

# Role of Harmaline in Inhibiting c-Myc, Altering Molecular Typing, and Promoting Apoptosis in Triple-Negative Breast Cancer

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**Objective:** Triple-negative breast cancer (TNBC) lacks effective targeted, endocrine therapeutic agents and the development of novel agents is costly and time-consuming. The objective of this study was to identify pharmaceuticals and natural products utilized in clinical practice that have the potential to inhibit the expression of Cellular-myelocytomatosis oncogene (c-Myc), based on a review of the current literature. The aim was to assess the effect of the specified drugs on c-Myc expression in TNBC cells, determine the most potent inhibitor, and evaluate its impact on TNBC cell proliferation, invasive migration, and apoptosis, as well as the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) at both the gene and protein levels. Explore its potential for treatment or adjuvant therapy for triple-negative breast cancer.

**Methods:** Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were used to quantify gene and protein expression levels. Flow cytometry was employed to measure cell proliferation and apoptosis, while the Transwell assay was utilized to assess cell invasion and migration.

**Results:** Harmaline emerged as the strongest inhibitor, significantly decreasing the expression of c-Myc at both the gene and protein levels in TNBC cells. It also inhibited cell proliferation, invasion, and migration while promoting apoptosis in TNBC cells. Additionally, there was a varying increase in the expression of ER and PR genes and proteins. While the expression of the HER-2 gene was elevated, there was no significant change in HER-2 protein levels. Notably, the expression of the phosphorylated HER-2 protein increased.

**Conclusion:** Harmaline was found to promote apoptosis and inhibit cell proliferation, invasion, and migration in TNBC cells by targeting the inhibition of c-Myc. It also induced the re-expression of the ER, PR, and HER-2 genes, as well as the ER and PR proteins.

**Keywords:** c-Myc, harmaline, molecular typing, triple-negative breast cancer

## Introduction

In recent years, with an increasing incidence rate, breast cancer has surpassed lung cancer to become the most common malignant tumor in women worldwide.<sup>1</sup> The proportion of young women affected by breast cancer in China is higher than in other countries,<sup>2</sup> posing a serious threat to the physical and mental health of women. Triple-negative breast cancer (TNBC), which accounts for 15%–20% of breast cancers, is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2).<sup>3</sup> TNBC lacks effective targeted therapy and endocrine therapy drugs. These aspects contribute to the aggressive nature of TNBC, poor prognosis, and a high recurrence of metastasis, often occurring 1–3 years post-surgery. The search for new therapeutic approaches in TNBC has been the focus of much research.<sup>4,5</sup>

In TNBC, the expression of the nuclear transcription factor c-Myc is significantly elevated and associated with poor prognosis compared to other breast cancer subtypes.<sup>6,7</sup> c-Myc is a member of the MYC family and is commonly expressed and abundant in proliferating cells.<sup>8,9</sup> The inhibition of c-Myc leads to the arrest of proliferation, induction of cellular senescence and promotion of apoptosis in tumor cells.<sup>10–13</sup> Other studies have shown that c-Myc is closely associated with the expression levels of ER, PR, and HER-2,<sup>14,15</sup> and in ER-positive breast cancers, the c-Myc expression level is correlated with endocrine therapy resistance;<sup>16</sup> in HER-2-positive breast cancers, the effect of their anti-HER-2 therapy is positively correlated with the level of phosphorylated HER-2(p-HER-2),<sup>17,18</sup> and c-Myc in turn interacts with HER-2 and enhances HER-2 expression.<sup>19</sup> Previous studies by the research team have found that knocking down c-Myc in TNBC cells can increase their apoptosis, inhibit invasion and migration, and inhibit the growth of solid tumors in animal experiments. Therefore, targeting c-Myc represents a potential strategy for developing targeted therapies.

The targeted inhibition of c-Myc has been extensively studied. However, due to the nuclear localization of c-Myc, which renders it inaccessible to antibodies and lacking enzyme sites where conventional small molecules can bind, there are no drugs that can directly target c-Myc. The expression of c-Myc is mainly reduced by mechanisms that directly inhibit its activity or indirectly regulate its protein stability.<sup>20,21</sup> For example, the function of c-Myc can be inhibited by targeting the BET family-related proteins,<sup>22</sup> or by stabilizing the structure of G-quadruplexes.<sup>23</sup> Although some existing small molecule and peptide inhibitors, such as MYCMI7,<sup>24</sup> sAJM589, and 10058-F4,<sup>25,26</sup> have shown promising results in animal models, their rapid clearance in vivo<sup>9</sup> and high cost have limited their clinical applications. Currently, Omomyc is the only inhibitor that has progressed to clinical Phase II trials.<sup>27</sup> As of now, there is no c-Myc inhibitor that has been approved for clinical use.

