Identification of lineariifolianoid A as a novel dual NFAT1 and MDM2 inhibitor for human cancer therapy

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Abstract

There is an increasing interest in development of novel anticancer agents that target oncogenes. We have recently discovered that nuclear factor of activated T cells 1 (NFAT1) is a novel regulator of the Mouse Double Minute 2 (MDM2) oncogene and the NFAT1-MDM2 pathway has been implicated in human cancer development and progression, justifying that targeting the NFAT1-MDM2 pathway could be a novel strategy for discovery and development of novel cancer therapeutics. The present study was designed to examine the anticancer activity and underlying mechanisms of action of lineariifolianoid A (LinA), a novel natural product inhibitor of the NFAT1-MDM2 pathway. The cytotoxicity of LinA was first tested in various human cancer cell lines in comparison with normal cell lines. The results showed that the breast cancer cells were highly sensitive to LinA treatment. We next demonstrated the effects of LinA on cell proliferation, colony formation, cell cycle progression, and apoptosis in breast cancer MCF7 and MDA-MB-231 cells, in dose-dependent and p53-independent manners. LinA also inhibited the migration and invasion of these cancer cells. Our mechanistic studies further indicated that its anticancer activities were attributed to its inhibitory effects on the NFAT1-MDM2 pathway and modulatory effects on the expression of key proteins involved in cell cycle progression, apoptosis, and DNA damage. In summary, LinA is a novel NFAT1-MDM2 inhibitor and may be developed as a preventive and therapeutic agent against human cancer.

Keywords: lineariifolianoid A, NFAT1, MDM2, breast cancer, p53, apoptosis

Introduction

Recent advances in research on the etiology and pathogenesis of human cancers have demonstrated that a number of oncogenes and tumor suppressor genes play a pivotal role in cancer onset, development, progression, and response to therapy¹⁻⁶, including the Mouse Double Minute 2 (MDM2) oncogene and TP53 tumor suppressor⁵⁻⁶. A multitude of targeted therapy approaches have been developed to inhibit the oncogenes⁷⁻⁹. The amplification and overexpression of MDM2 are common genetic events in human cancers and correlated with tumor initiation, growth, metastasis, and responses to chemotherapy and radiation. 

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thapy\(^{10\text{-}12}\). MDM2 plays a major role in cancer cell survival and proliferation, cell cycle progression, apoptosis, migration, and invasion by regulating a variety of signaling pathways\(^{13\text{-}16}\). We and others have demonstrated that MDM2 exerts its oncogenic activity via p53-dependent and p53-independent mechanisms, inspiring the development of several different MDM2 targeting approaches\(^{17\text{-}24}\). Not surprisingly, there is an increasing interest in developing specific, potent, and safe MDM2 inhibitors for cancer prevention and therapy.

There are several MDM2 inhibitors being developed to inhibit the MDM2-p53 interaction, protecting p53 from MDM2-mediated ubiquitination and proteasomal degradation\(^{17\text{-}19,23,25\text{-}26}\). However, concerns have been raised about the limitations on both the efficacy of these MDM2 inhibitors in p53-mutant cancers and the potential toxicity associated with p53 activation\(^{5\text{-}6,14}\). In continuous efforts to develop novel MDM2 targeting approaches and the specific and potent MDM2 inhibitors, we and others have identified antisenes oligonucleotides and siRNAs\(^{27\text{-}33}\), natural products\(^{22,34\text{-}39}\), and synthetic compounds\(^{30\text{-}31,40\text{-}41}\), in order to directly inhibit MDM2 expression and/or its activity, emphasizing the p53-independent mechanism. More recently, we have identified that the transcription factor nuclear factor of activated T cells 1 (NFAT1) activates MDM2 in a p53-independent manner\(^{42}\). NFAT1 has been demonstrated to play an important role in cancer development and progression and has been suggested as a promising molecular target for cancer treatment and prevention\(^{44\text{-}45}\).

