Teriflunomide, an immunomodulatory drug, exerts anticancer activity in triple negative breast cancer cells

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Abstract

Triple-negative breast cancer (TNBC) is defined as a group of primary breast cancers lacking expression of estrogen, progesterone, and human epidermal growth factor receptor-2 (HER-2) receptors, characterized by higher relapse rate and lower survival compared with other subtypes. Due to lack of identified targets and molecular heterogeneity, conventional chemotherapy is the only available option for treatment of TNBC, but non-discordant positive therapeutic efficacy could not be achieved. Here, we demonstrated that these TNBC cells were sensitive to teriflunomide, which was a well-known immunomodulatory drug for treatment of relapsing multiple sclerosis (MS). Potent anti-cancer effects in TNBC *in vitro*, including proliferation inhibition, cell cycle delay, cell apoptosis, and suppression of cell motility and invasiveness, could be achieved with this agent. Of note, we showed that multiple signals involved in TNBC proliferation, survival, migratory, and invasive potential were under regulation by teriflunomide. Among them, we identified down-regulation of growth factor receptors to abolish growth maintenance, suppression of c-Myc, and cyclin D1 to contribute to its anti-proliferative effect, modulation of components of cell cycle to induce S-phase arrest, degradation of Bcl-xL, and up-regulation of BAX via activation of MAPK pathway to induce apoptosis, and inhibition of epithelial-mesenchymal transition (EMT) process, matrix metalloproteinase-9 (MMP9) expression, and inactivation of Src/FAK to reduce TNBC migration and invasion. The results identified teriflunomide may be of therapeutic benefit for the more aggressive and difficult-to-treat breast cancer subtype, indicating the use of teriflunomide for clinical trials for treatment of TNBC patients.

Keywords: Triple negative breast cancer, teriflunomide, growth factor receptor, epithelial-mesenchymal transition, Src, cell cycle

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Introduction

Breast cancer is the most diagnosed female cancer and the second leading cause of cancer death in the United States.¹ According to analysis of gene expression, breast cancer is a heterogeneous disease consisting of a variety of entities, with various molecular subtypes and markedly distinct clinical behaviors.^{2,3} Triple-negative breast cancer (TNBC), accounting for less than 20% of breast cancer cases and representing 68.5% of basal-like subtype, is a group of primary breast cancers lacking the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (Her-2) on the basis of immunohistochemical staining.⁴ TNBC showed poorer prognosis than any other breast cancer subtypes, irrespective of clinical stages, demonstrating more aggressive clinical features

with higher recurrence rate and lower survival.⁵ TNBC is non-sensitive to currently available endocrine or Her-2-targeted therapies and is generally receiving chemotherapeutic regimens for systemic management, however, with variable efficacy. So far, there is no explicit guideline for TNBC treatment and, therefore, searching for novel pharmaceutical agents for TNBC is urgent and still a hot-spot in present clinical research.

Teriflunomide (Aubagio®), the principal active metabolite of leflunomide in first-pass metabolism, has well-known immunomodulatory and anti-inflammatory effects and has been approved by FDA for use in the treatment of relapsing multiple sclerosis (MS).⁶ The precise underlying mechanisms by which teriflunomide exerts immunomodulation in MS remain elusive. However, to our knowledge, by

suppressing the activity of proliferating T- and B-lymphocytes through dihydroorotate-dehydrogenase (DHODH) inhibition-dependent and independent manners, teriflunomide diminishes overall inflammatory response in MS.⁷ Of note, high plasma concentrations,⁸ delayed elimination half-life,⁹ benign safety and tolerability profiles,⁹ the convenience of oral administration, and extensive molecular targets¹⁰ make teriflunomide an attractive agent for further clinical utility in MS and even in a broader spectrum of non-MS disorders.

Despite the promising use of teriflunomide for MS, the application of this agent for the treatment of human tumors has only recently been considered. Previous researchers have shown that teriflunomide exhibited powerful antitumor activity in several human neoplasms, such as chronic lymphocytic leukemia (CLL),^{11,12} prostate cancer,¹³ melanoma,^{14,15} cutaneous cancer,¹⁶ multiple myeloma,¹⁷ and carcinoids.^{18,19} Teriflunomide may exert the observed anti-cancer effects by inhibition of cancer cell proliferation,^{14,18} induction of cell death,^{11,13,16,17} disturbance of cell cycle.^{12,16,17,19} The molecular mechanisms that have been identified for teriflunomide-mediated cellular events include down-regulation of anti-apoptotic proteins,¹¹ direct inhibition of receptor tyrosine kinases (RTKs),²⁰ abrogation of cancer stem cells,¹⁴ induction of mitochondrial disruption,¹³ and modulation of cell survival signaling.^{17,19} Although exact mechanisms involved in cytotoxic effect of teriflunomide are not clearly understood, of note, the available above studies may imply the DHODH-independent modulation plays a principal role in teriflunomide-mediated anti-tumor activity.^{11–13,15,17,20} Thus, teriflunomide may also be a potent agent for treatment of breast cancer and even TNBC. However, direct evidence of this hypothesis has not yet to be documented.

In the present study, we assessed, for the first time to our knowledge, the activity of teriflunomide and the underlying DHODH-independent mechanisms in TNBC cell lines: MDA-MB-468, BT549, and MDA-MB-231. Here, we reported that teriflunomide was capable of suppressing the growth of TNBC cell *in vitro* by inducing cell apoptosis and cell cycle arrest. We also showed that this compound could inhibit the cell motility and invasiveness. Furthermore, the information presented in this study indicated teriflunomide exerted modulatory effects on TNBC cells through multiple signaling pathways. Together, we think teriflunomide is a novel agent for the prevention of tumor progression and/or treatment of human TNBC.