To reduce the R&D cost of c-Myc inhibitors, we investigated natural compounds and drugs used in clinical practice that could inhibit c-Myc expression, to explore their potential as c-Myc inhibitors for the treatment of TNBC. By reviewing the literature, several natural compounds and drugs used in clinical practice have been found to inhibit the expression of c-Myc. Notable examples include:

1) Aspirin: Aspirin has been reported to reduce the risk of breast cancer in general, particularly hormone receptor-positive tumors, and breast cancer in situ in postmenopausal women.<sup>28</sup> It inhibits the proliferative capacity of cancer cells by reducing the abnormal expression of the c-Myc oncoprotein through the inhibition of platelet-induced molecular signaling.<sup>29</sup> 2) Primaquine: Primaquine belongs to the sulphonamide family. It reduces c-Myc transcript and protein levels via the nEGFR/STAT3 pathway and induces apoptosis in breast cancer cells through co-downregulation of c-Myc/Bcl-2.<sup>30</sup> Additionally, Mabeta et al found that the primaquine-cinnamic acid complex could inhibit the growth of breast cancer cells.<sup>31</sup> 3) Metformin: Metformin has demonstrated potential as an adjuvant anticancer drug.<sup>32</sup> In breast cancer, it has been found to inhibit cancer cells by impeding glucose metabolism and increasing immune responses.<sup>33</sup> Yang et al found that metformin-induced ferroptosis could significantly inhibit breast cancer growth.<sup>34</sup> Furthermore, metformin leads to the dephosphorylation of the c-Myc protein and thus reducing its functional role.<sup>35</sup> 4) Curcumin: Curcumin triggers apoptosis in breast cancer cells by inhibiting c-Myc back to its threshold level and through the MYC-E2F-1-BCL-2 axis pathway.<sup>36</sup> 5) Celastrol: Celastrol significantly inhibits the expression of c-Myc and Stat3 at both the transcriptional and protein levels. The inhibition of Stat3 expression also results in the downregulation of c-Myc expression.<sup>37</sup> 6) Diclofenac: Diclofenac inhibits TNBC proliferation by downregulating c-Myc and inhibiting glycolysis pathway.<sup>38</sup> 7) Harmaline: Harmaline is a  $\beta$ -carboline alkaloid with antitumor effects.<sup>39</sup> Harmine is a derivative of this compound and its anti-tumor effects have been widely studied.<sup>40</sup>

A review of the literature reveals that harmaline and its derivatives can inhibit the proliferation, migration, and invasion of breast cancer cells via multiple pathways.<sup>41,42</sup> Harmine has been shown to regulate the expression of c-Myc by modulating the activity of dual-specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2).<sup>43</sup> Furthermore, it has also been documented in the literature that harmine targets TAZ in breast cancer cells to reverse epithelial-mesenchymal transition (EMT) progression and thereby inhibit the proliferation and invasion of breast cancer cell lines.<sup>44</sup> In TNBC, harmine has been found to reduce the growth rate of TNBC cells through both endogenous and exogenous apoptotic mechanisms.<sup>45</sup>

In summary, c-Myc represents a potential target for the treatment of TNBC. Although there have been studies on c-Myc inhibitors, there are currently no c-Myc inhibitors available for clinical use, primarily due to their limited in vivo efficacy. Our objective in this study was to identify the most potent inhibitors of c-Myc expression in TNBC from among seven natural compounds reported in the literature to have c-Myc inhibitory effects, as well as those that have been used

in clinical settings. The aim was to use the most potent inhibitors to further investigate their effects on the functions and phenotypes of TNBC. Additionally, we sought to conduct preliminary explorations on the potential for these compounds to target c-Myc and their utility as adjuvants to improve the prognosis of patients with TNBC.

## Materials and Methods

### Cell Culture and Grouping

The MDA-MB-231 human breast cancer cells we used were from Pricella Biotechnology Co.Ltd, and the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium with 10% fetal bovine serum (FBS) at 37°C in an environment of 95% air and 5% CO<sub>2</sub>.

The experiment was structured into the following groups:

Blank control group, negative control group, c-Myc knockdown group (c-Myc-shRNA group), harmaline group, and c-Myc-shRNA+harmaline group.

### Construction of a TNBC Cell Line with Stable Low Expression of c-Myc Lentivirus Construction

The target gene shRNA was inserted into the lentiviral plasmid vector GV248 (shRNA). The target vector and other auxiliary packaging original vector plasmid (pspx2) were subjected to high-purity, endotoxin-free extraction. They were then co-transfected into 293T cells using plasmid transfection reagents. Six hours after transfection, the cells were provided with a complete medium. The cell supernatant enriched with lentiviral particles was collected after 48 and 72 hours of incubation. Subsequently, the viral supernatant was concentrated by ultracentrifugation, and the viral titer was subsequently calculated. Thus, the GV248-target gene-shRNA lentiviral vector was obtained.

### Construction of Stabilized Cell Lines

1. The target cells were inoculated into 48-well plates until they reached a confluence of 70%–80%.
2. After the cells adhered to the wall, puromycin was added to identify the lowest lethal concentration of empty cells. The drug concentration gradient was set at 1 µg/mL, 2.5 µg/mL, 5 µg/mL, and 10 µg/mL (final concentration) for the screening.
3. The lowest drug concentration at which all the cells died after 48 hours of drug treatment was selected as the final concentration of the drug for the subsequent selection of the stabilized strain.
4. The target cells were inoculated into 24-well plates and infected with lentivirus after the cells had adhered to the surface. On the second day post-infection, puromycin was added to the cells for screening. The expression of tracer genes in the surviving cells was observed under a microscope, and the positivity rate was estimated.
5. The digested cells were seeded into 96- or 384-well plates, and the density of inoculated cells was adjusted to 1–5 cells/well. The inoculation density was tailored based on the percentage of cells expressing tracer genes. For instance, if 90% of cells were positive, the inoculation density was adjusted to 1.5 cells per well.
6. The cells were cultured for approximately two weeks, with the medium changed every other day. Once the cells in the well plates formed clones, the clones expressing the tracer gene were selected. The mRNA levels of the target gene were verified using the real-time PCR method. Subsequently, the positive cell clones were selected and expanded in culture until the desired cell volume was achieved.

