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Eupatorium lindleyanum DC. sesquiterpene fraction F1012-2 regulates p53/NF-κB signaling pathways in human breast cancer

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Abstract: F1012-2, a novel sesquiterpene lactone isolated from the Chinese herbal medicine *Eupatorium lindleyanum* DC, exhibits an antitumor effect. In this study, we investigated the anticancer activities of F1012-2 on ten human breast cancer lines and demonstrated significantly lower IC_{50} values for triple-negative breast cancer (TNBC) than for non-TNBC cell lines. The transcription factors p53 and nuclear factor-κB (NF-κB) are important regulators of tumorigenesis. F1012-2 not only depleted mutant p53, but also activated wild-type p53. F1012-2 reduced the expression of phosphorylated p65 and p105 NF-κB family members and coregulated p53, NF-κB members and their dependent targets. To further clarify the key role of p53, lentivirus small hairpin RNA (shRNA) infection was used to knockdown p53 in MDA-MB-231 cells. F1012-2 significantly reduced the inhibitory effect on cell proliferation and apoptosis, while the levels of p53, NF-κB family members and their dependent genes were not significantly different. F1012-2 exhibited a significant antitumor effect and reduced the expression of p53 in MDA-MB-231 xenografts. Taken together, our results show that F1012-2 exhibited an inhibitory effect on TNBC and affected the regulation of p53/NF-κB signaling pathways.

Keywords: *Eupatorium lindleyanum* DC; F1012-2; triple-negative breast cancer; p53; nuclear factor-κB (NF-κB)

INTRODUCTION

Breast cancer (BC) remains the prevalent cancer-related cause of disease burden for women [1]. It can be classified into unique molecular subtypes based on protein expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2) [2]. BC that lacks expression of ER, PR and Her2 are classified as triple negative (TN), accounting for nearly one-fifth of cases, and result in poor clinical outcomes [3].

Nuclear transcription factor NF-κB is involved in inflammatory processes in various diseases from cancer to innate immunity [4]. The NF-κB family is comprised of p65 (RelA), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52), while p105 is cleaved to form p50 [5]. Protein p53, the "guardian of the genome", plays a central role in tumor suppression through the transcriptional regulation of its downstream targets [6]. p53 is frequently mutated during carcinogenesis, and in at least 80% of TNBC [7]. Wild-type and mutant p53 have opposing functions, with wild-type p53 suppressing cancer development, whereas mutant p53 promotes cancer by acting with other oncogenes [8]. Recently, mutant p53 has become an attractive target for cancer therapy. Replicative stress and DNA damage were shown to lead to the coregulation of p53 and NF-κB through crosstalk between these transcription factors [9]. Wild-type p53 can positively regulate NF-κB-dependent anti-apoptotic genes, such as B-cell lymphoma-extra large (Bcl-xL) protein. As a powerful oncogene, mutant p53 can activate NF-κB, thus promoting tumor formation independent of external stimulation, enhancing the anti-apoptotic activity of NF-κB [10].

Eupatorium lindleyanum DC. (called by local residents in China "Ye-Ma-Zhui"), has been reported to possess antiinflammatory [11], antioxidant [12] and antitumor activities [13]. F1012-2 is a sesquiterpene lactone isolated from the Chinese herbal medicine *Eupatorium lindleyanum* DC. Previously we found that F1012-2 exhibited a significant anticancer effect and induced cell cycle arrest, apoptosis and ROS-mediated

DNA damage [14]. To elucidate the mechanism of F1012-2 action, we further investigated its effect on the crosstalk between p53 and NF-κB signaling pathways. For this purpose, we (i) systematically investigated the anticancer effect of F1012-2 *in vitro* and *in vivo*; (ii) detected the expression levels of p53, NF-κB and their members and targets; (iii) explored the co-expression of p53 and NF-κB target genes.

MATERIALS AND METHODS

Ethics statement

Animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University and were carried out in accordance with the guidelines of the Laboratory Animal Research Center of Zhejiang Chinese Medical University.

Cell culture and reagents

Human breast cancer cell lines were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology, China Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12), supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA). Cells were grown with 5% CO_2 at 37°C in a humidified atmosphere. F1012-2 was provided by Dr. Yang Bo (Zhejiang Chinese Medical University, Hangzhou, China). F1012-2 is comprised of three compounds, eupalinolide G, eupalinolide I and eupalinolide J. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 7-AAD kit and Annexin V-FITC apoptosis detection kit were purchased from BD Biosciences (NY, USA). The primary antibodies were p53 (#2527 and #2524), p73 (#14620), BCL2 associated X protein (BAX; #5023), Bcl-xL (#2764), NF-κB p65 (#8242), phospho-NF-κB p65 (p-p65, #3033), NF-κB1 p105 (#4717) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #5174) (Cell Signaling Technology, Danvers, MA, USA), NF-κB1 p50 (#354R-100) (BioVision, California, USA), anti-p53 (Ab-3) (mutant) mouse mAb (PAb240; OP29L) and Pab1620 (OP29) (Merck Millipore, Burlington, MA, USA). The membranes were incubated with secondary antibody of goat anti-mouse IgG (immunoglobulin)-horseradish peroxidase (HRP; #43593), goat anti-rabbit IgG-HRP (#98164) (Cell Signaling Technology, Danvers, MA, USA), goat anti-mouse IgG-FITC (sc-2010) (Santa Cruz Biotech, CA, USA) or goat anti-rabbit IgG Texas Red (ab6719) (Abcam, Cambridge, UK) at room temperature for 2 h. Chemiluminescent detection was performed by electrochemiluminescence (ECL; Bio-Rad, CA, USA).

