A Novel Taspine Derivative, HMQ1611, Inhibits Breast Cancer Cell Growth via Estrogen Receptor α and EGF Receptor Signaling Pathways

Yingzhuan Zhan¹, Yanmin Zhang¹, Cuicui Liu¹, Jie Zhang¹, Wanli W. Smith², Nan Wang¹, Yinnan Chen¹, Lei Zheng¹, and Langchong He¹

Abstract

Breast cancer is a common cancer with a leading cause of cancer mortality in women. Currently, the chemotherapy for breast cancer is underdeveloped. Here, we report a novel taspine derivative, HMQ1611, which has anticancer effects using in vitro and in vivo breast cancer models. HMQ1611 reduced cancer cell proliferation in four human breast cancer cell lines including MDA-MB-231, SK-BR-3, ZR-75-30, and MCF-7. HMQ1611 more potently reduced growth of estrogen receptor α (ERα)-positive breast cancer cells (ZR-75-30 and MCF-7) than ERα-negative cells (MDA-MB-231 and SK-BR-3). Moreover, HMQ1611 arrested breast cancer cell cycle at S-phase. In vivo tumor xenograft model, treatment of HMQ1611 significantly reduced tumor size and weight compared with vehicles. We also found that HMQ1611 reduced ERα expression and inhibited membrane ERα-mediated mitogen—activated protein kinase (MAPK) signaling following the stimulation of cells with estrogen. Knockdown of ERα by siRNA transfection in ZR-75-30 cells attenuated HMQ1611 effects. In contrast, overexpression of ERα in MDA-MB-231 cells enhanced HMQ1611 effects, suggesting that ERα pathway mediated HMQ1611’s inhibition of breast cancer cell growth in ERα-positive breast cancer. HMQ1611 also reduced phosphorylation of EGF receptor (EGFR) and its downstream signaling players extracellular signal—regulated kinase (ERK)1/2 and AKT activation both in ZR-75-30 and MDA-MB-231 cells. These results showed that the novel compound HMQ1611 had anticancer effects, and partially via ERα and/or EGFR signaling pathways, suggesting that HMQ1611 may be a potential novel candidate for human breast cancer intervention. Cancer Prev Res; 1–10. ©2012 AACR.

Introduction

Breast cancer is a common cancer with high mortality among women. Currently the chemotherapy of breast cancer is underdeveloped although there are some molecules that have been identified as drug targets including estrogen receptor α (ERα). ERα plays an important role in tumor formation and progression. More than 70% of breast tumors have been diagnosed as ERα-positive expression (1–3). ERα is a member of the large superfamily of nuclear receptors acting as a ligand-activated transcription factor (4–7), which binds to 17β-estradiol (E2) and combines with specific estrogen response elements (ERE) in the promoters of target genes in the nucleus. In addition to this genomic mechanism of ERα, increasing experimental evidence has showed its nongenomic effect, which can be rapidly activated by estrogen (8–12). The membrane ERα can also transactivate insulin-like growth factor 1 receptor (IGF-1R) and EGF receptor (EGFR)/HER2, which leads to the activation of the downstream extracellular signal-regulated kinases (ERK) signaling cascade (13–15). For ERα-positive breast cancer, anti-ER treatment strategies involve selective estrogen receptor modulators (SERMs), selective estrogen receptor downregulators, and disruption of estrogen synthesis (16–18). However, the long-term anti-ER treatment results in drug resistance due to activation of HER2/EGFR growth factor receptor pathway (19–21). Evidence suggests that EGFR overactivation is frequently related to the development of cancer and increase of tumor cell proliferation (22). There are at least 30% of breast cancers expressing EGFR (23, 24). The EGFR is a transmembrane tyrosine kinase that belongs to the HER/ErbB protein family (25). Ligands such as EGF and TGFα binding to EGFR result in receptor homo- or heterodimerization with another member of the HER/ErbB family, followed by autophosphorylation of the tyrosine residues of the intracellular domain of EGFR/HER1 (26). These tyrosine phosphorylated sites allow proteins to bind through their Src homology 2 (SH2) domains leading to the activation of the downstream...
signaling cascades such as the Ras/mitogen-activated protein kinase (MAPK) pathway, the phosphoinositide-3 kinase (PI3K)/Akt pathway and the phospholipase-Cγ/protein kinase C (PLCγ/PKC) pathway (27–29). These signal pathways regulate cellular proliferation, angiogenesis, and inhibition of apoptosis, which play critical roles in breast cancer pathogenesis. Drugs blocking both the ERα and the EGFR pathways have been reported to enhance antitumor effects compared with those agents that only affect one pathway (30, 31). Thus, development of the novel drugs targeting both ERα and EGFR signaling becomes a promising cancer treatment strategy.