### Detection of Cell Proliferation and Apoptosis Using Flow Cytometry

Cell sample preparation: the culture medium from the treated cells was aspirated into a suitable centrifuge tube. The adherent cells were washed twice with PBS, and an appropriate amount of trypsin, pre-warmed to 37°C, was added to digest the cells. The cells were incubated at 37°C until they detached from the substrate. An equal volume of complete medium was added. The cell culture solution was collected and gently mixed until homogeneous, then transferred to a centrifuge tube and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, and the cells were washed with 5 mL of PBS. They were then centrifuged at 1200 rpm for 5 minutes, and the cells were washed again.

A total of 100  $\mu\text{L}$  of Annexin V conjugate was added to the collected cells, which were then gently resuspended. The cells were passed through a 200-mesh sieve to create a single-cell suspension. A total of 5  $\mu\text{L}$  of Annexin V-FITC and 5  $\mu\text{L}$  of propidium iodide (PI) staining solution were added, gently mixed, and incubated at room temperature for 15 minutes, protected from light. Subsequently, 400  $\mu\text{L}$  of Annexin V conjugate solution was added to the tube. Flow cytometry was conducted within 30 minutes. Green fluorescence was detected for Annexin V-FITC, and red fluorescence was detected for PI.

## Transwell Detection of Cell Invasion and Migration

A total of 100  $\mu\text{L}$  of diluted Matrigel was added vertically to the center of the bottom of the upper chamber of the Transwell apparatus. The chamber was incubated for 4 to 5 hours to allow the Matrigel to solidify into a gel.

Cells were inoculated into the prepared Transwell chambers that had been treated with Matrigel. The inoculation was performed according to the experimental grouping design. After inoculation, the chambers were carefully removed with tweezers, washed twice with PBS, and transferred to wells pre-filled with approximately 4% formaldehyde for fixation for 20 minutes at room temperature. Subsequently, the chambers were taken out, washed twice with PBS, and transferred to wells pre-filled with methanol for permeabilization for 20 minutes at room temperature. Finally, the chambers were transferred to wells pre-filled with Giemsa staining solution (A:B = 1:3), stained for 5 minutes at room temperature, and protected from light. The chambers were then removed, washed twice with PBS, and the cells on the membrane surface at the bottom of the upper chamber were carefully wiped off with a wet cotton swab. The chambers were then dried with the bottom side up. The cells were transferred to a slide, counted under the microscope, and the results were recorded and analyzed.

## Detection of Gene Expression Using qRT-PCR

RNA was extracted from cell lysates using the Trizol method. The reference reaction system for cDNA synthesis included the following components: Total RNA: 7  $\mu\text{L}$ , Or Random Primer (0.1  $\mu\text{g}/\mu\text{L}$ ): 1  $\mu\text{L}$ ,  $2\times$  TS Reaction Mix: 10  $\mu\text{L}$ , TransScript@RT/RI Enzyme Mix: 1  $\mu\text{L}$ , gDNA Remover: 1  $\mu\text{L}$ , and RNase-free Water: to make up a total volume of 20  $\mu\text{L}$ . The reaction mixture was subjected to reverse transcription for cDNA synthesis, followed by fluorescence quantification using the SYBR Green method to quantify gene expression levels. The primer information is listed below (Table 1).

**Table 1** Primer Information Table for Fluorescence Quantitative mRNA Assay

| Primer name  | Sequences, (5' to 3')   | Product size |
|--------------|-------------------------|--------------|
| MYC-F1       | AACACACAACGTCTTGGAGC    | 193bp        |
| MYC-R1       | CCGCAACAAGTCCTCTTCAG    |              |
| MYC-F1       | AACACACAACGTCTTGGAGC    | 193bp        |
| MYC-R1       | CCGCAACAAGTCCTCTTCAG    |              |
| ER-F2        | CCATATCCACCGAGTCCTGG    | 184bp        |
| ER-R2        | CACCACGTTCTTGCACTTCA    |              |
| PR-F1        | ATGGAAGGGCAGCACAATA     | 186bp        |
| PR-R1        | GAGAGCAACAGCATCCAGTG    |              |
| HER2-F1      | GGTGGTCTTTGGGATCCTCA    | 171bp        |
| HER2-R1      | ACCTTCACCTTCCTCAGCTC    |              |
| hsa GAPDH_F2 | GGAGCGAGATCCCTCCAAAAT   | 197bp        |
| hsa GAPDH_R2 | GGCTGTTGTCATACTTCTCATGG |              |

## Detection of Protein Expression Using Western Blot

Total protein extraction: the cell samples were washed with PBS, and then 300  $\mu$ L of Radioimmunoprecipitation Assay (RIPA) lysate was added and thoroughly mixed. After being placed at 4°C for 60 minutes and homogenized, the samples were centrifuged at 12,000 rpm for 15 minutes at 4°C, and the supernatant containing the total protein was collected.

Subsequently, gel preparation, spiking, and electrophoresis were carried out. The electrophoresis was initiated with an 80V constant voltage to facilitate the separation of the gel using bromophenol blue, followed by a constant pressure of 100V for 90 minutes.