Materials and methods

Cell culture and drug treatment

The human TNBC cell lines MDA-MB-468, BT549, and MDA-MB-231 were purchased from American Type Culture Collection (ATCC) and cultured in RPMI 1640 (Gibco, Breda, The Netherlands) with 10% FBS at 37°C and 5% CO₂ (MDA-MB-468 and BT549) or in Leibovitz L15 (Gibco) with 10% FBS at 37°C in 100% room air (MDA-MB-231), respectively. Teriflunomide was kindly provided by Cinkate Corporation, Oak Park, IL, USA,

dissolved in DMSO as stock solution at concentration of 200 mM.

Sulforhodamine B assay

Cytotoxicity of teriflunomide on TNBC cells were determined using Sulforhodamine B (SRB) method. Cells were seeded in 96-well plate (Corning, Acton, MA, USA) at a concentration of 2000 × cells/well. After 24 h, cells were incubated for additional 24 h, 48 h, 72 h, and 96 h with various concentrations of teriflunomide and then fixed by 200 µL cooled trichloroacetic acid (Sinopharm Reagent, Shanghai, China) for 1 h at 4°C. The plates were washed with distilled water, air dried, stained with SRB solution (150 µL) at 0.4% (w/v) in 1% acetic acid for 30 min at room temperature, and then washed with 1% acetic acid and dried. The bound SRB was solubilized with 100 µL/well of 10 mM Tris base for 15 min. The optical density (OD) was read by an automated microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) at wavelength of 560 nm. Relative survival was calculated using the equation: (OD test/OD con) × 100%.

Clonogenic assay

TNBC cells were plated at the concentration of 500 × cells/well in 6-well plates and, the next day, were treated with various concentrations of teriflunomide. Cells were allowed to form colonies in complete media with teriflunomide for 2 weeks. And then, colonies were fixed with solution of acetic acid and methanol (1:3) for 10 min, stained with 0.5% crystal violet for 15 min and counted.

Flow cytometric assay

For cell cycle analysis, TNBC cells were treated with 25, 50, and 100 µM teriflunomide for 48 h, then harvested and fixed by cooled 75% ethanol for 1 h. Fixed cells were stained with propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO, USA) and DNase-free RNase (Sigma-Aldrich) for 15 min at RT, and then analysed in FACSCalibur analyzer (Becton-Dickinson, San Jose, CA, USA). Data were analysed using Modfit software (Verity Software House, Topsham, ME, USA). For apoptosis analysis, after receiving teriflunomide at concentrations of 50 and 100 µM for 2 days, TNBC cells were detached with EDTA-free trypsin, washed with cooled PBS, and stained by 5 µL Annexin V and 5 µL PI in 1 × loading buffer (BD Pharmingen, San Diego, CA, USA) for 5 at RT in dark. Analyses were determined in FACSCalibur analyzer (Becton-Dickinson).

Transwell assay

TNBC cell migration and invasion were determined with or without matrigel-coated transwell Boyden chambers. TNBC cells were trypsinized, resuspended, and placed into upper chambers (8 × 10⁴ cells/well) in FBS-free medium and complete medium was added into lower chamber as chemo-attractant, with various concentrations of teriflunomide for 18 h (migration) or 24 h (invasion). After incubation, cells on the upper chamber were removed with cotton buds, migratory or invasive cells were fixed

with cooled 90% ethanol for 1 h at 4°C, and stained with 0.1% crystal violet (Sinopharm Reagent).

Western blot and antibodies

After receiving treatment of teriflunomide, TNBC cells were harvested and lysed in RIPA (Beyotime Institute of Biotechnology, Beijing, China) with protease inhibitor (Roche Applied Science, Indianapolis, IN, USA). Total protein levels were determined using BCA Protein assay (Beyotime). Then all samples were separated by SDS-PAGE, transferred to NC membranes (Millipore, Bedford, MA, USA), and probed by respective primary antibodies and HRP-conjugated secondary antibodies. Proteins were visualized using ECL detection system (GE Healthcare, Piscataway, NJ, USA). The primary antibodies used were c-Myc, cyclin D1, P27, p-P38 (Thr180/Tyr182), p-ERK1/2 (Thr202/Tyr204), p-JNK (Thr183/Tyr185), p-RB, Vimentin, Slug, Snail, Raf-1, EGFR, IGF-1R and FGFR4 (Cell Signaling Technology, Danvers, MA, USA), E-cadherin, Bax, Bcl-xL, cyclin A2, cyclin B1, P-FAK(Tyr397) and FAK(Epitomics, Burlingame, CA, USA), p-Src(Tyr418) (Abcam,

Cambridge, MA, USA), and matrix metalloproteinase-9 (MMP9; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistics

Statistical significance was determined by Student's *t*-test using GraphPad Prism Software, and *P* values < 0.05 was considered significant between the teriflunomide-treated and control groups.