We have recently proposed to develop MDM2 inhibitors via targeting the NFAT1-DM2 signaling pathway\(^{39,42}\), leading to the identification of lineariifolianoid A (LinA) (Fig. 1) through the screening of our in-house compound library (Data not shown). LinA is a naturally occurring sesquiterpenoid dimer from the aerial parts of *Inula lineariifolia* Turcz., which has been used in traditional Chinese medicine "Jinfeicao" for treating digestive disorders, bronchitis, inflammation, and diabetes\(^{35\text{-}48}\). In our previous study, LinA was discovered as a specific and potent MDM2 inhibitor, but the precise mechanisms by which LinA inhibits MDM2 remain unclear\(^{22}\). The present study was designed to evaluate the *in vitro* cytotoxicity of LinA in a variety of cancer cell lines and the underlying mechanism(s) of action. Since breast cancer cells were highly sensitive to the treatment of this compound, our major efforts were devoted to further investigate its anticancer activity in breast cancer cells. It was hoped that our results from the present study would provide a basis for further development of LinA for human breast cancer therapy in preclinical and clinical settings.

### Materials and methods

#### Chemicals, antibodies, and reagents

LinA was prepared as described in our previous study\(^{49}\). All chemicals and solvents used in the present study were of highest grade available. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). The penicillin/streptomycin preparation was bought from Corning (Manassas, VA, USA). The anti-human NFAT1 (1/NFAT-1) antibody was purchased from BD Biosciences (San Jose, CA, USA). The antibodies against human MDM2 (Ab-2), p21 (Ab-1), and p-H2AX (Ser139) were sourced from EMD Chemicals (Gibbstown, NJ, USA). The antibodies against human p53 (DO-1), Cdk2 (M2), Cdk4 (H-22), Cdk6 (C-21), cyclin D1 (DCS-6), cyclin E (HE12), c-Myc (0.N.222), Bax (N-20), Bcl-2 (100), PARP (H-250), Chk1 (G-4), Chk2 (B-4), and ATR (N-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against human p-Chk1 (Ser317), p-Chk2 (Thr68), and p-p53 (Ser15) were purchased from Cell Signaling Technology (Danvers, MA, USA). The anti-human β-actin (AC-15) antibody was bought from Sigma (St. Louis, MO, USA) and the goat anti-mouse IgG (H+L) and goat anti-rabbit IgG (H+L) antibodies were obtained from Bio-Rad (Hercules, CA, USA).

#### Cells and cell culture

Human normal and cancer cell lines used in the present study were purchased from American Type Culture Collection (Rockville, MD, USA). All cell culture media were supplemented with 10% FBS and 1% penicillin/streptomycin, except for those indicated otherwise. Human breast cancer cell lines tested in the present study included MCF7, MCF7 p53 knockdown (MCF7 p53\(^{-}\)), MDA-MB-468, and MDA-MB-231. The MCF7 and MDA-MB-231 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM). The MCF7 p53\(^{-}\) cells were developed and maintained as reported previously\(^{30,49}\). The MDA-MB-468 cells were grown in DMEM/Ham’s F12 media. Human prostate cancer cell lines included PC3, LNCaP, and DU145. PC3 cells were