Membrane transfer: after sodium dodecyl-sulfate polyacrylamide gel (SDS-PAGE) electrophoresis, the polyvinylidene fluoride (PVDF) membrane was soaked in methanol for 10 seconds and rinsed in distilled water for 1 minute. Subsequently, the polyacrylamide gel, filter paper, and treated PVDF membrane were immersed in the transfer buffer for 10 minutes. The components were assembled into the transmembrane “sandwich” for the protein transfer process. Post-transfer, the PVDF membrane was washed three times with water for 10 minutes each.

The membrane was sealed with 5% skimmed milk powder for 1 hour and then washed three times with Tris-buffered saline with 0.1% Tween<sup>®</sup> 20 detergent (TBST) for 10 minutes each time. Subsequently, incubation with primary and secondary antibodies was performed according to the manufacturer’s instructions.

A color development solution was prepared, and 2 mL of it was added to the membrane. Subsequently, the reaction was detected and imaged using a chemiluminescence imaging instrument.

## Statistical Analysis

All data were entered into Excel spreadsheets. The gene expression data obtained by qRT-PCR and Western blot experiments were expressed as the mean  $\pm$  standard deviation (). The data for each group were statistically analyzed using SPSS 26.0 software. If the data followed a normal distribution, one-way analysis of variance (ANOVA) was employed to assess statistical significance among the groups. Where variances were homogenous, multiple comparisons were conducted using the least significant difference (LSD) method. If the variance was heterogeneous, multiple comparisons were performed using the Tamhane method. If the data did not conform to a normal distribution, log transformation was applied to normalize the data before conducting statistical analysis using the one-way ANOVA method as described above. A significance level of  $P < 0.05$  was considered to indicate statistical significance. Statistical graphs were generated using GraphPad Prism 9.5 software, with statistical significance denoted by \* for  $P < 0.05$ .

## Results

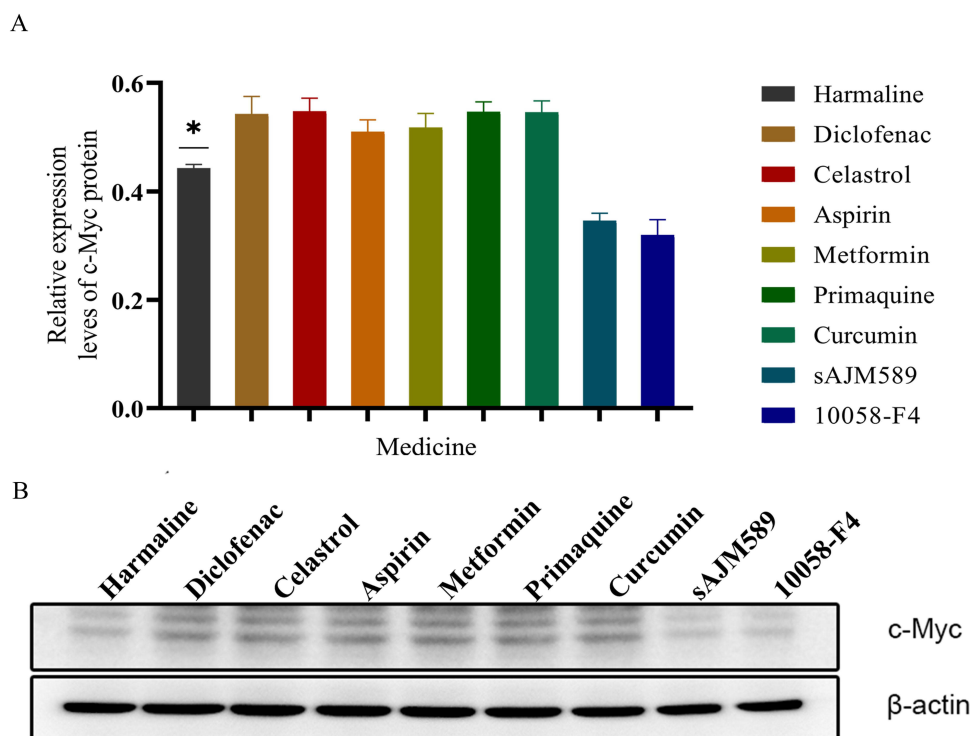
### Harmaline Was the Most Potent Inhibitor of c-Myc in TNBC

In this study, the following seven drugs that can inhibit c-Myc expression were initially selected based on available literature to evaluate their inhibitory efficacy on c-Myc expression TNBC cells: harmaline, diclofenac, celastrol, aspirin, metformin, primaquine, and curcumin—all these are from natural compounds and drugs that have been used in clinical settings. Two small-molecule c-Myc inhibitors, saJM589 and 10058-F4, were used as controls for comparative analysis.

Subsequently, MDA-MB-231 cells were treated individually with these nine compounds. A Western blot assay was conducted to assess their inhibition of c-Myc protein expression in TNBC. The results indicated that harmaline ranked second only to the small-molecule inhibitors saJM589 and 10058F4 in inhibiting c-Myc protein expression in TNBC (0.443 vs 0.346 and 0.320, respectively;  $P < 0.05$ ). Harmaline exhibited the most potent inhibitory effect on c-Myc compared to the other drugs (0.443 vs 0.543, 0.548, 0.510, 0.518, 0.547, and 0.546;  $P < 0.05$ , Figure 1). Given these results, harmaline was selected to investigate its impact on TNBC cell proliferation, invasive migration, apoptosis, and the expression of the ER, PR, and HER-2 genes and proteins.