Results

Teriflunomide suppresses the growth of TNBC

To investigate cytotoxic effect on cell proliferation, we treated the TNBC cell lines MDA-MB-468, BT549, and MDA-MB-231 with increasing concentrations of teriflunomide for 96 h and performed an SRB assay. As revealed in Figure 1(a), teriflunomide potently repressed cell viabilities in dose- and time-dependent manners. In addition, the significant cytotoxicity could not be observed in initial phase of treatment, and in fact the TNBC cells proliferation was reduced by only 19.88% in MDA-MB-468, 6.60% in BT549,

Figure 1 Teriflunomide inhibited proliferation of TNBC cells. (a) TNBC cells were treated with varying concentrations of teriflunomide, cell proliferation and IC50 were determined by SRB assay on days 1, 2, 3, and 4. Each value represented the mean $\pm$ SD (*n* = 3); (b) Teriflunomide suppressed colony formation in TNBC cells. Cells were treated with indicated concentrations of teriflunomide and allowed to form colonies in fresh medium for 14 days. Photomicrographic differences and relative cell number (mean $\pm$ SD, *n* = 3) in colony forming are shown. Asterisks indicate *P* < 0.05. (A color version of this figure is available in the online journal.)

and 7.29% in MDA-MB-231 even at the highest concentration of 200 μ M. The anti-proliferation effect continued to increase over the next 72 h and the mean IC₅₀ estimated from three independent experiments gave a concentration of 31.36 μ M for MDA-MB-468 cells, 31.83 μ M for BT549 cells, and 59.72 μ M for MDA-MB-231 cells at the time point of 96 h post-treatment.

Teriflunomide induces loss of clonogenic survival in TNBC

To simulate the long-term effect of teriflunomide on cell growth *in vivo*, we then verified if teriflunomide could affect the ability of TNBC cells to form colonies *in vitro*. After 14 days, exposure of all three TNBC cell lines to teriflunomide was associated with significant repression of colony formation in a dose-dependent manner (Figure 1b). TNBC cells formed a large number of colonies, which were clearly visible; however, when cells were grown for 2 weeks in the presence of different concentrations of teriflunomide, number and size of TNBC cells colonies were markedly decreased. In three TNBC cell lines, consistent with SRB data as shown in Figure 1(a), teriflunomide at 100 μ M caused a dramatic reduction of the colony number compared with untreated cells.

Teriflunomide induces S-phase arrest in TNBC

We then investigated the effect of teriflunomide on cell cycle distribution of TNBC cells following treatment with this agent over a 48-h period. The analysis (Figure 2) revealed cell cycle distribution of untreated cells showing 54.95%, 51.96%, and 48.77% in G₁, 17.52%, 20.08%, and 17.84% in G₂/M, 27.52%, 27.97%, and 33.42% cells in S-phase for MDA-MB-468, BT549 and MDA-MB-231, respectively. It is interesting to note that when BT549 cells were treated with teriflunomide at the lower concentration of 25 μ M for 48 h, the proportion of cells in G₁ was reduced to 42.29% and that in G₂/M down to 9.57%, and 48.14% occurred in S-phase, whereas at the highest drug concentration a relatively marked accumulation of MDA-MB-468 and MDA-MB-231 cells in S-phase (41.54% and 47.56%) occurred. Taken together, these results suggested that teriflunomide treatment for 48 h induced the entrapment of TNBC cells in the S-phase and concomitant reduction of cells in both G₁- and G₂/M-phase, in a dose-dependent manner, resulting in an effective loss of cell cycle transition, and therefore, leading to diminished cell growth.

Teriflunomide induces apoptosis in TNBC

To determine whether cell death was attributable to apoptosis, TNBC cells were treated with teriflunomide at 50 μ M and 100 μ M for 48 h. As evident from Figure 3, in TNBC cells exposed to 50 μ M teriflunomide, only slight percentage increases of PI-positive cells could be found, whereas at high concentration, significant increases of necrotic cells, about twofold increases, could be observed. Regarding early apoptosis, only BT549 cells with teriflunomide treatment showed approximately twofold increase in Annexin-V+/PI- staining. Thus, high doses of teriflunomide for 2 days induced significant necrosis and minor apoptosis in

TNBC cells, which were in agreement with its anti-proliferative activity.

Teriflunomide inhibits migration in TNBC

Additionally, the effect of teriflunomide on TNBC cell migration was tested using a Boyden chamber assay. Because long-term treatment of teriflunomide was toxic, we investigated the inhibitory effect on migration and invasion of TNBC cells in short duration. Results in Figure 4 indicated that the migratory abilities were decreased significantly in dose-dependent manner, to different extents, after incubation of TNBC cells with various concentrations of teriflunomide for 18 h. The agent could inhibit cell motility by greater than 40% at 100 μ M and even 80% at 200 μ M compared with that in untreated cells, showing noticeable migration defects, even counting the minor inhibition of cell proliferation, respectively.

Teriflunomide inhibits invasion in TNBC

We next assayed whether teriflunomide could change the capacity of invasion of TNBC cells. As shown in Figure 5, incubated with teriflunomide at concentration of 100 μ M and 200 μ M, TNBC cells showed diminished invasion through a reconstituted basement membrane (Matrigel) in transwell assays after 24 h, and invasiveness was reduced considerably in a concentration-dependent manner. These results suggested that teriflunomide inhibited migration and invasion *in vitro* in TNBC cells.

Teriflunomide down-regulates proliferative proteins in TNBC

To exam whether inhibition of proliferation of TNBC cells by teriflunomide was due to the suppression of key regulators involved in cell growth, such as cyclin D1 and c-Myc, we evaluated their protein levels after receiving teriflunomide treatment. Our analysis showed that the expression of cyclin D1 and c-Myc was decreased significantly in BT549 and MDA-MB-231 cells at the concentration of 50 μ M and the maximum inhibition could be found at 100 μ M for 96 h (Figure 6). However, in MDA-MB-468 cells, we did not observe the similar dose-dependent down-regulation of cyclin D1 and c-Myc: decreased markedly at a lower concentration of 25 μ M and slightly raised at serial concentrations but still in lower level compared with that in untreated cells.