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**Fig. 1** The chemical structure of LinA.
cultured in Ham's F12 medium. LNCaP and DU145 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium. Human lung cancer A549 cells were grown in RPMI 1640. Human pancreatic cancer cell lines included HPAC, Panc-1, and BxPC3. HPAC cells were grown in DMEM/Ham's F12. Panc-1 and BxPC3 cells were cultured in RPMI 1640. Human colon cancer HCT116 and HCT116 p53 knockdown (HCT116 p53−/−) cells were grown in McCoy’s 5A. Human osteosarcoma U2OS cells were grown in DMEM. Human ovarian cancer SKOV-3 cells were maintained in McCoy’s 5A and OVCAR-3 cells were grown in RPMI 1640 supplemented with 20% FBS, 10 mmol/L of sodium pyruvate, 10 mmol/L of HEPES, 10 mg/L of bovine insulin, and 4.5 g/L of glucose. Human liver cancer HepG2 and Hep3B cells were grown in DMEM. Human glioma U87MG cells were grown in Eagle's minimum essential medium. Human embryonic kidney HEK293 cells were cultured in DMEM. Human breast epithelial MCF10A cells were grown in DMEM/Ham's F12 containing 5% horse serum, 20 ng/mL of EGF, 0.5 mg/mL of hydrocortisone, 100 μg/mL of choler toxin, 10 μg/mL of insulin, and 1% penicillin/streptomycin. The inductible MDM2 overexpression (OE) and NFAT1 OE MCF7 cell lines were established and maintained as reported previously[38,42]. In brief, both cell lines were grown in DMEM containing 10 μg/mL of blasticidin and 200 μg/mL of zeocin (Invitrogen, Grand Island, NY, USA). Both MDM2 and HA-NFAT1 plasmids were transfected into cells using the same protocols as we reported previously[35,42].

Cell viability assay

The cytotoxicity of LinA was assessed as described in our previous studies[46,50]. Briefly, the cells were seeded into 96-well plates (3-4×10^3 cells/well) for 24 h and then treated with various concentrations of LinA (0-50 μmol/L) for additional 72 hours. After exposure of cells to LinA, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL; Sigma) was added into each well for a 3-hour incubation at 37°C. Absorption was measured at 570 nm using a SYNERGY Mx microplate reader (BioTek, Winooski, VT, USA). The cell survival percentages were calculated by comparisons with the DMSO treated cells.

Cell proliferation assay

The effects of LinA on cell proliferation were evaluated as described previously[21,49]. In brief, the cells were seeded into 6-well plates (2-3 × 10^4 cells/well) and treated with various concentrations of LinA (0, 5, and 10 μmol/L) for 24 hours. The cells were harvested and fixed in physiological saline (0.9% NaCl) and 95% ethanol at 4°C overnight. The cells were then incubated with RNase (Sigma), stained with propidium iodide (Sigma), and analyzed by flow cytometry.

Cell cycle analysis

The effects of LinA on cell cycle distribution were analyzed as described previously[20,49]. In brief, the cells were seeded into 6-well plates (2-3 × 10^4 cells/well) and treated with various concentrations of LinA (0, 5, and 10 μmol/L) for 24 hours. The cells were harvested and fixed in physiological saline (0.9% NaCl) and 95% ethanol at 4°C overnight. The cells were then incubated with RNase (Sigma), stained with propidium iodide (Sigma), and analyzed by flow cytometry.

Cell apoptosis assay

The effects of LinA on cell apoptosis were evaluated as described in our previous studies[46,52]. In brief, the cells (2-3 × 10^5 cells/well) were exposed to various concentrations of LinA (0, 5, 10, and 25 μmol/L) for 48 hours. Then, the cells were collected, suspended in Annexin V binding buffer, and incubated with Annexin V-FITC and propidium iodide in the dark at room temperature for 30 minutes. Both early apoptosis and late apoptosis were determined by flow cytometry.

Wound healing assay

The effects of LinA on cell migration were analyzed as reported previously[20,38]. In brief, the monolayer of MDA-MB-231 cells was grown to confluence in 6-well plates and scratched in each well by using a pipette tip. The cells were then exposed to LinA (2 or 5 μmol/L), and five fields of each wound were monitored and photographed at 0, 8, and 24 hours.

Transwell invasion assay

The effects of LinA on cell invasion were examined as described in our previous study[53]. In brief,
MDA-MB-231 cells was serum starved overnight and then seeded onto the upper well of Boyden chamber (1-2×10^4 cells/well). After incubation for 6 hours, the cells were exposed to LinA (2 or 5 µmol/L) for another 24 hours. The cells on the upper surface were then removed by wiping with a cotton swab and the cells adhering to the filter were stained with Mayer's Hematoxylin and Eosin solution and photographed under a phase-contrast Olympus microscope (Olympus America Inc). The positive staining area was measured using image analysis software.