### Harmaline Inhibited TNBC Proliferation and Invasive Migration and Promoted Apoptosis

Flow cytometry results showed that, compared with the blank control and negative control groups, there was a significant decrease in the cell proliferation rate of the knockdown c-Myc group while the apoptosis rate significantly increased ( $P <$



**Figure 1** (A) c-Myc protein expression in MDA-MB-231 cells following treatment with nine drugs (\*:  $P < 0.05$ ); (B) c-Myc protein banding in MDA-MB-231 cells following treatment with nine drugs.

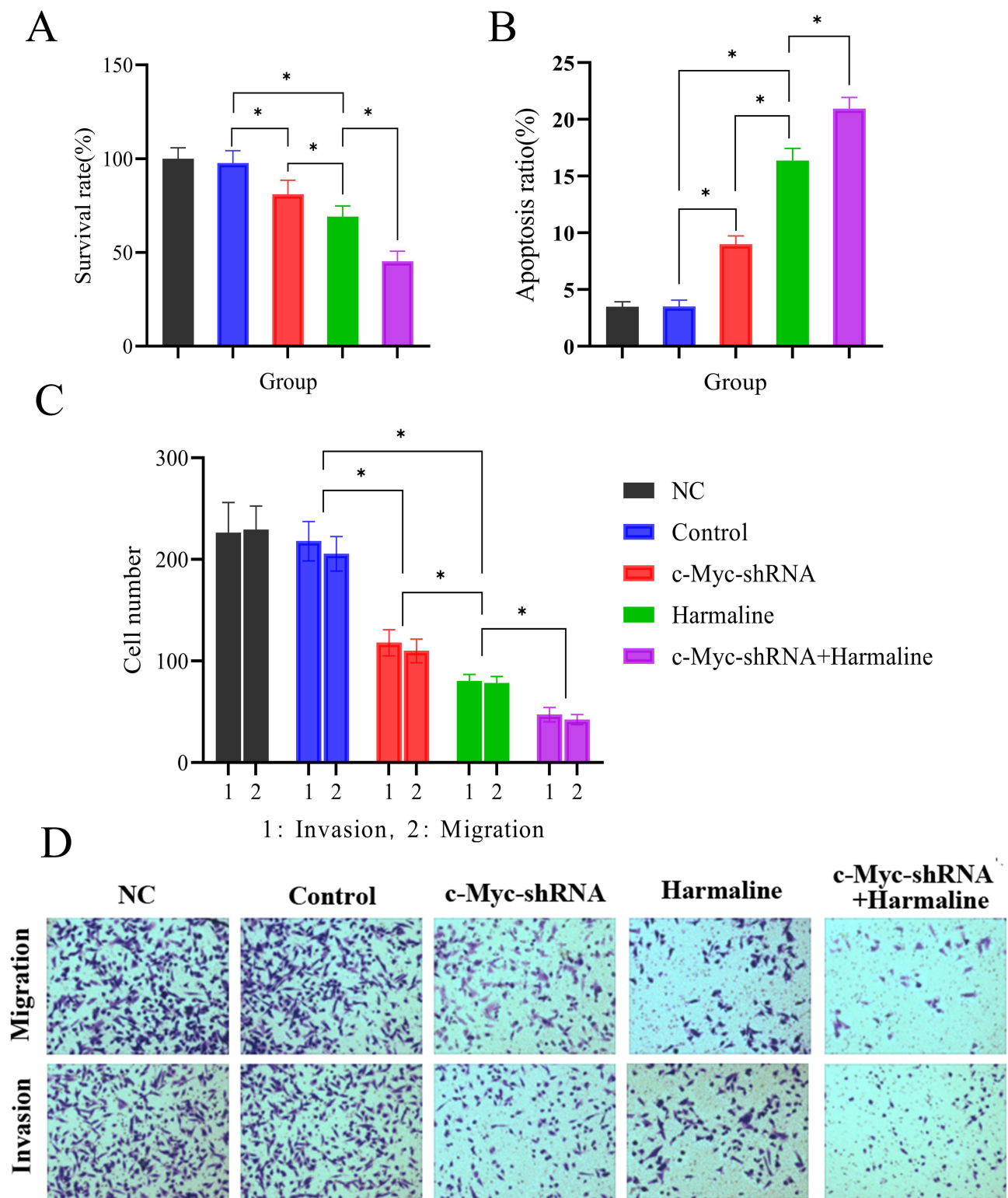
0.05). Additionally, compared with the blank control, negative control, and knockdown c-Myc groups, the cell proliferation rate in the harmaline group was significantly reduced, and the apoptosis rate was significantly increased ( $P < 0.05$ ). Furthermore, cell lines with knockdown c-Myc that were treated with harmaline showed a further reduction in the cell proliferation rate and a significant increase in the apoptosis rate ( $P < 0.05$ , Figure 2A and B).

The Transwell assay revealed that the number of membrane-permeable cells in the knockdown c-Myc group was significantly reduced, and the cell invasion and migration ability were weakened ( $P < 0.05$ ) compared with the blank control and negative control groups. Moreover, there was a significant reduction in the number of membrane-permeable cells in the harmaline group, and the cell invasion and migration ability were significantly attenuated ( $P < 0.05$ ) compared with the blank, negative control, and c-Myc knockdown groups. Furthermore, there was a significant reduction in the number of membrane-permeable cells in c-Myc knockdown cell lines treated with additional harmaline ( $P < 0.05$ , Figure 2C and D).