Teriflunomide modulates cell cycle regulators in TNBC

We then investigated the mechanisms of teriflunomide-induced S-phase arrest by focusing on the expression level of cell cycle regulators such as p27, RB, and cyclins. As shown in Figure 7, upon teriflunomide treatment, an evident dose-dependent decrease in the level of p27 was observed in all three TNBC cell lines, indicating loss of ability to cause cells to arrest in the G₁ phase of cell cycle. Next, in contrast to cyclin D1 in Figure 6, cyclin A, important for progression through S-phase and G₂/M transition, showed significant increases in TNBC cells following exposure to teriflunomide and thus implying a delay in S-phase. Moreover, cyclin B1, an M-phase kinase, and RB hyper-phosphorylation, the G₁ blocker, showed no changes in TNBC cells. These findings

Figure 2 Teriflunomide blocked TNBC cells in S-phase. MDA-MB-468, BT549, and MDA-MB-231 cells were treated for 48 h with vehicle or teriflunomide (25, 50 and 100 μM). The fraction of cells in G1, S and G2/M phase was analyzed by flow cytometry and data of one representative experiment ($n = 3$) are graphed. (A color version of this figure is available in the online journal.)

confirmed the results observed in cell cycle analysis and further indicated the perturbation of cell cycle progression arose at putative S-phase checkpoint.

Teriflunomide regulates cell survival signals via activation of MAPK pathway in TNBC

To investigate the molecular mechanisms underlying the pro-apoptotic activity in TNBC cells, we evaluated the

effect of teriflunomide on the key regulators involved in cell death. As shown in Figure 8, the treatment of MDA-MB-468 and BT549 cells with teriflunomide caused significant increase in the expression of pro-apoptotic protein Bax, accompanied by marked decrease of anti-apoptotic Bcl-xL, meaning the anti- versus pro-apoptotic balance has been shifted. And, interestingly, in MDA-MB-231 cells, at lower concentrations of 25 μM and 50 μM, the trend in response to teriflunomide was found opposite to that in other two

Figure 3 Teriflunomide induced apoptosis in TNBC cells. Flow cytometric analyses of Annexin-V/PI staining show the induction of apoptosis in TNBC cells with 50 and 100 μ M teriflunomide for 2 days. Percentages of cells undergoing early and late apoptosis from one representative experiment ($n=3$) are presented. (A color version of this figure is available in the online journal.)

TNBC cell lines described previously, which, however, was restored finally coupled to the increasing concentration to 100 μ M. We then addressed the question whether the teriflunomide-induced apoptosis was associated with the activation of MAPKs; the activity of ERK, p38, and JNK in the teriflunomide-treated cells was examined. The results showed that teriflunomide dose-dependently activated ERK, p38, and JNK in three TNBC cell lines, more evident in MDA-MB-468 cells, indicating involvement of MAPK pathway in cell death induction.

Teriflunomide leads to suppression of marker signals involved in migratory and invasive potential in TNBC

The data shown in Figures 5 and 6 clearly demonstrate the ability of teriflunomide to attenuate the motility and invasion of TNBC cells. To gain an insight into the underlying mechanisms, we analyzed the expression of several

proteins associated with epithelial-mesenchymal transition (EMT) and metastasis. As shown in Figure 9, E-cadherin, the epithelial cell marker, was elevated in three TNBC cell lines, more notably in BT549, in a dose-dependent fashion under 24-h treatment of 50 μ M, 100 μ M, and 200 μ M of teriflunomide. Meanwhile, expression levels of mesenchymal cell marker Vimentin, as well as the key EMT inducer Slug and Snail, were all decreased in teriflunomide-treated TNBC cells under the same conditions. Collectively, teriflunomide inhibited the EMT process in TNBC cells and induced the cells to gain epithelial properties in a dose-dependent manner.

The key endopeptidase, MMP9, involved in ECM degradation in invasion process, was further tested in teriflunomide-treated TNBC cells and found to be suppressed in a dose-dependent manner. In addition, we also assessed the status of FAK/Src, which are crucial upstream molecules in

Figure 4 Teriflunomide blocked migration of TNBC cells. MDA-MB-468, BT549, and MDA-MB-231 cells were treated with the indicated drug at 50, 100, and 200 μ M and assessed for migration by transwell analysis after 18 h. The representative images and analysis of the migrating cells were shown (mean $\pm$ SD, $n = 3$). Asterisks indicate $P < 0.05$. (A color version of this figure is available in the online journal.)

Figure 5 Teriflunomide blocked invasion of TNBC cells. With matrigel-coated transwell system, after receiving treatment of teriflunomide at concentrations of 100 and 200 μ M for 24 h, invaded TNBC cells were stained. Representative images and analysis of cell invasion are displayed (mean $\pm$ SD, $n = 3$). Asterisks indicate $P < 0.05$. (A color version of this figure is available in the online journal.)

modifying tumor metastasis. Of note, we found teriflunomide substantially suppressed the phosphorylation of both FAK and Src, in a dose-dependent manner, indicating the inactivation of FAK-Src complex in TNBC by teriflunomide.

Teriflunomide leads to down-regulation of onco-proteins involved in growth maintenance in TNBC

TNBC tumors express several receptors, such as insulin-like growth factor receptor (IGF1R), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 4 (FGFR4), showed to augment proliferative potential through activation of c-Raf protein serine-threonine kinase. As shown in Figure 10, we confirmed the constitutive expression of Raf-1, EGFR, and IGF1R in all three TNBC

cell lines and detected that it was suppressed by treatment with teriflunomide for 96 h in dose-dependent manner, with maximum inhibition occurring at 100 μ M. By contrast, we noticed that FGFR4 was only in accumulation of significant levels in BT549 cells, however decreased drastically by teriflunomide in dose-dependent manner as well. These data implied that the cytotoxicity of teriflunomide on TNBC was partially through the inhibition of expression of TNBC-specific growth factor receptors.