Cell viability assay

The viability of cells was measured by the MTT assay [15]. The cells were seeded on a 96-well plate (3×10^3 cells per well) to allow attachment, and incubated overnight in a medium containing 10% FBS at 37°C. The cells were treated with the indicated concentrations of F1012-2 for 72 h. MTT solution (5 mg/mL) was added to each well for 4 h and formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm with a cell imaging multi-mode reader (BioTek, CA, USA). The IC_{50} values of F1012-2 for each cell line were calculated by GraphPad Prism 5.0 software (San Diego, CA, USA).

Western blotting analysis

Western blotting was carried out as described previously [14,16]. Cells were seeded on 6-well culture dishes (8×10^5 cells/dish) and treated with the indicated concentrations of F1012-2 for 24 h. Cells were harvested, washed twice with ice-cold PBS, lysed by incubation in RIPA buffer for 30 min on ice, and then centrifuged at 16100 ×g at 4°C for 15 min. Supernatants were collected, and the bicinchoninic acid (BCA) method for protein quantification was used. Equal amounts of denatured proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), blocked with 5% non-fat milk at room temperature for 1 h and incubated with the respective specific primary antibodies overnight at 4°C. The membranes were washed three times with Tris-buffered saline with 5% Tween-20 (TBST) solution and incubated with a horseradish peroxidase

(HRP)-conjugated secondary antibody, goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP at room temperature for 2 h. Chemiluminescent detection was performed by ECL (Bio-Rad, California, USA).

Immunofluorescence Staining

After exposure to F1012-2 for 24 h, the cells were fixed with 4% paraformaldehyde and permeabilized in 0.5% Triton X-100. After blocking with 2% BSA, p53 and Bcl-xL antibodies were incubated overnight. The cells were incubated with secondary antibody goat anti-mouse IgG-FITC or goat anti-rabbit IgG Texas Red for 1 h on the second day. Finally, 4′, 6-diamidino-2-phenylindole (DAPI) staining for 10 min and direct photography were performed [17].

p53 knockdown experiments

shRNAs for p53 were obtained from Genechem (Shanghai, China) and used to package into lentivirus. The target sequences of p53-shRNA were GCGCACAGAGGAAGAGAATCT and CTGGAAGACTCCA-GTGGTAAT. The breast cancer cells were transfected with lentivirus-shRNA targeting p53, and the cells were treated with F1012-2.

Annexin V-FITC/PI double staining assay

Apoptotic cell death in TNBC cells was quantified by flow cytometry using an apoptosis detection kit (Becton Dickinson, NJ, USA). The assay was performed as previously described [13].

***In vivo* animal study**

The animal study was carried out as described [13] . MDA-MB-231 cells (2×10^6 cells in 100 μL DMEM/F12 medium) were injected subcutaneously into 4- to 5-week-old BALB/c female mice (Shanghai Experimental Animal Center, Shanghai, China) and randomly divided into 2 groups. When the tumor volume reached 50 mm^3, the control group received an intraperitoneal (i.p.) injection of vehicle control (CremophorEL:ethanol:saline=5:5:90) every second day and the treatment group received 15 mg/kg and 25 mg/kg of F1012-2 (i.p.) on alternate days. The tumor size was measured every other day, and the formula used to calculate the tumor volume was: volume=($width^2\times length$)/2. After 20 days, the mice were killed, and the tumors were isolated for histological analysis.

Immunohistochemical assay

The formalin-fixed and paraffin-embedded tumor tissue samples were cut into 5-μ-thick sections. After the slices were baked, deparaffinized and rehydrated, they were incubated in hydrogen peroxide for 10 min. Subsequently, the slides were incubated with the p53 primary antibody at 4°C. The next day, the slides were incubated with HRP-labeled polymer. Finally, the tumor tissue sections were stained with 3,3'-diaminobenzidine (DAB; ZSGB-BIO, Beijing, China), counterstained with hematoxylin, dehydrated and covered with a cover glass.

Statistical analysis

All data are expressed as the mean±SD from at least three independent experiments. Statistical significance was analyzed with Student's t-test. The difference between the means was statistically significant if $P<0.05$.