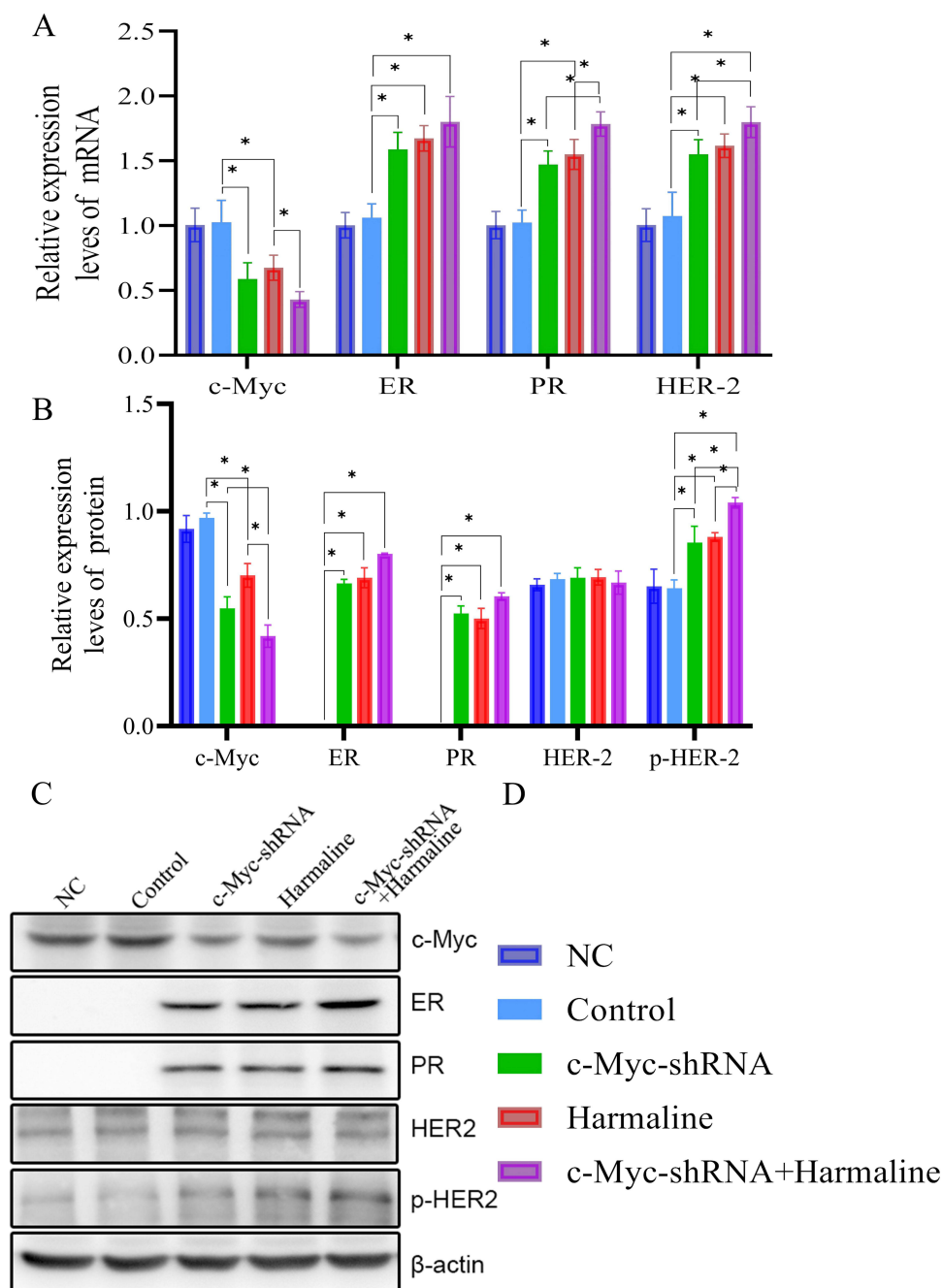
These findings suggest that harmaline was effective in reducing cellular proliferation and invasive migration across all experimental groups while enhancing their apoptotic ability.

## Harmaline Attenuated c-Myc Expression in TNBC Cells and Altered Molecular Typing

The results of qRT-PCR indicated that the expression levels of ER, PR, and HER-2 genes were significantly upregulated in TNBC cells following the knockdown of c-Myc, as compared to the blank control and negative control groups ( $P < 0.05$ ). Harmaline intervention resulted in a significant reduction in the expression of the c-Myc gene, while the expression of ER, PR, and HER-2 genes increased in TNBC cells when compared with the blank control and negative control groups ( $P < 0.05$ ). Further treatment with harmaline in the cell lines with c-Myc knockdown resulted in a further significant decrease in c-Myc gene expression and a further elevation of PR gene expression as opposed to the use of harmaline alone ( $P < 0.05$ ). Additionally, PR and HER-2 gene expression were significantly elevated ( $P < 0.05$ ) compared with knockdown of c-Myc alone (Figure 3A).



**Figure 2** (A) Histogram of cell proliferation rates of MDA-MB-231 cells in the blank control, negative control, c-Myc-shRNA, harmaline, and c-Myc-shRNA+harmaline groups as detected by flow cytometry (\*:  $P < 0.05$ ); (B) Histogram of the apoptosis rates of MDA-MB-231 cells in the blank control, negative control, c-Myc-shRNA, harmaline, and c-Myc-shRNA+harmaline groups as detected by flow cytometry (\*:  $P < 0.05$ ). (C) Histogram of the number of membrane-permeable cell counts in MDA-MB-231 cells assessed using a Transwell assay in the blank control, negative control, c-Myc-shRNA, harmaline, and c-Myc-shRNA+harmaline groups; 1: cell invasion, 2: cell migration (\*:  $P < 0.05$ ). (D) Cell migration (top) and invasion (bottom) in the blank control, negative control, c-Myc-shRNA, harmaline, and c-Myc-shRNA+harmaline groups as detected by a Transwell assay of MDA-MB-231 cell lines.