Discussion

Women with TNBC, a distinct subgroup lacking estrogen and progesterone receptors and amplification of HER2 gene, do not benefit from endocrine therapy or

Figure 6 Teriflunomide suppressed pro-proliferation molecules in TNBC cells. MDA-MB-468, BT549, and MDA-MB-231 cells were treated with teriflunomide (25, 50 and 100 μM) for 4 days and then subjected to western blotting using anti-c-Myc and anti-cyclin D1 antibodies. Representative data are presented ($n=3$)

trastuzumab. Chemotherapy is currently the mainstay systemic medical treatment; however, TNBC patients still have high risk for recurrence and disease progression after receiving regimens of chemotherapy.²¹ Lack of responses to some of the most effective therapies available for breast cancer treatment warrants new therapeutic strategies to improve the prognosis of TNBC.²² In the present study, we provided evidence that teriflunomide might have chemopreventive potential against TNBC by affecting multiple cell signaling molecules. We found that this immunomodulatory drug suppressed the expression of various growth factor receptors such as EGFR, IGF1R, and FGFR4 in TNBC cells and gene products involved in tumor cell survival and proliferation. Teriflunomide also modulated marker signals involved in EMT and invasion of TNBC cells and inhibited activation of FAK/Src complex.

Teriflunomide has been reported to inhibit the proliferation of human melanoma cells in culture and in xenotransplant animal models either alone or in combination with BRAF inhibitor through abrogating the transcriptional elongation of Myc target genes.¹⁴ However, the lethal effect of this agent has not been systematically investigated in TNBC. In TNBC cells, not only the inhibition of one of the Myc target genes cyclin D1 but unexpected direct suppression of Myc could also be observed. In multiple myeloma cells, cyclin D1 was found to be one target molecule of teriflunomide.¹⁷ Our data also supported recent findings that TNBC cells exhibited elevated Myc expression, as well as altered expression of Myc regulatory gene, resulting in activation of Myc pathway^{23,24} Importantly, depletion of cyclin D1 or Myc has been found to elicit dramatic effects on the viability of TNBC,^{23,24} indicating the cytotoxicity of teriflunomide was tightly associated with down-regulation of these two key regulators.

Consistent with the data published by other groups,^{11,13,16,17} we also showed that teriflunomide led to induction of apoptosis processes in TNBC cells. After teriflunomide treatment of TNBC cells, BAX was up-regulated, whereas Bcl-xL was down-regulated, displacing the balance between pro- and anti-apoptotic BCL-2 family members towards apoptosis.²⁵ And, in myeloma cells, teriflunomide could also elevate BAX and suppress Bcl-xL significantly.¹⁷ Of note, in some refractory cancers, such as fludarabine-resistant CLL¹¹ and endocrine-resistant breast cancer,²⁶

Figure 7 Teriflunomide altered the expression of cell cycle-related proteins in TNBC cells. All cells were treated with 25, 50, and 100 μM teriflunomide for 96 h. After treatment, the expression of p27, cyclin A, cyclin B1, and p-RB were measured by western blot analysis. Representative data are presented ($n=3$)

Figure 8 Teriflunomide modulated cell survival signals and MAPK pathway in TNBC cells. Lysates from teriflunomide (25, 50, and 100 μ M) treatment for 96 h with western blot revealed significant reduction in expression of Bcl-xL, increase in BAX and activation of ERK, p38, and JNK. Representative data are presented ($n = 3$)

Figure 9 Teriflunomide suppressed EMT process and invasion of TNBC cells. 50, 100, and 200 μ M of teriflunomide was added to culture of TNBC cells; 24 h later, proteins of EMT markers (E-cadherin, Vimentin, Slug, Snail), invasion marker (MMP9), and FAK/Src were determined by western blot. Representative data are presented ($n = 3$)

Bcl-xL was induced in acquisition of drug resistance and teriflunomide reversed its expression. Bcl-xL, an apoptosis-counteracting member of Bcl-2 family, was expressed at higher levels in TNBC, a type of clinically refractory breast cancer, and contributed to tumor progression and resistance to chemotherapy provoked apoptosis²⁷ and, conversely, Bcl-xL suppression by either Bcl-xL antisense or its inhibitors (ABT-737 and ABT-263) showed promise for inducing apoptosis in TNBC cells and increasing sensitivity

to killing by anti-cancer drugs.²⁸ Along these lines, the substantial decrease of Bcl-xL total protein suggested its degradation in response to teriflunomide contribution to apoptosis in TNBC cells. Interestingly, low-dose leflunomide, the parent compound of teriflunomide, even reduced apoptosis induced by several anticancer agents in erythroleukemia cells by reducing MAPK activities and activating PI3K/Akt pathway, showing protective effects opposite to that in TNBC at 100 μ M.²⁹ Thus, further experiments, in

Figure 10 Teriflunomide suppressed onco-proteins in driving enhanced growth of TNBC cells. All cells were incubated with increasing concentration of teriflunomide and expression of Raf-1, EGFR, IGF-1R, and FGFR4 were assessed by western blot. Representative data are presented ($n = 3$)

variable doses and in different cancer context, are needed to thoroughly clarify the distinct effects and mechanisms of teriflunomide or leflunomide. The phosphorylated form of ERK, p38, or JNK has been shown to act in mediating apoptosis in a wide variety of cancer types in response to cytotoxic stimuli,^{30,31} and in regulating cytokines secretion after receiving teriflunomide in rheumatoid arthritis (RA),³² indicating that activation of these kinases contributed to the pro-apoptotic activity of teriflunomide in TNBC cells.

