μM), SP600125 (10 μM) or SB203580 (10 μM) for 1 h were exposed to TNF-α (10 ng·mL^{-1}) for 3 h and analysed by Western blotting. (C) The effect of p38 MAPK down-regulation on Nur77 expression. MCF-7 cells transfected with p38 MAPK siRNA or control scramble siRNA were treated with vehicle control or TNF-α for the indicated time and analysed for the expression of Nur77 and p38 MAPK by Western blotting. (D) The effect of honokiol on TNF-α activation of IKK and JNK. Cells treated with vehicle control, TNF-α (10 ng·mL^{-1}) and/or honokiol (10 μM) for 1 h and analysed by Western blotting. (E) Honokiol inhibits TNF-α activation of NF-κB. MCF-7 cells transfected with the NF-κB luciferase reporter gene were treated with TNF-α (10 ng·mL^{-1}), honokiol (10 μM) or their combination for 12 h. Each transfection also included β-galactosidase for normalization of the luciferase activity. Medium containing 0.1% DMSO was used as vehicle control. RLU, relative luciferase unit.

of honokiol on the TNF-α signalling could be attributed to its regulation of the recruitment of adaptor proteins by TNFR1. The co-immunoprecipitation assay showed that immunoprecipitation of TNFR1 resulted in co-immunoprecipitation of TRAF2 and RIPK1 in cells treated with TNF-α (Figure 6B). When cells were co-treated with honokiol, the interaction of RIPK1 but not TRAF2 with TNFR1 was largely inhibited. These results demonstrated that inhibition of TNF-α signalling by honokiol was probably due to its interference of TNFR1 recruitment of RIPK1.

Discussion and conclusions

Our results demonstrated that TNF-α could strongly and rapidly induce the expression of Nur77 mRNA and protein in breast cancer cells (Figure 1), similar to its effect in macrophages (Pei *et al.*, 2006; Shao *et al.*, 2010). The role of inflammation-induced Nur77 has been well described in inflammatory cells, showing its critical role in macrophage activation and the development of atherosclerosis (Zhang, 2007; Pearen and Muscat, 2010; Lee *et al.*, 2011; McMorrow and Murphy, 2011; Deutsch *et al.*, 2012; Mohan *et al.*, 2012; To *et al.*, 2012; Kurakula *et al.*, 2014). In cancer cells, Nur77 is implicated in the regulation of survival and apoptosis (Zhang, 2007; Lee *et al.*, 2011; McMorrow and Murphy, 2011; Deutsch *et al.*, 2012; Mohan *et al.*, 2012; To *et al.*, 2012; Kurakula *et al.*, 2014). Several lines of evidence are presented here showing that TNF-α-induced Nur77 serves as a survival factor in breast cancer cells. TNF-α-induced Nur77 protein was mainly found in the nucleus (Figure 1C and D), while knocking down Nur77 enhanced the killing effect of TNF-α in MCF-7 cells (Figure 2A and B). Moreover, stable expression of Nur77 in MCF-7 cells promoted its growth in nude mice (Figure 2D and E). The survival function of Nur77 appears to be clinically relevant, as our results showed that Nur77 was overexpressed in tumour tissues than the corresponding tumour surrounding tissues (Figure 2F). These data are consistent with our previous observation that Nur77 is highly expressed in both