**Western blotting analysis**

In brief, the cells were seeded into 6-cm dishes at 3-4 × 10^5 cells per dish for a 24-hour incubation and treated with various concentrations of LinA (0, 5, 10, and 25 µmol/L) for another 24 hours. Then, the cells were lysed in NP-40 lysis buffer containing a protease inhibitor mixture (Sigma) and the samples were subjected to Western blotting analysis for examining the expression levels of protein of interest, using the methods described previously.[39,53].

**Statistical analysis**

All the quantitative data were expressed as mean ± SEM from at least three independent experiments. Significance of the differences in comparisons of multiple groups was determined by one-way ANOVA and post-hoc pairwise test when there was only one variable. Two-way ANOVA was used when there were two variables. The differences were considered statistically significant at P < 0.05.

**Results**

**LinA exerts remarkable cytotoxicity in various cancer cells with minimal activity in normal cells**

The in vitro cytotoxicity of LinA was tested in 20 cell lines representing nine types of human cancer (breast, prostate, lung, pancreatic, colon, ovarian, and liver cancer, sarcoma, and glioblastoma) and two normal cell lines at different concentrations (0 to 50 µmol/L) for 72 hours. The cell viability and the IC_{50} values are shown in Table 1. Notably, the normal cell lines (HEK293 and MCF10A) were much less sensitive to this compound, indicating a selective cytotoxicity of LinA against cancer cells. The breast cancer cell lines, including MCF7 (p53 wild-type), MCF7 p53<sup>-/-</sup>, MDA-MB-231 (p53 mutant), and MDA-MB-468 (p53 mutant), were among the most sensitive cell lines, with the IC_{50} values ranging from 4.4 to 9.1 µmol/L, indicating that its anti-breast cancer activity was not dependent on p53 status, which is important for discovery of a novel anti-MDM2 inhibitor.

**LinA inhibits cell proliferation and colony formation in breast cancer cells**

As shown in Fig. 2A, LinA exhibited its antiproliferative effects in both MCF7 and MDA-MB-231 cells in a concentration-dependent manner, regardless of p53 status. At a high concentration of 25 µmol/L, LinA inhibited cell proliferation by 43% (P < 0.01) and 50% (P < 0.01) in MCF7 and MDA-MB-231 cells, respectively. Similarly, LinA also inhibited cell colony formation in both cell lines, in a concentration-dependent manner (Fig. 2B).

**LinA delays exit from G2/M phase in breast cancer cells**

As shown in Fig. 2C, LinA delays exit from G2/M phase of the cell cycle in a concentration-dependent manner in both MCF7 and MDA-MB-231 cells, indicating that the effect was independent of p53. LinA at 5 µmol/L markedly increased the number

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**Table 1** Cytotoxicity of lineariifolianoid A against normal and cancer cell lines

| Cell type     | Cell line   | IC_{50} (µmol/L) |
|---------------|-------------|-----------------|
| Breast cancer | MCF7        | 4.5 ± 0.3       |
|               | MCF7 p53<sup>-/-</sup> | 9.1 ± 0.9       |
|               | MDA-MB-468  | 4.4 ± 0.3       |
|               | MDA-MB-231  | 7.8 ± 0.6       |
| Prostate cancer | PC3        | 3.7 ± 0.5       |
|               | LNCaP       | 4.5 ± 0.7       |
|               | DU145       | 8.4 ± 0.5       |
| Lung cancer   | A549        | 8.2 ± 0.9       |
|               | H385        | 4.6 ± 0.4       |
| Pancreatic cancer | HPAC  | 6.4 ± 0.6       |
|               | Panc-1      | 4.3 ± 0.3       |
|               | BxPC3       | 5.3 ± 0.3       |
| Colon cancer  | HCT116      | 9.1 ± 0.8       |
|               | HCT116 p53<sup>-/-</sup> | 28.8 ± 2.2     |
| Osteosarcoma  | U2OS        | 8.2 ± 0.8       |
| Ovarian cancer | SKOV-3   | 7.5 ± 0.7       |
|               | OVCAR-3     | 17.3 ± 1.5      |
| Liver cancer  | HepG2       | 17.7 ± 1.2      |
|               | Hep3B       | 5.9 ± 0.5       |
| Glioma        | U87MG       | 3.2 ± 0.2       |
| Human embryonic kidney cells | HEK293 | 45.5 ± 3.1      |
| Human breast epithelial cells | MCF10A | 37.2 ± 3.5      |