The anti-proliferative effect of teriflunomide was associated with a change of cell cycle distribution in S-phase. However, exposure to low micromolar concentration of teriflunomide could promote cell cycle arrest in G1-phase in multiple myeloma cells,¹⁷ endocrine-resistant breast cancer cells,²⁶ and chronic lymphocytic leukemia cells,¹² in S-phase in prostate cancer cells,¹³ premalignant and malignant cutaneous keratinocytes,³³ or in G2/M phase in gastrointestinal carcinoid cells,¹⁹ indicating tumor-type-dependent regulation of cell cycle by teriflunomide and, when our data in TNBC were also included, implying the S-phase delay in solid cancers more likely. In prostate cancer, DHODH inhibition-dependent mechanism was found to be involved in teriflunomide-related S-phase arrest.¹³ However, in TNBC cells, we focused on the cell cycle regulators. RB tumor suppressor is a critical negative regulator of multiple cellular processes and, when hypophosphorylated, can inhibit cell progression along the cell cycle through G1 into S and thus block DNA replication. Conversely, inactivation of RB protein after phosphorylation contributes to cancer development and progression, and is one of the most fundamental events in cancer biology.³⁴ In all three untreated TNBC cell lines, the hyperphosphorylation status of RB occurred and teriflunomide treatment did not affect the status of phosphorylation significantly, indicating the first checkpoint of G1/S is compromised allowing cells to enter into S-phase freely. We also examined expression of the other two putative regulators of G1/S checkpoint after receiving teriflunomide, the cyclin D1 and p27. Cyclin D1 binds with CDK4, forming cyclin D1/CDK4 complex, and initiates the phosphorylation of RB, disrupting RB-mediated transcriptional repression of target genes involved in cell progression through G1.^{35,36} In the presence

of teriflunomide, a significant suppression of cyclin D1 in TNBC cells was observed, with concomitant decrease in level of p27, an inhibitor of catalytic activity of CDK4, leading to the persistence of RB in the inactivated status. In contrast to cyclin D1, cyclin A, accompanied by CDK2, an important mediator of drug-induced accumulation of cells in the S phase and apoptosis,^{37,38} showed significant increase in TNBC cell lines following cell exposure to teriflunomide and thus indicating a delay in the S-phase. In accordance with the involvement of the ERK activity in regulation of Cyclin A-dependent S-phase arrest,³⁷ we also confirmed the activation of ERK in teriflunomide-treated TNBC cells. Moreover, cyclin B1, in complex with CDK1, functioning as the M-phase regulator,³⁹ was not significantly affected by teriflunomide in TNBC cells, indicating no perturbation of G2/M phase.

In this study, we also demonstrated for the first time that teriflunomide could diminish the highly invasive and migratory potential of TNBC cells. Increasing evidences have suggested that in breast cancer, malignant cancer cells undergoing an EMT became more motile, especially in the most lethal and aggressive subtype, TNBC.^{21,40} EMT, a developmental process with loss of epithelial cell polarity and acquisition of mesenchymal phenotype, can be regulated by several transcription factors, including Snail and Slug which repress E-cadherin transcription and increase Vimentin expression, and contributes to migratory and invasive properties in TNBC.⁴¹ Strategies selectively targeting EMT-associated molecules have emerged as putative therapeutic approaches in TNBC⁴² and as shown in our study, teriflunomide could become a promising new agent against EMT. Since the Src/FAK complex, also one molecular marker for TNBC,⁴⁰ plays a prominent role in cell invasion and in other tumor progression-associated events, including EMT and development of metastasis,⁴² we analyzed the effect of teriflunomide on the activity of this complex and found the agent could inhibit its phosphorylation events significantly. With respect to MMPs, not in cancers but in rheumatoid synovial fibroblasts, some of them were inhibited by teriflunomide efficiently.⁴³ MMP9 has previously been identified as a downstream target of Src/FAK signaling.⁴⁴ Consistent with this and with the critical role of

MMP9 in invasion, teriflunomide may suppress TNBC invasion by blocking MMP9 expression.

Several studies linked TNBC to some growth factor receptors, such as EGFR⁴⁵ and IGF1R,⁴⁶ which were considered as major oncogenic factors and attractive therapeutic targets in the management of TNBC patients. Aberrant activation of these receptors has been reported to be involved in dysfunctional biological activities, such as tumor proliferation, decreased apoptosis and differentiation, and increased angiogenesis, migration, and metastasis, and lead to dysregulation of multiple malignancy driving molecules, including cyclin D1, c-Myc, and Src/FAK.^{45,46} However, unexpectedly, receptor-targeted inhibitors in TNBC have not been confirmed promising efficacy in clinical trials, partially caused by multiple compensatory mechanisms and complexity of growth factor receptor pathways, indicating selection of additional targets for combinational therapy or available multiple-target agents. Actually, in contrast to single target inhibition treatment, teriflunomide offered the possibility to optimize anticancer treatment by fighting TNBC on different molecules. Interestingly, we also revealed that teriflunomide could suppress the expression of FGFR4 (but not FGFR1-3), whose knockdown showed anti-survival effect in TNBC cells in a human kinome RNAi library screen, and silencing of its ligand triggered ERK activation.⁴⁷

Collectively, for the first time, our findings revealed that a multitude of TNBC malignancy driving proteins could be recognized by teriflunomide, including signaling pathways involved in proliferation, cell cycle distribution, anti-apoptotic potential, migration, and invasion. Overall, teriflunomide might represent further promising therapeutic options for TNBC, but still needs to be evaluated in appropriate phase III clinical trials, and significant new targets of teriflunomide can be identified with further studies.