**Figure 3** (A) qRT-PCR analysis of c-Myc, ER, PR, and HER-2 gene expression in MDA-MB-231 cells in the blank control, negative control, c-Myc-shRNA, harmaline, and c-Myc-shRNA+harmaline groups (\*:  $P < 0.05$ ). (B) Western blot analysis of c-Myc, ER, PR, HER-2, and p-HER-2 protein expression levels in MDA-MB-231 cells in the blank control, negative control, c-Myc-shRNA, harmaline, and c-Myc-shRNA+harmaline groups (\*:  $P < 0.05$ ). (C) Western blot analysis of c-Myc, ER, PR, HER-2, and p-HER-2 protein bands in MDA-MB-231 cells of the blank control, negative control, c-Myc-shRNA, harmaline, and c-Myc-shRNA+harmaline groups. (D) Grouping.

Western blot analysis revealed that the expression of ER and PR proteins significantly increased in TNBC cells following c-Myc knockdown compared to the blank and negative control groups ( $P < 0.05$ ). When compared with the blank control group and negative control group, harmaline intervention resulted in a significant decrease in the expression of the c-Myc protein in TNBC cells ( $P < 0.05$ ). Additionally, the expression of ER and PR proteins was significantly elevated in comparison with the blank control group and negative control group ( $P < 0.05$ ). Further intervention with harmaline in the c-Myc knockdown cell lines showed a further reduction in c-Myc protein expression ( $P < 0.05$ ) with no

significant changes in ER and PR ( $P > 0.05$ ) compared to the harmaline alone and knocked-down c-Myc alone groups. HER-2 protein expression showed no significant change ( $P > 0.05$ ) across all groups (Figure 3B and C).

HER-2-positive breast cancers are usually more aggressive, but our results were contrary to this. We then reviewed the literature. It has been reported in earlier literature that the expression of phosphorylated HER-2 (p-HER-2) protein is negatively correlated with the expression of HER-2 protein. Increased expression of phosphorylated HER-2 protein leads to better outcome and prognosis.<sup>17,18</sup> Therefore, in our experiment, we used Western blot analysis to detect the expression level of phosphorylated HER-2 in TNBC cells. The results showed that, compared with the blank control group and negative control group, knocking down c-Myc in TNBC cells led to a significant elevation in the expression of the p-HER-2 protein ( $P < 0.05$ ). The expression level of p-HER-2 protein increased when Harmaline was used alone to intervene in TNBC cells ( $P < 0.05$ ). Furthermore, intervening with harmaline in TNBC cell lines with knocked-down c-Myc resulted in a further significant increase in the expression of the p-HER-2 protein ( $P < 0.05$ ) (Figure 3B and C).

These findings suggest that the decreased HER-2 protein expression in TNBC may be associated with its phosphorylation.

## Discussion

TNBC is notably resistant to immune and targeted therapies due to the absence of ER, PR, and HER-2 expression. This, combined with the lack of effective treatment modalities and its high aggressiveness, results in a poor prognosis for patients with TNBC. Consequently, there is an urgent need for the development of new therapeutic targets and agents.

As a proto-oncogene, c-Myc is involved in various aspects of tumorigenesis and development. The expression level of c-Myc has been found to be correlated with the prognosis of many types of tumors, including TNBC.<sup>6,7</sup> Furthermore, the proliferative, invasive, and migratory abilities of TNBC decrease after knocking down c-Myc,<sup>46</sup> as demonstrated by our findings in this study. While there has been extensive research on the effects of targeting c-Myc, direct inhibition of c-Myc expression remains unfeasible currently. However, it is possible to modulate c-Myc expression by inhibiting its activity or interfering with its transcription and translation processes. Therefore, c-Myc presents a promising new target for TNBC. Indirect targeting of c-Myc to treat TNBC can be used as an adjuvant strategy to enhance the therapeutic efficacy of existing drugs or to improve overall treatment outcomes.

Ideally, the aberrant transcription activity of c-Myc could be halted by targeting c-Myc indirectly through strategies such as using drugs to prevent its dimerization with Max, thereby preventing the c-Myc-MAX dimer from binding to DNA. However, the intrinsically disordered nature of unbound c-Myc presents significant challenges for drug development. Although several small-molecule c-Myc inhibitors that can significantly reduce c-Myc expression at the cellular level have been identified, these compounds are generally characterized by low bioavailability, high toxicity, and a short half-life in vivo.<sup>47</sup> Due to these limitations, there is currently only one small-molecule c-Myc inhibitor, Omomyc—which is reverse-transcribed from part of the structure of c-Myc—that has entered Phase II clinical trials.<sup>48</sup> As of now, there are no effective c-Myc inhibitors available for clinical use.

Our literature review revealed certain drugs and natural compounds used in clinical settings that can also inhibit c-Myc expression. The primary advantage of these agents is that their toxicity and in vivo utilization in the human body have been verified. In this study, seven of these drugs and compounds previously reported in the literature to inhibit the expression of c-Myc were selected, with two small-molecule inhibitors additionally chosen as controls. Thus, a total of nine drugs were applied to TNBC cells individually to identify the most potent inhibitors. The results showed that harmaline significantly inhibited c-Myc protein levels in TNBC, and its effect was comparable to that of small-molecule c-Myc inhibitors (10058F4 and sAJM589).