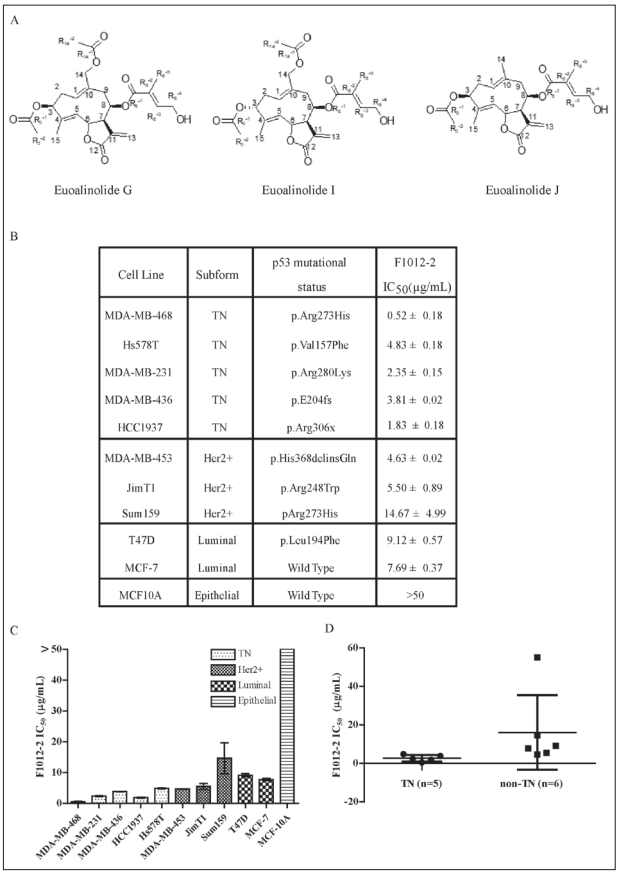
RESULTS

F1012-2 exhibited a better inhibitory effect on TNBC cells

The MTT assay was used to evaluate the anticancer activities of F1012-2 on human breast cell lines, including TN, Her2+, luminal and epithelial breast cells. As shown in Fig. 1, IC_{50} values ranged from 0.52-4.83 μg/mL for TNBC cells, from 4.63-17.05 μg/mL for other breast cancer cells, and ≥50 μg/mL for the non-tumor breast cell line (MCF 10A).

F1012-2 reversely regulated the protein expression of p53

To verify the effect of F1012-2 on the regulation of p53 expression in human breast cancer cells, Western blotting analysis was performed. F1012-2 downregulated the expression of mutant p53 protein in MDA-MB-231 (R280K) and MDA-MB-468 (R273H) cell



Cell Line	Subform	p53 mutational status	F1012-2 IC_{50}(μg/mL)
MDA-MB-468	TN	p.Arg273His	0.52 ± 0.18
Hs578T	TN	p.Val157Phe	4.83 ± 0.18
MDA-MB-231	TN	p.Arg280Lys	2.35 ± 0.15
MDA-MB-436	TN	p.E204fs	3.81 ± 0.02
HCC1937	TN	p.Arg306x	1.83 ± 0.18
MDA-MB-453	Her2+	p.His368dclinsGln	4.63 ± 0.02
JimT1	Her2+	p.Arg248Trp	5.50 ± 0.89
Sum159	Her2+	pArg273His	14.67 ± 4.99
T47D	Luminal	p.Leu194Phe	9.12 ± 0.57
MCF-7	Luminal	Wild Type	7.69 ± 0.37
MCF10A	Epithelial	Wild Type	>50

Fig. 1. Effect of F1012-2 on the growth of breast cell lines. **A** – Chemical structures of three F1012-2 compounds eupalinolide G, eupalinolide I and eupalinolide J. **B** – Breast cell lines used in this investigation, their molecular subtype, p53 mutational status, and IC_{50} values±SD for F1012-2. **C** – Mean IC_{50} values±SD were calculated using GraphPad Prism software following the MTT assay. **D** – Scatter-plot representation of the relationship between F1012-2 IC_{50} values and breast cell lines. Data points represent the mean of three independent experiments. TN – triple-negative.