Various normal and cancer cell lines were treated with LinA (0-50 µmol/L) for 72 hours for the determination of cell viability and IC_{50} values. All assays were performed in triplicate and repeated three times.
of cells in G2/M phase in both cell lines ($P < 0.05$). Moreover, in comparison to the control, treatment of cells with 10 µmol/L of LinA led to an accumulation of cells in G2/M phase by 33.1% ($P < 0.01$) and 48.5% ($P < 0.01$) in MCF7 and MDA-MB-231 cells, respectively.

**LinA induces apoptosis in breast cancer cells**

As shown in Fig. 2D, LinA induced significant apoptosis in a concentration-dependent manner, while MDA-MB-231 cells were less sensitive than MCF7 cells. After treatment with 25 µmol/L of LinA for 48 hours, 53.2% of MCF7 cells and 14.3% of MDA-MB-231 cells underwent apoptosis, which were significantly higher than that of control cells (both $P < 0.01$).

**LinA inhibits cell migration and invasion in breast cancer cells**

As shown in Fig. 3A, untreated MDA-MB-231 cells migrated into the wound area rapidly in a 24-hour treatment period, whereas LinA at the sub-lethal concentrations (2 and 5 µmol/L) significantly inhibited cell migration in a concentration-dependent manner. Similarly, Transwell invasion assay showed that LinA remarkably reduced MDA-MB-231 cell invasion (Fig. 3B). In comparison to the control cells, LinA at 2 and 5 µmol/L reduced the invasion of MDA-MB-231 cells by 41% ($P < 0.05$) and 87% ($P < 0.01$), respectively.

**LinA inhibits the NFAT1-MDM2 pathway independent of p53**

We next explored possible molecular mechanisms of LinA-induced anticancer effects. As shown in Fig. 4A, LinA decreased the protein expression of NFAT1 and MDM2 in both MCF7 and MDA-MB-231 cells, in a concentration-dependent manner. LinA also increased the expression level of wild-type p53 in MCF7 cells, but it did not show any effects on the mutant p53 in MDA-MB-231 cells.
LinA modulates the expression of key proteins associated with cell cycle progression, apoptosis, and DNA damage response

To further explore the mechanisms of action for anticancer activity of LinA, we next examined the effects of LinA on the expression of key proteins involved in regulating cell cycle progression, apoptosis, and DNA damage response. In both MCF7 and MDA-MB-231 cells, LinA significantly increased the expression level of p21 and decreased the levels of Cdk2, Cdk4, Cdk6, cyclin D1, cyclin E, and c-Myc compared to the control (Fig. 4B). The compound also markedly activated Bax and cleavage of PARP, and decreased Bcl-2 levels in both cell lines (Fig. 4C). The inhibition of ATR by LinA also led to the phosphorylation of Chk1 at Ser317, Chk2 at Thr68, p53 at Ser15, and H2AX at Ser139 (Fig. 4D).

MDM2 overexpression reduces anti-breast cancer activity of LinA

To demonstrate the importance of MDM2 in LinA’s anti-breast cancer activity, we compared the effects of LinA on parent and inducible MDM2 overexpression MCF7 cells. As shown in Figs. 5A-5B, Tet treatment resulted in MDM2 overexpression (Fig. 5A), which increased colony formation (Fig. 5B) and attenuated the inhibitory effects of LinA on MDM2 expression (Fig. 5A) and colony formation (Fig. 5B). Similar results were obtained in MDA-MB-231 cells. As shown in Figs. 5C-5F, transient transfection of an MDM2 plasmid caused MDM2 overexpression (Fig. 5C), leading to increased colony formation (Fig. 5D), enhanced cell migration (Fig. 5E) and invasion (Fig. 5F), and mitigated the inhibitory effects of LinA (Figs. 5C-5F) in the cells.