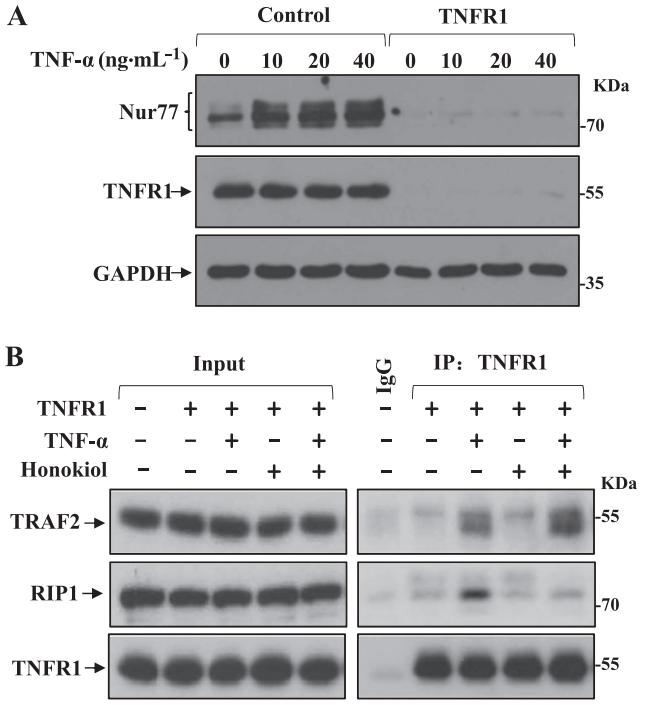

Figure 6

Honokiol inhibits TNFR1 recruitment of adaptor RIPK1 (RIP1). (A) TNFR1 is essential for TNF-α induction of Nur77 expression. MCF-7 cells transfected with TNFR1 siRNA or control scramble siRNA were treated with the indicated concentrations of TNF-α for 3 h and analysed for the expression of Nur77 and TNFR1 by Western blotting. (B) Honokiol inhibits TNF-α-induced TNFR1 interaction with RIPK1 (RIP1). MCF-7 cells treated with honokiol for 1 h before being exposed to TNF-α for 15 min. Interaction of TNFR1 with TRAF2 and RIPK1 were analysed by co-immunoprecipitation assay using anti-TNFR1 antibody. Immuniprecipitates were analysed for the presence of TRAF2 and RIPK1 by Western blotting. Medium containing 0.1% DMSO was used as vehicle control.

hormone-dependent and hormone-independent breast cancer cell lines (Wu *et al.*, 1997a). Interestingly, all nine patients with elevated Nur77 expression in their tumour tissues were diagnosed with triple negative breast cancer (not shown), demonstrating an association between Nur77 overexpression and the development of the malignant disease. A recent profiling of the nuclear receptor superfamily placed Nur77 as one of the five nuclear receptors that are overexpressed in breast tumours, representing a nuclear receptor signature with discriminant and prognostic value for the disease (Muscat *et al.*, 2013). How Nur77 exerts its survival function in breast cancer cells remains to be determined. It has been shown that Nur77 could enhance the expression of IKK (Pei *et al.*, 2006) or directly modulate NF-κB activity (de Leseleuc and Denis, 2006) to up-regulate the expression of antiapoptotic genes. However, we did not observe any effect of ectopic Nur77 expression on IκBα expression in the absence or presence of TNF-α (not shown), arguing against its effect on IKK activity in breast cancer cells. A recent study demonstrated that Nur77 strongly promoted TGFβ signalling in breast cancer cells by interacting with SMAD7 (Zhou *et al.*, 2014), providing a plausible mechanism for the oncogenic effect of Nur77 in breast cancer.

TNF-α can trigger either cell survival or cell death signalling (Mocellin and Nitti, 2008; Balkwill, 2009). However, its killing effect is often attenuated in cancer cells due to its activation of survival signalling such as the NF-κB pathway. Thus, the balance of TNF-α-induced survival-signalling and death-signalling is pivotal in determining the fate of TNF-α-responding cells (Mocellin and Nitti, 2008; Balkwill, 2009). Our results showing that knocking down Nur77 enhanced the killing effect of TNF-α (Figure 2A and B) suggested that Nur77 represents one of the important factors that mediate the survival function of TNF-α. Thus, targeting Nur77 may represent a new strategy to convert TNF-α signalling from survival to death. Several classes of small molecules that target Nur77 have demonstrated therapeutic activities against breast cancer. Retinoid X receptor (RXR)-selective retinoids inhibited the growth of breast cancer cells by inducing the expression of RARβ through RXR/Nur77 heterodimer (Wu *et al.*, 1997a). The atypical retinoid AHPN (also called CD437) (Dawson *et al.*, 2001) and the octaketide cytosporone B (Zhan *et al.*, 2008) could induce cancer cell apoptosis by inducing Nur77 nuclear export and mitochondrial targeting, while 1,1-bis(3-indolyl)-1-(p-substituted phenyl) methanes induced apoptosis of MCF-7 cells by modulating Nur77 transactivation function (Chintharlapalli *et al.*, 2005). Significantly, 6-mercaptopurine, an activator of Nur77, is currently in phase II clinical trials for advanced breast cancer (Nicum *et al.*, 2014).