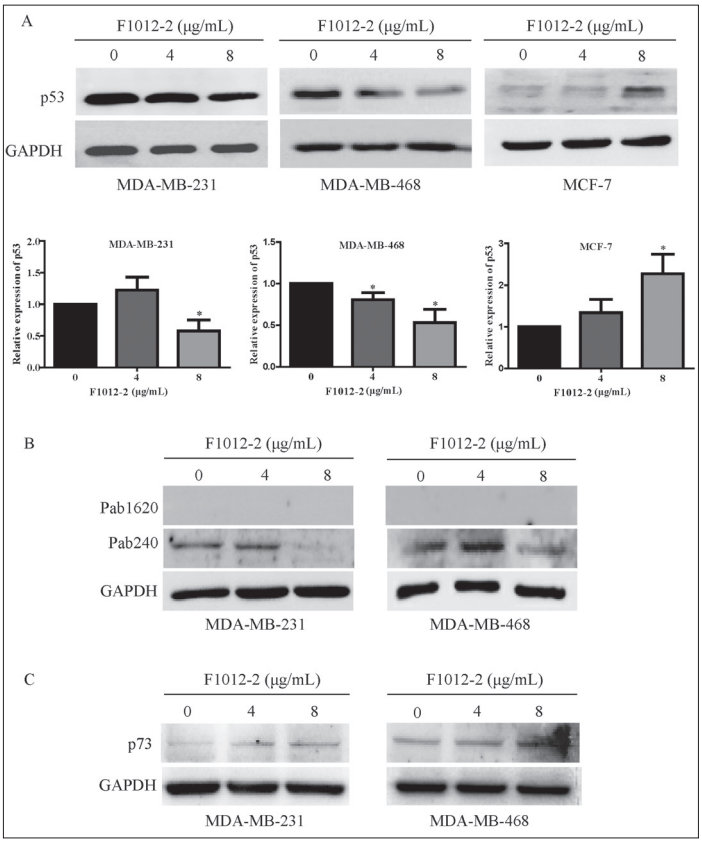


Fig. 2. Effect of F1012-2 treatment on the levels of mutant p53, wild-type p53 and p73. **A** – F1012-2 reversed the expressions of mutant p53 and wild-type p53 in human breast cancer cells. **B** – Pab1620 was used to detect wild-type p53, and Pab240 to detect mutant p53 in TNBC cells. **C** – The expression levels of p73 detected in TNBC cells. Cells were treated with F1012-2 for 0, 4, and 8 μg/mL.

lines and upregulated the expression of wild-type p53 protein in MCF-7 cancer cells (Fig. 2A). The p53 conformation-specific antibody Pab240 was used to detect mutant p53, and Pab1620 to detect wild-type p53, as described [18]. As can be seen in Fig. 2B, although the expression of mutant p53 protein was significantly reduced in the p53-mutated cell lines MDA-MB-231 and MDA-MB-468, the expression of wild-type p53 protein was not increased. Finally, we detected the expression level of p73, one of the p53 family members that has a high degree of structural and functional similarity with p53, in p53-mutated cell lines after treatment with F1012-2. Our results indicated that there was no obvious change of p73 (Fig. 2C).

F1012-2 regulated the NF-κB signaling pathway

It was reported that p53 and NF-κB coregulate processes in replicative stress and DNA damage [9]. Additionally, mutant p53 can activate the NF-κB signaling pathway by enhancing anti-apoptotic activity, and wild-type p53 can positively regulate NF-κB-dependent anti-apoptotic genes, such as Bcl-xL. We therefore investigated the effect of F1012-2 on the NF-κB signaling pathway [10]. Western blotting showed that F1012-2 significantly decreased the expression levels of the NF-κB family members p-p65 and p105 and had no effect on the expression of p65 and p50 (Fig. 3A). F1012-2 increased the expression levels of BCL-XL (an anti-apoptotic protein) and BAX (a pro-apoptotic protein) (Fig. 3B).

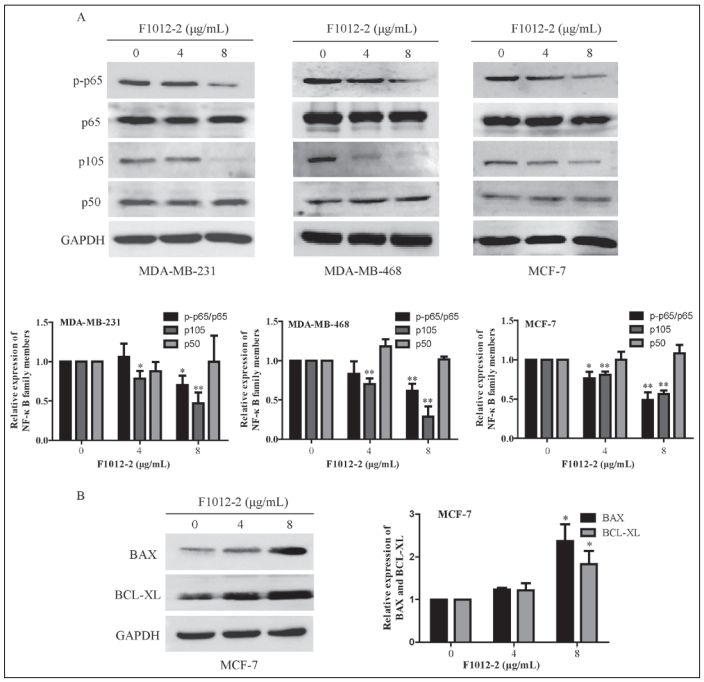


Fig. 3. Effect of F1012-2 treatment on the expression of NF-κB family members (**A**), and BAX and Bcl-xL (**B**).