NFAT1 overexpression activates MDM2 and decreases LinA’s anticancer activity

To demonstrate the crucial role of the NFAT1 and NFAT1-MDM2 pathway in LinA’s anticancer activity, we further compared the inhibitory effects of LinA on parent and NFAT1 OE MCF7 and MDA-MB-231 cells. As shown in Figs. 6A-6D, NFAT1 overexpression increased MDM2 expression (Figs. 6A & 6C) and colony formation (Fig. 6B & 6D) and decreased the inhibitory effects of LinA (Figs. 6A-6D) in both cell lines. In NFAT1 overexpressing MDA-MB-231 cells, NFAT1 overexpression also increased cell migration (Fig. 6E) and invasion (Fig. 6F), and mitigated LinA’s inhibitory effects.

Discussion

In the present study, we investigated anticancer activity and the molecular mechanisms of the newly identified NFAT1-MDM2 inhibitor LinA. We have demonstrated several important points that would be of great importance in further research and development of this compound as an anticancer agent. First, LinA displayed potent cytotoxicity against a broad spectrum of human cancer cell lines; breast cancer cell lines were among the most sensitive cell lines, which gave a good explanation for the focus on this cancer type. Second, normal cells were much less sensitive to LinA, suggesting a selective cytotoxicity of this compound. Third, LinA inhibited cell colony formation, delayed exit from G2/M phase of the cell cycle, and induced apoptosis in breast cancer cells in a concentration-dependent manner, regardless of p53 status of the cells. Fourth, the
compound reduced breast cancer cell migration and invasion at sub-lethal concentrations in vitro. Fifth, LinA inhibited both NFAT1 and MDM2 expression, and modulated the expression of key proteins related to cell cycle progression, apoptosis, and DNA damage response. Finally, enforced MDM2 or NFAT1 expression remained effective when the cells were exposed to LinA. **Fig. 7** depicts the possible mechanisms of action for LinA.

Significant advances have recently been made in our understanding of genetic alterations in human cancers, including the amplification, overexpression, and mutation of key oncogenes (e.g., *MDM2* and *NFAT1*) as well as the mutation and deletion of tumor suppressors (e.g., *TP53* and *BRCA1*)[1-2,4-6,54]. Targeted therapies that inhibit the oncogenes and/or reactivate the tumor suppressors offer great promise for patients with advanced cancer[7-9]. The targeted therapies may also enhance chemotherapy-induced cytotoxicity and boost responses to immunotherapy, improving the clinical outcomes of chemotherapy and immunotherapy for patients with advanced diseases[7]. There is an increasing interest in developing novel approaches to inhibit the *MDM2* oncogene via p53-dependent and p53-independent mechanisms[5-6,13-16]. Considering our recent discovery in the NFAT1-MDMD2 pathway that