An important finding reported in the current study is that honokiol could target Nur77 to sensitize MCF-7 cells to the death effect of TNF-α. Although honokiol did not show any apparent apoptotic effect, its combination with TNF-α strongly induced apoptosis of MCF-7 cells (Figure 3B). Similar to previous reports (Liu *et al.*, 2008; Tian *et al.*, 2013; Avtanski *et al.*, 2014), honokiol potently inhibited the growth of MCF-7 cells in nude mice (Figure 3C), probably due to its effect on TNF-α produced *in vivo*. Our results that induction of PARP cleavage by honokiol/TNF-α combination was associated with inhibition of TNF-α-induced Nur77 expression (Figure 3D) and that the apoptotic (Figure 4A and B) and the antitumour (Figure 4C) effects of the combination were blunted by Nur77 overexpression further confirmed the survival function of Nur77. These results also suggested an effective way of inducing cancer cell apoptosis by inhibiting Nur77 expression. Honokiol has been shown to synergistically induce apoptosis of breast cancer cells when combined with other anticancer agents, including adriamycin (Hou *et al.*, 2008), the HER-2 inhibitor lapatinib and the mTOR inhibitor rapamycin (Liu *et al.*, 2008) and etoposide (Tian *et al.*, 2013). The ability of honokiol to inhibit Nur77 expression may contribute to its sensitizing effect in breast cancer cells, which remains to be studied.

By using specific signalling pathway inhibitors, we found that inhibition of TNF-α activation of IKK and JNK largely inhibited TNF-α induction of Nur77 expression (Figure 5A and B), demonstrating that both NF-κB and AP-1 are involved. Our results also showed that honokiol was an effective inhibitor of TNF-α activation of IKK and JNK (Figure 5D), revealing a mechanism for its suppression of TNF-α-induced Nur77 expression. Recent studies have demonstrated that honokiol is a potent anti-inflammatory agent through undefined mechanism (Tse *et al.*, 2005; Vaid *et al.*, 2010; Li *et al.*, 2011; Tian *et al.*, 2013). Our observation that honokiol inhibited TNFR1 interaction with RIPK1 (Figure 6B)

suggested that the anti-inflammatory effect of honokiol might involve the interference of TNFR1 recruitment of its adaptor proteins. Our current finding that honokiol in combination with TNF-α-induced apoptosis of MCF-7 cells by inhibiting Nur77 expression also revealed a critical role of Nur77 in bridging inflammation and cancer.

In summary, our results demonstrate that TNF-α strongly induces Nur77 expression in breast cancer cells, which in turn acts to mediate its survival function. Our studies also identify honokiol as a negative regulator of TNF-α induction of Nur77 expression. With its ability to sensitize breast cancer cells to the death effect of TNF-α and proven human safety profile, honokiol represents a promising agent for breast cancer therapy that warrants further clinical development.