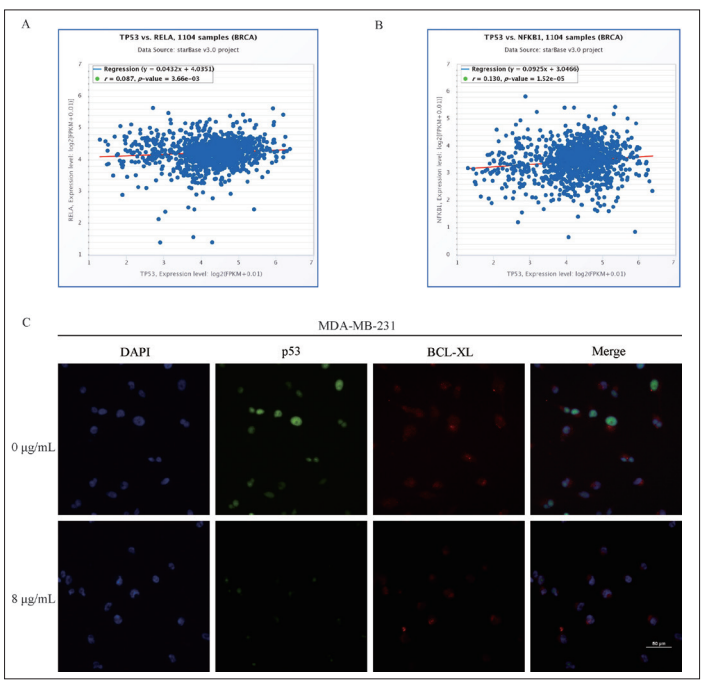


Fig. 4. Co-expression of p53 and NF-κB family members. **A, B** — Results obtained using the ENCORI Pan-Cancer Analysis Platform. **C** – Immunofluorescence assay used to detect the co-expression levels of p53 and Bcl-xL.

F1012-2 regulated the co-expression of p53 and NF-κB signaling pathways

The co-expression of NF-κB members and p53 was analyzed by the ENCORI Pan-Cancer Analysis Platform. As shown in Fig. 4A and B, there was a correlation between p53 and NF-κB family members p65 and p105/p50. Then, we detected the expression levels of p53 and Bcl-xL using an immunofluorescence assay that revealed that F1012-2 led to joint accumulation of p53 and Bcl-xL in MDA-MB-231 cells (Fig. 4C).

F1012-2 targeted p53 in inducing breast cancer cell death

To further confirm the role of p53 in F1012-2-induced cell death, p53 shRNA was introduced to block p53 in MDA-MB-231 cells. As shown in Fig. 5A, the expression of p53 protein was successfully inhibited after lentivirus transfection of p53-shRNA (shp53). The cells of the negative control (NC) and shp53-16 and shp53-17 were treated with F1012-2 for 24 h and 48 h, and cell viability was estimated. Cell viability in p53 knockdown cells was found to be higher than in NC cells after F1012-2 treatment (Fig. 5B). To confirm whether the effect of F1012-2 on cell apoptosis was p53-dependent, annexin V-FITC/7AAD staining was used and results were obtained by flow cytometry analysis. The number of apoptotic cells was increased in NC cells while there was no significant increase in shp53-16 cells (Fig. 5C).

F1012-2 showed minor regulation of the NF-κB signaling pathway after p53 knockdown

After p53 knockdown, Western blotting showed that there was no obvious expression change of BAX, NF-κB members and target Bcl-xL in shp53-16 in response to F1012-2 treatment (Fig. 6).

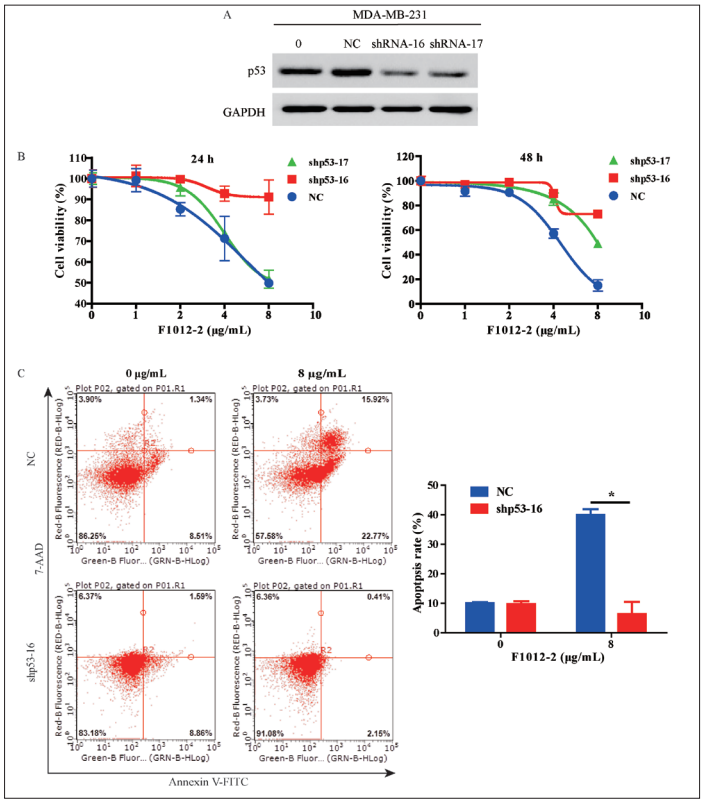


Fig. 5. The role of p53 in F1012-2-induced cell apoptosis of breast cancer. **A** – Expression of p53 after transfection of shp53. **B** – Viability of MDA-MB-231 cells treated with F1012-2 detected by the MTT assay after transfection with shp53. **C** – NC and shp53 MDA-MB-231 cells stained with Annexin V-FITC/7AAD and analyzed for fluorescence by flow cytometry after treatment with F1012-2. Data are expressed as the mean±SD, * P<0.05 vs. NC. NC – negative control.