Harmaline was initially used in the adjuvant treatment of gastrointestinal tumors, with positive outcomes. It was also found to inhibit the proliferation, migration, and invasion of breast cancer cells through various pathways. Its derivatives, such as harmine, exhibited superior anti-tumor effects and lower side effect profiles.<sup>39–44</sup> In the present study, harmaline was utilized as a target drug to further investigate its effects on TNBC cells. Concurrently, lentiviral transfection techniques were employed to establish TNBC cell lines with low c-Myc expression to serve as a control. This approach was used to observe the changes in the function of TNBC cells and the expression of receptors, with the aim of exploring the potential of harmaline to enhance the efficacy of treating TNBC cells by targeting c-Myc or serving as an adjuvant drug.

Our results showed that harmaline significantly inhibited the proliferation, invasion, and migration of TNBC cells and accelerated the apoptosis of TNBC cells, showing greater efficacy than in TNBC cells with low c-Myc expression. In the experiment, we also found that when the cells with low c-Myc expression were additionally treated with harmaline, the proliferation, invasion, and migration of TNBC cells were further decreased, and apoptosis was enhanced. This suggests that harmaline may influence the proliferation, invasive migration, and apoptosis of TNBC cells by targeting pathways other than c-Myc.

When we examined the TNBC cell phenotype, variations in the expression levels of ER and PR receptors in TNBC cells were observed. Following harmaline intervention, the ER and PR genes and proteins were re-expressed, while the expression pattern of the HER-2 gene and protein differed. The HER-2 gene expression was upregulated after harmaline intervention, but there was no significant change in the expression of HER-2 protein levels.

HER-2-positive breast cancer is often highly invasive, however, in this study, Harmaline promoted apoptosis and inhibited proliferation, invasion and migration of TNBC cells, and at the same time elevated the expression of HER-2 gene but there was no significant change in HER-2 protein. The efficacy of the HER-2-targeted drug trastuzumab has been reported to be dependent on the phosphorylation of HER-2 in HER-2-negative breast cancer cell lines.<sup>17</sup> Patients with positive phosphorylated HER-2 (p-HER-2) who were treated with the anti-HER-2 drug trastuzumab exhibited longer progression-free survival (PFS) and better treatment efficacy.<sup>18</sup> In this study, the p-HER-2 protein expression level was further examined in the cells of each experimental group. The results showed that p-HER-2 protein expression levels were significantly elevated after harmaline intervention and Knockdown of c-Myc. Additionally, the p-HER-2 protein levels were further elevated in TNBC cells with knocked-down c-Myc after treatment with harmaline. These differences were statistically significant, aligning with reports in the literature.<sup>17,18</sup> So we think that after the high expression of HER-2 gene in this study, theoretically its protein expression should also be elevated. However, due to some mechanism, the HER-2 protein was phosphorylated to become phosphorylated HER-2 protein. This led to the phenomenon that HER-2 gene was highly expressed but promoted apoptosis and inhibited proliferation, invasion and migration of TNBC cells. The mechanisms involved deserve to be explored further. This finding further confirms the potential of harmaline as an effective c-Myc inhibitor for the treatment of TNBC and provides a basis for exploring the mechanisms underlying the re-expression of ER, PR, and HER-2.

## Conclusion

In this study, Harmaline had the strongest inhibitory effect on c-Myc compared to the other six drugs screened and was comparable in effect to the c-Myc inhibitors saJM589 and 10058F4. Harmaline was found to enhance apoptosis as well as inhibit the proliferation, invasive and migration of TNBC cells. It also facilitated the re-expression of ER and PR genes and proteins and the re-expression of HER-2 genes and p-HER-2 proteins by targeting and inhibiting c-Myc. However, there was no significant change in HER-2 protein expression. Further exploration of the mechanism by which Harmaline derivatives induce typing changes by targeting c-Myc in TNBC may open up new possibilities for improving therapeutic outcomes in TNBC.

## Abbreviations

c-Myc, Cellular-myelocytomatosis oncogene; TNBC, Triple-Negative Breast Cancer; ER, Estrogen Receptor; PR, Progesterone Receptor; HER-2, Human Epidermal growth factor Receptor-2; miRNA, microRNA; E-box, CACGTG; lncRNA, Long non-coding RNA; Stat3, Signal Transducer And Activator Of Transcription 3; GLUT1, Glucose transporter type 1; HK, Hexokinase; DYRK2, Dual-specificity tyrosine phosphorylation-Regulated Kinase; TAZ, transcriptional co-activator with PDZ-binding motif; Bcl-2, B-cell lymphoma-2; ERK, Extracellular regulated protein kinases; Akt, protein kinase B; EMT, Epithelial-Mesenchymal Transition; shRNA, Short hairpin RNA; qRT-PCR, Quantitative reverse transcription polymerase chain reaction; WB, Western Blot; p-HER-2, Phosphorylated-Human epidermal growth factor receptor -2; N-Myc, MYCN Proto-Oncogene; L-Myc, MYCL Proto-Oncogene; bHLHZip, basic-helix-loop-helix-leucine zipper.

## Ethics Approval

This study did not involve human or animal subjects, and thus, no ethical approval was required. The study protocol adhered to the guidelines established by the journal.

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## Disclosure

The authors declare that they have no competing interests.

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