Authors' contributions: All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript; MJ and ZX conceived and designed the experiments; MJ and MG wrote the manuscript; OH, WZ, QZ, and XX conducted the experiments; HL, DM, and MG contributed reagents/materials; OH and WZ contributed equally to this work.

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REFERENCES

- Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* 2014;**64**:9–29
- Comprehensive molecular portraits of human breast tumours. *Nature* 2012;**490**:61–70
- Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumours. *Nature* 2000;**406**:747–52
- Prat A, Adamo B, Cheang MC, Anders CK, Carey LA, Perou CM. Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. *Oncologist* 2013;**18**:123–33
- Boyle P. Triple-negative breast cancer: epidemiological considerations and recommendations. *Ann Oncol* 2012;**23**(Suppl 6): vi7–12
- Ali R, Nicholas RS, Muraro PA. Drugs in development for relapsing multiple sclerosis. *Drugs* 2013;**73**:625–50
- Oh J, O'Connor PW. Teriflunomide. *Neurol Clin Pract* 2013;**3**:254–60
- Tallantyre E, Evangelou N, Constantinescu CS. Spotlight on teriflunomide. *Int MS J* 2008;**15**:62–8
- Wiese MD, Rowland A, Polasek TM, Sorich MJ, O'Doherty C. Pharmacokinetic evaluation of teriflunomide for the treatment of multiple sclerosis. *Expert Opin Drug Metab Toxicol* 2013;**9**:1025–35
- Oh J, O'Connor PW. Safety, tolerability, and efficacy of oral therapies for relapsing-remitting multiple sclerosis. *CNS Drugs* 2013;**27**:591–609
- Dietrich S, Kramer OH, Hahn E, Schafer C, Giese T, Hess M, Tretter T, Rieger M, Hülle J, Zenz T, Ho AD, Dreger P, Luft T. Leflunomide induces apoptosis in fludarabine-resistant and clinically refractory CLL cells. *Clin Cancer Res* 2012;**18**:417–31
- Ringshausen I, Oelsner M, Bogner C, Peschel C, Decker T. The immunomodulatory drug Leflunomide inhibits cell cycle progression of B-CLL cells. *Leukemia* 2008;**22**:635–8
- Hail N Jr, Chen P, Bushman LR. Teriflunomide (leflunomide) promotes cytostatic, antioxidant, and apoptotic effects in transformed prostate epithelial cells: evidence supporting a role for teriflunomide in prostate cancer chemoprevention. *Neoplasia* 2010;**12**:464–75.
- White RM, Cech J, Ratanasirintrawoot S, Lin CY, Rahl PB, Burke CJ, Langdon E, Tomlinson ML, Mosher J, Kaufman C, Chen F, Long HK, Kramer M, Datta S, Neuberger D, Granter S, Young RA, Morrison S, Wheeler GN, Zon LI. DHODH modulates transcriptional elongation in the neural crest and melanoma. *Nature* 2011;**471**:518–22
- O'Donnell EF, Koppapapu PR, Koch DC, Jang HS, Phillips JL, Tanguay RL, Kerkvliet NI, Kolluri SK. The aryl hydrocarbon receptor mediates leflunomide-induced growth inhibition of melanoma cells. *PLoS One* 2012;**7**:e40926
- Hail N Jr, Chen P, Rower J, Bushman LR. Teriflunomide encourages cytostatic and apoptotic effects in premalignant and malignant cutaneous keratinocytes. *Apoptosis* 2010;**15**:1234–46
- Baumann P, Mandl-Weber S, Volkl A, Adam C, Bumeder I, Oduncu F, Schmidmaier R. Dihydroorotate dehydrogenase inhibitor A771726 (leflunomide) induces apoptosis and diminishes proliferation of multiple myeloma cells. *Mol Cancer Ther* 2009;**8**:366–75
- Somnay Y, Chen H, Kunnimalaiyaan M. Synergistic effect of pasireotide and teriflunomide in carcinoids in vitro. *Neuroendocrinology* 2013;**97**:183–92
- Cook MR, Pinchot SN, Jaskula-Sztul R, Luo J, Kunnimalaiyaan M, Chen H. Identification of a novel Raf-1 pathway activator that inhibits gastrointestinal carcinoid cell growth. *Mol Cancer Ther* 2010;**9**:429–37
- Xu X, Shen J, Mall JW, Myers JA, Huang W, Blinder L, Saclarides TJ, Williams JW, Chong AS. In vitro and in vivo antitumor activity of a novel immunomodulatory drug, leflunomide: mechanisms of action. *Biochem Pharmacol* 1999;**58**:1405–13
- Perou CM. Molecular stratification of triple-negative breast cancers. *Oncologist* 2011;**16**(Suppl 1): 61–70
- Baselga J, Gomez P, Greil R, Braga S, Climent MA, Wardley AM, Kaufman B, Stemmer SM, Pègo A, Chan A, Goeminne JC, Graas MP, Kennedy MJ, Ciruelos Gil EM, Schneeweiss A, Zube A, Groos J, Melezinková H, Awada A. Randomized phase II study of the anti-epidermal growth factor receptor monoclonal antibody cetuximab with cisplatin versus cisplatin alone in patients with metastatic triple-negative breast cancer. *J Clin Oncol* 2013;**31**:2586–92
- Horiuchi D, Kusdra L, Huskey NE, Chandriani S, Lenburg ME, Gonzalez-Angulo AM, Creasman KJ, Bazarov AV, Smyth JW, Davis SE, Yaswen P, Mills GB, Esserman LJ, Goga A. MYC pathway activation in