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**Fig. 4** Effects of LinA on the NFAT1-MDM2 pathway and on the expression of key proteins related to cell cycle progression, apoptosis, and DNA damage response in breast cancer cells. Human breast cancer MCF7 and MDA-MB-231 cells were exposed to various concentrations of LinA for 24 hours. Then, the expression levels of (A) NFAT1, MDM2, and p53, and the key proteins related to (B) cell cycle progression, (C) apoptosis, and (D) DNA damage response were examined by Western blotting assay. All assays were performed in triplicate and repeated three times.
activates MDM2 in a p53-independent manner\cite{4,39,42}, we proposed to develop NFAT1-MDM2 inhibitors for cancer therapy, leading to the identification of LinA. In our recent study with cell-based screening assays for identifying novel NFAT1-MDM2 inhibitors, LinA was identified as a novel dual NFAT1 and MDM2 inhibitor from our in-house compound library (Data not shown). We demonstrated potent cytotoxicity of LinA against a wide spectrum of human cancer cell lines, of which breast cancer cell lines exhibited high sensitivity to LinA. These findings are consistent with our previous results that the NFAT1-MDM2 pathway plays a crucial role in breast cancer cell growth in vitro and in vivo\cite{39,42}. We further demonstrated the potent inhibitory effects of Lin A on NFAT1 and MDM2 in both p53-wildtype and -mutant breast cancer cell lines. Given these observations, we then demonstrated the critical role of the NFAT1-MDM2 pathway in LinA’s anti-breast cancer activity using MDM2 or NFAT1 overexpressing MCF7 and MDA-MB-231 cells. In the overexpression studies,
the enforced MDM2 or NFAT1 expression was established and remained effective when the cells treated with LinA, including decreased colony formation and reduced cell migration and invasion. Interestingly, compared with the parent cells, MDM2 or NFAT1 overexpression reduced the inhibitory effects of LinA on colony formation, cell migration, and cell invasion. Although the exact mechanisms of action are not completely understood, the possible reason may be related to high level of MDM2 or NFAT1, which compete for the limited available compound (LinA) in the assay system. The current overexpression studies may not represent the situation of real MDM2 or NFAT1 overexpression in clinical setting, but may still provide evidence for LinA binding to its targets (MDM2 and NFAT1). Nevertheless, further investigations are required for establishing the link between LinA’s activity and its inhibitory effects on the NFAT1-MDM2 pathway.

With respect to the therapeutic potency of LinA, our results indicated that this compound exerted its anticancer activity at relatively low concentration, as elucidated in cell viability, colony formation, cell cycle...
distribution, and apoptosis assays. However, the effects of LinA on cell proliferation were achieved at very high concentration, especially in MDA-MB-231 cells. The dose-response effects could just be a function of cytotoxicity and not a real function in cell proliferation. Depended on the observation, the major mechanisms of cell death might be apoptosis induction in MCF7 cells and cell cycle regulation in MDA-MB-231 cells. In addition, we demonstrated the preventive effects of LinA on cell migration and invasion at sub-lethal concentrations, indicating that LinA might possess therapeutic potential in metastatic cancers. Given the potent anticancer activity, we further examined the effects of LinA on various key proteins involved in regulating cell cycle progression, apoptosis, and DNA damage response. The majority of the proteins have been reported to be associated with the MDM2-p53 and NFAT1-MDM2 pathways. The results are similar to previous observations on other MDM2 inhibitors that were developed to inhibit MDM2 expression. However, the present study just provided the proof-of-concept \textit{in vitro} data for the new compound, but it had certain limitation. In the future, various animal models such as orthotopic mouse models and metastatic models should be employed to evaluate the \textit{in vivo} efficacy of LinA and characterize its toxicological profile. Additional techniques such as pharmacological and genetic interventions should be utilized to generate more evidence for demonstrating LinA as a specific NFAT1-MDM2 dual inhibitor.

In summary, we have identified LinA, a natural sesquiterpenoid dimer, as a novel NFAT1-MDM2 inhibitor and demonstrated its potent anticancer activity in breast cancer cells. Our results suggest that LinA may be a potential therapeutic agent for the treatment and prevention of human cancers. Future studies should further demonstrate its activity \textit{in vivo}, when used alone and/or in combination with other conventional cancer chemotherapies. If we could confirm its anticancer effects in preclinical settings, it can be developed as a clinical candidate for breast cancer therapy. It should be pointed out that most advanced breast cancers do not express wild-type p53; our newly identified p53-independent MDM2 inhibitor may offer new hope for the cancer patients without functional p53 expression.

\textbf{Fig. 7} The schematic diagram depicts the possible mechanisms of action of LinA against cancer cells. LinA inhibits cancer cell growth and induces cell cycle arrest and apoptosis by inhibiting the NFAT1-MDM2 pathway and modulating the expression of key proteins involved in regulating cell cycle progression, apoptosis, and DNA damage.
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