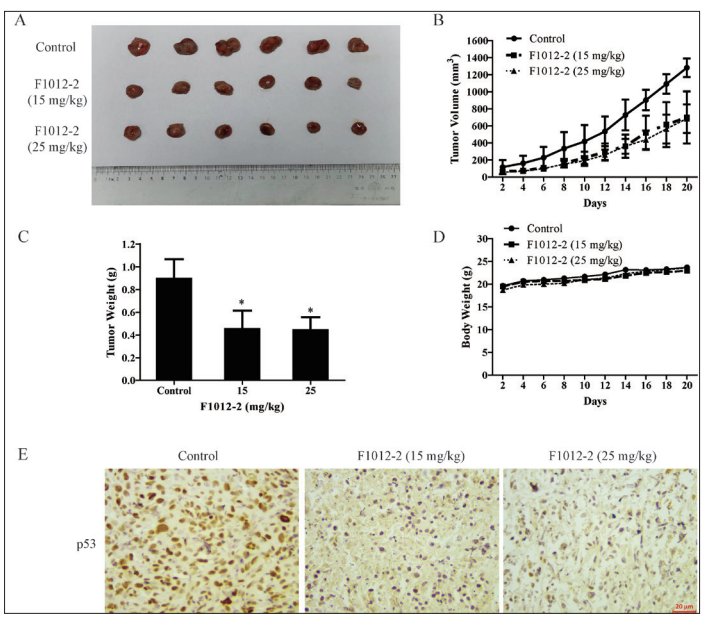


Fig. 7. F1012-2 inhibited breast cancer growth *in vivo*. **A** – Tumor images. **B** – Tumor sizes. **C** – Tumor weights. **D** – The average body weight of mice with xenografts. **E** – Immunohistochemistry was performed to detect the expression of p53 in tumor tissues. * P<0.05 vs. Control. Data are expressed as the mean±SD.

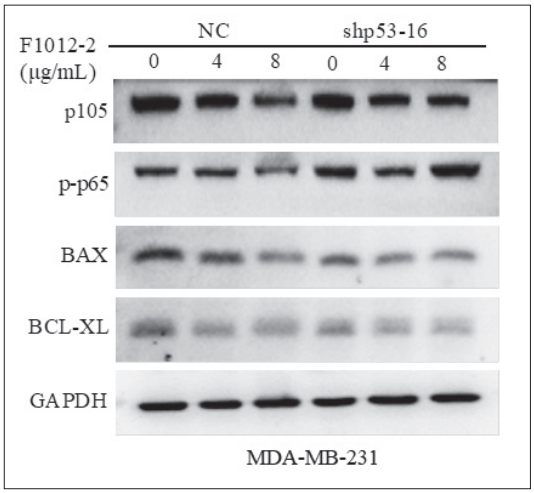


Fig. 6. Expression levels of BAX, NF-κB members and target Bcl-xL in response to F1012-2 treatment by p53 knockdown.

F1012-2 suppressed tumor growth in the mouse xenograft tumor model and regulated the expression of p53

Consistent with our previous results, after treatment with 15 mg/kg and 25 mg/kg of F1012-2 for 20 days, tumor growth was significantly inhibited (Fig. 7A-C). In addition, there was no observed sign of toxicity, as judged by parallel monitoring of body weight (Fig. 7D). Furthermore, the level of p53 in the tumor tissues of F1012-2 treatment was significantly decreased compared to the control group, as detected by immunohistochemistry and in accordance with the *in vitro* results (Fig. 7E).

DISCUSSION

In our previous study, we reported that F1012-2, a sesquiterpene lactone isolated from the Chinese herbal medicine *Eupatorium lindleyanum* DC, exhibits anticancer effects [13]. In the present study, we investigated the anticancer activities of F1012-2 in human breast cell lines, including TN, Her2+, luminal and epithelial breast cells. Our results indicated that the IC_{50} values were significantly lower for TNBC than for non-TNBC cell lines after F1012-2 treatment, which suggested that the

TNBC cell lines possess greater sensitivity to F1012-2. Moreover, we conducted xenograft tumor studies and found that F1012-2 also significantly suppressed the growth of MDA-MB-231-driven tumors, consistent with our previous study. These results shed new light for further investigation of the mechanism of F1012-2 as an anticancer agent.

p53 is the most frequently mutated gene in breast cancer, especially in TNBC [19]. The gain-of-function oncogenic activity of mutant p53 correlates with higher rates of metastasis and poor survival; therefore, mutant p53 may be a potential target for TNBC treatment. Our results indicated that F1012-2 regulated mutant p53 and wild-type p53 in human breast cancer cells. p53 is a tumor suppressor that loses its tumor suppressive function after mutation. The main consequence of p53 mutations is a change in protein conformation. However, there was no mutant conformational change of p53 protein after treatment with F1012-2 in breast cancer cells. p73, as one of p53 family members, has a high degree of structural and functional similarity with p53, which makes it a potential target of interest in cancers lacking wild-type p53 [20]. Our results indicated that there was also no change in p73 expression level. These results implied that F1012-2 might mediate other signaling pathway that regulate p53.