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Author contributions

X-k. Z. and Q. C. C. conceived and designed the experiments. L. X., F. J., X. Z., G. A., X. S., A. R., J. W., L. C. and Y. Z. developed the methodology. L. X., F. J., X. Z., G. A., X. S., M. M., Y. X., A. R., J. W., L. C. and Y. Z. collected the data. L. X., F. J., X. Z., G. A., X. S., A. R., J. W., L. C., Y. Z., Q. C. C., X-k. Z., Y. X., J. L., Z. Z., G. W. and H. Z. analysed and interpreted the data. Q. C. C., X-k. Z., L. X. and F. J. wrote and reviewed the manuscript. X-k. Z. and Q. C. C. supervised the study.

Conflict of interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13375

Figure S1 Chromatographic analysis for the purity of honokiol. The purified product was injected into HPLC in a Shimadzu LCsolution system with PDA detector and a Syncronis C18 column (φ4.6 mm × 25 cm), eluting with methanol-H_2O (78:22) at the flow rate of 1 ml·min^{-1} and detecting at 294 nm.

Figure S2 Effect of actinomycin D, cycloheximide, and honokiol on TNF-α induction of Nur77 expression. (A) Effect of actinomycin D. MCF-7 cells pretreated with actinomycin D (0.5 μg·ml^{-1}) for 1 h were exposed to TNF-α (10 ng·ml^{-1}) and/or honokiol (10 μM) for 3 h, and the expression of Nur77 was analysed by Western blotting. (B) Effect of cycloheximide. MCF-7 cells pretreated with cyclohexlmide (20 μM) for 1 h were exposed to TNF-α (10 ng·ml^{-1}) and/or

honokiol (10 μM) for 3 h. Medium containing 0.1% DMSO was used as vehicle control. The expression of Nur77 was analysed by Western blotting.

Figure S3 Expression of Nur77 in human breast tumor tissues (T) and the corresponding tumour surrounding tissues (NT). Tumour tissues and the corresponding tumour surrounding tissues from 13 breast cancer patients were analysed for the expression of Nur77 and RXRα by Western blotting.

Figure S4 Induction of Nur77 expression by TNF-α and its suppression by honokiol in MDA-MB231 breast cancer cells. MDA-MB231 cells pretreated with honokiol (10 μM) for 1 h were exposed to TNFα (10 ng·ml^{-1}) for 3 h. Medium containing 0.1% DMSO was used as vehicle control. Expression of Nur77 was analysed by Western blotting.

Figure S5 Differential effect of honokiol on Nur77 expression induced by TNF-α and IL-1β in cancer cells. HeLa (A) and MCF-7 (B) cells were pretreated with or without honokiol (10 μM) in for 1 h before exposed to TNF-α (10 ng·ml^{-1}) or IL-1β (10 ng·ml^{-1}) for another 3 h. Medium containing 0.1% DMSO was used as vehicle control. The expression of Nur77 was analysed by Western blotting. The results showed that honokiol failed to inhibit IL-1β-induced Nur77 expression in both cell lines, while TNF-α-induced Nur77 expression was inhibited.

Figure S6 Induction of apoptosis by honokiol is associated with its inhibition of Nur77 expression in HepG2 liver cancer cells. HepG2 cells were treated with honokiol (10 μM) for the indicated time. Medium containing 0.1% DMSO was used as vehicle control. PARP cleavage and Nur77 expression were analysed by Western blotting.

Figure S7 Characterization of chemical inhibitors. (A) Effect of Bay-11-7082 and MG132 on TNF-α-induced IKKβ phosphorylation and IκBα degradation. MCF-7 cells pretreated with MG132 (10 μM) or Bay11-7082 (10 μM) for 1 h were exposed to TNF-α (10 ng·ml^{-1}) for another 30 min. The expression of p-IKKβ and IκBα was analysed by Western blotting. (B) Effect of SB203580 and SP600125 on phosphorylation of JNK and p38 MAPK. MCF-7 cells pretreated with SB203580 (10 μM) and SP600125 (10 μM) for 1 h were exposed to TNF-α (10 ng·ml^{-1}) for another 30 min. The expression of p-JNK and P-p38 MAPK was analysed by Western blotting. Medium containing 0.1% DMSO was used as vehicle control.