- triple-negative breast cancer is synthetic lethal with CDK inhibition. *J Exp Med* 2012;**209**:679–96
24. Dai M, Al-Odaini AA, Fils-Aime N, Villatoro MA, Guo J, Arakelian A, Rabbani SA, Ali S, Lebrun JJ. Cyclin D1 cooperates with p21 to regulate TGFbeta-mediated breast cancer cell migration and tumor local invasion. *Breast Cancer Res* 2013;**15**:R49
 25. Korsmeyer SJ. BCL-2 gene family and the regulation of programmed cell death. *Cancer Res* 1999;**59**:1693s–700s
 26. Huang O, Xie Z, Zhang W, Lou Y, Mao Y, Liu H, Jiang M, Shen KW. A771726, an anti-inflammatory drug, exerts an anticancer effect and reverses tamoxifen resistance in endocrineresistant breast cancer cells. *Oncol Rep* 2014;**32**:627–34
 27. Amundson SA, Myers TG, Scudiero D, Kitada S, Reed JC, Fornace AJ Jr. An informatics approach identifying markers of chemosensitivity in human cancer cell lines. *Cancer Res* 2000;**60**:6101–10
 28. O'Toole SA, Beith JM, Millar EK, West R, McLean A, Cazet A, Swarbrick A, Oakes SR. Therapeutic targets in triple negative breast cancer. *J Clin Pathol* 2013;**66**:530–42
 29. Leger DY, Liagre B, Beneytout JL. Low dose leflunomide activates PI3K/Akt signalling in erythroleukemia cells and reduces apoptosis induced by anticancer agents. *Apoptosis* 2006;**11**:1747–60
 30. Wada T, Penninger JM. Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* 2004;**23**:2838–49
 31. Cagnol S, Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death-apoptosis, autophagy and senescence. *FEBS J* 2010;**277**:2–21
 32. Vergne-Salle P, Leger DY, Bertin P, Treves R, Beneytout JL, Liagre B. Effects of the active metabolite of leflunomide, A77 1726, on cytokine release and the MAPK signalling pathway in human rheumatoid arthritis synoviocytes. *Cytokine* 2005;**31**:335–48
 33. Hail N Jr, Chen P, Kepa JJ, Bushman LR. Evidence supporting a role for dihydroorotate dehydrogenase, bioenergetics, and p53 in selective teriflunomide-induced apoptosis in transformed versus normal human keratinocytes. *Apoptosis* 2012;**17**:258–68
 34. Dick FA, Rubin SM. Molecular mechanisms underlying RB protein function. *Nat Rev Mol Cell Biol* 2013;**14**:297–306
 35. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer* 2011;**11**:558–72
 36. Bienvenu F, Jirawatnotai S, Elias JE, Meyer CA, Mizeracka K, Marson A, Frampton GM, Cole MF, Odom DT, Odajima J, Geng Y, Zagodzina A, Jecrois M, Young RA, Liu XS, Cepko CL, Gygi SP, Sicinski P. Transcriptional role of cyclin D1 in development revealed by a genetic-proteomic screen. *Nature* 2010;**463**:374–8
 37. Yang TY, Chang GC, Chen KC, Hung HW, Hsu KH, Sheu GT, Hsu SL. Sustained activation of ERK and Cdk2/cyclin-A signaling pathway by pemetrexed leading to S-phase arrest and apoptosis in human non-small cell lung cancer A549 cells. *Eur J Pharmacol* 2011;**663**:17–26
 38. Romani AA, Desenzani S, Morganti MM, La Monica S, Borghetti AF, Soliani P. Zoledronic acid determines S-phase arrest but fails to induce apoptosis in cholangiocarcinoma cells. *Biochem Pharmacol* 2009;**78**:133–41
 39. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature* 2004;**432**:316–23
 40. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, Pietersen JA. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest* 2011;**121**:2750–67
 41. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;**144**:646–74
 42. Engebraaten O, Volla HK, Borresen-Dale AL. Triple-negative breast cancer and the need for new therapeutic targets. *Am J Pathol* 2013;**183**:1064–74
 43. Migita K, Miyashita T, Ishibashi H, Maeda Y, Nakamura M, Yatsushashi H, Ida H, Kawakami A, Aoyagi T, Kawabe Y, Eguchi K. Suppressive effect of leflunomide metabolite (A77 1726) on metalloproteinase production in IL-1beta stimulated rheumatoid synovial fibroblasts. *Clin Exp Immunol* 2004;**137**:612–6
 44. Kim YH, Kwon HJ, Kim DS. Matrix metalloproteinase 9 (MMP-9)-dependent processing of betaig-h3 protein regulates cell migration, invasion, and adhesion. *J Biol Chem* 2012;**287**:38957–69
 45. Burness ML, Grushko TA, Olopade OI. Epidermal growth factor receptor in triple-negative and basal-like breast cancer: promising clinical target or only a marker? *Cancer J* 2010;**16**:23–32
 46. Castano Z, Marsh T, Tadipatri R, Kuznetsov HS, Al-Shahrour F, Paktinat M, Greene-Colozzi A, Nilsson B, Richardson AL, McAllister SS. Stromal EGF and igf-I together modulate plasticity of disseminated triple-negative breast tumors. *Cancer Discov* 2013;**3**:922–35
 47. Kai HT, Boon ST, Heng LC, Ammu KR, Rozita R, Soon KC. RNAi screening of human kinome identified fibroblast growth factor receptor 4 (FGFR4) as potential molecular target in basal-like breast cancers (BLBC). *Cancer Res* 2013; [abstract]. In: *Proceedings of the 104th Annual Meeting of the American Association for Cancer Research*; 6–10 Apr 2013; Washington, DC. Philadelphia (PA): AACR; *Cancer Res* 2013; **73**(8 Suppl): Abstract nr 1134. doi:10.1158/1538-7445.AM2013-1134

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