NF-κB is a critical regulator of immunity, inflammation, apoptosis and metastasis [21-23]. Together with DNA damage, response factors including tumor suppressor p53 and NF-κB become activated [24]. We previously reported that F1012-2 induced DNA damage in TNBC [14]. In the present study, F1012-2 not only regulated the expression of p53, but also significantly decreased the expression levels of NF-κB family members p-p65 and p105 and had no effect on the expression of p65 and p50. These results indicated that F1012-2 coregulated p53 and NF-κB signaling pathways, thus providing multiple targets for therapeutic intervention in TNBC. Furthermore, the B cell lymphoma-2 (Bcl-2) family is a pivotal arbiter of mitochondria-mediated apoptosis and can be divided into anti-apoptotic members that include Bcl-2 and BCL-XL and pro-apoptotic member such as BAX [25]. Consistent with the literature, we verified that F1012-2 increased the expression level of Bcl-xL and decreased the expression level of BAX in TNBC cells. It was reported that co-activated NF-κB and p53 modulate BAX (a p53 target)/Bcl-xL (a NF-κB target) expression [26-28]; we therefore hypothesized that F1012-2 inhibited the growth of breast cancer through a signal network of NF-κB and p53, as suggested by the results obtained herein. Interestingly, we found a correlation between p53 and NF-κB family members p65 and p105/p50 using the ENCORI Pan-Cancer Analysis Platform; furthermore, F1012-2 caused joint accumulation of p53 and Bcl-xL in MDA-MB-231 cells, as seen in the immunofluorescence assay. These results indicated that F1012-2 regulated crosstalk between p53 and NF-κB signaling pathways. To further confirm the important role of p53 in F1012-2-induced cell death, p53 shRNA was introduced to block p53 in MDA-MB-231 cells. After p53 knockdown, cell viability was found to be higher and there was no significant increase of apoptotic cells, suggesting that p53 appears to be the target of F1012-2. Moreover, there was no obvious expression change of BAX, NF-κB members and targets in p53 knockdown cells. Collectively, these results indicated that F1012-2 regulated the p53/NF-κB signaling pathways in human breast cancer.

In conclusion, the presented findings demonstrate that the *E. lindleyanum* DC sesquiterpene fraction F1012-2 exhibits an inhibitory effect on TNBC cell lines. The potential mechanism of F1012-2 may be by regulating the crosstalk between p53 and NF-κB signaling pathways. In this study, we evaluated the role of p53 on the NF-κB signaling pathway by blocking p53. In future, further investigation is needed to confirm the role of the NF-κB signaling pathway.

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Conflicts of interest disclosure: The authors declare that they have no conflicts of interest.

Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Wang%20et%20al_7883_Data%20Report.pdf

REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. CA Cancer J Clin. 2022;72(1):7-33. https://doi.org/10.3322/caac.21708.
2. McCarthy AM, Friebel-Klingner T, Ehsan S, He W, Welch M, Chen J, Kontos D, Domchek SM, Conant EF, Semine A, Hughes K, Bardia A, Lehman C, Armstrong K. Relationship of established risk factors with breast cancer subtypes. Cancer Med. 2021;10(18):6456-67. https://doi.org/10.1002/cam4.4158.
3. Kwapisz D. Pembrolizumab and atezolizumab in triple-negative breast cancer. Cancer Immunol Immunother. 2021;70(3):607-17. https://doi.org/10.1007/s00262-020-02736-z.
4. Poma P. NF-κB and Disease. Int J Mol Sci. 2020;21(23):9181. https://doi.org/10.3390/ijms21239181.
5. Antonangeli F, Natalini A, Garassino MC, Sica A, Santoni A, Di Rosa F. Regulation of PD-L1 Expression by NF-κB in Cancer. Front Immunol. 2020;11:584626. https://doi.org/10.3389/fimmu.2020.584626.
6. Sargolzaei J, Etemadi T, Alyasin A. The P53/microRNA network: A potential tumor suppressor with a role in anticancer therapy. Pharmacol Res. 2020;160:105179. https://doi.org/10.1016/j.phrs.2020.105179.
7. Duffy MJ, Synnott NC, O'Grady S, Crown J. Targeting p53 for the treatment of cancer. Semin Cancer Biol. 2020;79:58-67. https://doi.org/10.1016/j.semcancer.2020.07.005.
8. Kollareddy M, Martinez LA. Distinct classes of flavonoids and epigallocatechin gallate, polyphenol affects an oncogenic mutant p53 protein, cell growth and invasion in a TNBC breast cancer cell line. Cells. 2021;10(4):797. https://doi.org/10.3390/cells10040797.
9. Carrà G, Lingua MF, Maffeo B, Taulli R, Morotti A. P53 vs NF-κB: the role of nuclear factor-kappa B in the regulation of p53 activity and vice versa. Cell Mol Life Sci. 2020;77(22):4449-58. https://doi.org/10.1007/s00018-020-03524-9.
10. Schneider G, Krämer OH. NFκB/p53 crosstalk-a promising new therapeutic target. Biochim Biophys Acta. 2011;1815(1):90-103. https://doi.org/10.1016/j.bbcan.2010.10.003.
11. Chu C, Yao S, Chen J, Wei X, Xia L, Chen D, Zhang J. *Eupatorium lindleyanum* DC flavonoids fraction attenuates lipopolysaccharide-induced acute lung injury in mice. Int Immunopharmacol. 2016;39:23-33. https://doi.org/10.1016/j.intimp.2016.06.032.
12. Li C, Chen S, Sha J, Cui J, He J, Fu J, Shen Y. Extraction and purification of total flavonoids from *Eupatorium lindleyanum* DC and evaluation of their antioxidant and enzyme inhibitory activities. Food Sci Nutr. 2021;9(5):2349-63. https://doi.org/10.1002/fsn3.1999.
13. Tian S, Chen Y, Yang B, Lou C, Zhu R, Zhao Y, Zhao H. F1012-2 inhibits the growth of triple negative breast cancer through induction of cell cycle arrest, apoptosis, and autophagy. Phytother Res. 2018;32(5):908-22. https://doi.org/10.1002/ptr.6030.
14. Dai L, Tian S, Zhang J, Lu M, Zhu J, Zhao H. F1012-2 Induced ROS-mediated DNA damage response through activation of MAPK pathway in triple-negative breast cancer. Biomed Res Int. 2021;2021:6650045. https://doi.org/10.1155/2021/6650045.
15. Zhu Z, Shen W, Tian S, Yang B, Zhao H. F3, a novel active fraction of *Valeriana jatamansi* Jones induces cell death via DNA damage in human breast cancer cells. Phytomedicine. 2019;57:245-54. https://doi.org/10.1016/j.phymed.2018.12.041.
16. Tian S, Jiang F, Zhang K, Zhu X, Jin B, Lu J, Ding Z. Flavonoids from the leaves of *Carya cathayensis* Sarg. inhibit vascular endothelial growth factor-induced angiogenesis. Fitoterapia. 2014;92:34-40. https://doi.org/10.1016/j.fitote.2013.09.016.
17. Kupper H, Storz H. Double staining technique using a combination of indirect and direct immunofluorescence with monoclonal antibodies. Acta Histochem. 1986;78(2):185-8. https://doi.org/10.1016/s0065-1281(86)80052-6.
18. Sasaki M, Nie L, Maki CG. MDM2 binding induces a conformational change in p53 that is opposed by heat-shock protein 90 and precedes p53 proteasomal degradation. J Biol Chem. 2007;282(19):14626-34. https://doi.org/10.1074/jbc.M610514200.
19. Duffy MJ, Synnott NC, Crown J. Mutant p53 in breast cancer: potential as a therapeutic target and biomarker. Breast Cancer Res Treat. 2018;170(2):213-9. https://doi.org/10.1007/s10549-018-4753-7.
20. Klein AM, Biderman L, Tong D, Alaghebandan B, Plumber SA, Mueller HS, van Vlimmeren A, Katz C, Prives C. MDM2, MDMX, and p73 regulate cell-cycle progression in the absence of wild-type p53. Proc Natl Acad Sci USA. 2021;118(44):e2102420118. https://doi.org/10.1073/pnas.2102420118.
21. Fan Y, Mao R, Yang J. NF-κB and STAT3 signaling pathways collaboratively link inflammation to cancer. Protein Cell. 2013;4(3):176-85. https://doi.org/10.1007/s13238-013-2084-3.
22. Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol. 2002;2(10):725-34. https://doi.org/10.1038/nri910.
23. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-κB signaling pathways. Nat Immunol. 2011;12(8):695-708. https://doi.org/10.1038/ni.2065.
24. Dunphy G, Flannery SM, Almine JF, Connolly DJ, Paulus C, Jønsson KL, Jakobsen MR, Nevels MM, Bowie AG, Unterholzner L. Non-canonical activation of the DNA sensing adaptor STING by ATM and IFI16 mediates NF-κB signaling after nuclear DNA damage. Mol Cell. 2018;71(5):745-60.e5. https://doi.org/10.1016/j.molcel.2018.07.034.

25. Leibowitz B, Yu J. Mitochondrial signaling in cell death via the Bcl-2 family. Cancer Biol Ther. 2010;9(6):417-22. https://doi.org/10.4161/cbt.9.6.11392.
26. Lee TL, Yeh J, Friedman J, Yan B, Yang X, Yeh NT, Van Waes C, Chen Z. A signal network involving coactivated NF-kappaB and STAT3 and altered p53 modulates BAX/BCL-XL expression and promotes cell survival of head and neck squamous cell carcinomas. Int J Cancer. 2008;122(9):1987-98. https://doi.org/10.1002/ijc.23324.
27. Schneider G, Henrich A, Greiner G, Wolf V, Lovas A, Wieczorek M, Wagner T, Reichardt S, von Werder A, Schmid RM, Weih F, Heinzel T, Saur D, Krämer OH. Cross talk between stimulated NF-kappaB and the tumor suppressor p53. Oncogene. 2010;29(19):2795-806. https://doi.org/10.1038/onc.2010.46.
28. Wu X, Deng Y. Bax and BH3-domain-only proteins in p53-mediated apoptosis. Front Biosci. 2002;7:d151-6. https://doi.org/10.2741/a772.