Taspine was initially identified by screens of *Radix et Rhizoma leonticis* (Hong Mao Qi in Chinese) using cell membrane chromatography by Li and He (32). Taspine has many pharmacologic actions such as bacteriostasis, antibiosis, antivirus, anti-inflammatory, antitumor, and anticancer effects (33). Previously, we found that taspine displayed good affinity characteristics in a cell membrane chromatography model stably overexpressing EGFR (34) and could be used as a lead compound for searching novel anticancer agents. We have designed and synthesized a series of ring-opened and biphenyl taspine derivatives with increasing activity and solubility (35–37). With the biologic assay screens of taspine derivatives, we found that compound HMQ1611 (Fig. 1A) exhibits the most potent pharmacologic effects (37). In this study, we further characterized the anticancer effects of HMQ1611 in 4 breast cancer cell lines and an in vivo mouse model. We further investigated the signaling mechanisms underlying HMQ1611 inhibition of breast cancer growth. We found that HMQ1611 inhibited breast cancer growth by blocking both the ERα and the EGFR pathways. These findings suggest that HMQ1611 can be a very promising candidate for breast cancer intervention.

Materials and Methods

**Drugs**

Taspine derivate HMQ1611 was synthesized at our laboratory in the Natural Drug Research and Engineering Center of Xi’an Jiaotong University (Shaanxi Province, PR China) as described (ref. 37; Fig. 1A).

**Cell lines**

ZR-75-30, MCF-7, SK-BR-3, and MDA-MB-231 breast cancer cell lines were obtained from Shanghai Institute of Cell Biology (Shanghai, PR China) in the Chinese Academy of Sciences in 2010. The cumulative culture length of these cells was fewer than 6 months after resuscitation. Cell line authentication was conducted by the provider via DNA fingerprinting, mycoplasma tests, and isozyme detection. In our laboratory, cell morphology, growth curve, and mycoplasma contamination were regularly checked to ensure the absence of contamination during the culture. MCF-7 and ZR-75-30 are ERα-positive expression cell lines (38–41). SK-BR-3 and MDA-MB-231 are ERα-negative cell lines (42, 43). ZR-75-30, MCF-7, and MDA-MB-231 cells were maintained in RPMI-1640 supplemented with 10% (v/v) FBS and SK-BR-3 cells were cultured in Dulbecco’s Minimal Essential Medium (DMEM) supplemented with 20% FBS. All cell lines were incubated at 37°C in a 5% CO2 incubator with saturated humidity.

**Cell viability assay and cell-cycle analysis**

Exponentially growing cells were plated into 96-well plate (Costar), 24 hours after seeding, cells were incubated in the absence or presence of HMQ1611 for the indicated times. The cell viability was evaluated by WST-1 kit (Roche) according to the manufacturer’s instructions.

For cell-cycle analysis, ZR-75-30 cells were treated with HMQ1611 at different concentrations for 48 hours. At the end of treatment, cells were trypsinized and fixed in ice-cold 70% ethanol overnight at 4°C, washed with PBS and stained with RNase and propidium iodide for 30 minutes away from light. At last, cell cycle was analyzed with the flow cytometer (Becton, Dickinson and Company).

**Plasmids and siRNA transfection**

MDA-MB-231 cells were plated at a density of 1 × 10⁴ cells per well in a 24-well plate and then transfected with pReceiver-M60-ERα-GFP construct (GeneCopoeia) for 24 hours using the TurboFect in vitro transfection reagent (Fermentas) according to the manufacturer’s protocol. Transfection efficiency was monitored with GFP. ERα expression levels were detected using Western blot analysis. The transfected cells were seeded for proliferation assays.

A double-stranded siRNA against ERα and nonspecific siRNA (control siRNA) were obtained from Shanghai GenePharma Co., Ltd. ZR-75-30 cells were seeded in a 6-well plate at a density of 5 × 10⁵ cells per well and transfected with the siRNA against ERα for 24 hours at a final concentration of 50 nmol/L with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Transfection with a control siRNA was served as a negative control. Cells were subjected to RT-PCR to detect gene expression and Western blotting to protein expression. The transfected cells were seeded for proliferation assays.

**Immunoblotting analysis**

Cells were harvested and lysed in RIPA Lysis Buffer (Appylen Technologies) supplemented with protease inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets (Roche). The cell lysates were centrifuged at 12,000 × g at 4°C for 10 minutes. Protein concentration of the supernatants was determined by a protein assay kit (Bio-Rad Laboratories). Equivalent amount of protein was resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in TBS containing 0.05% Tween-20 (TBST) and 5% nonfat powdered milk and then probed with different primary and secondary antibodies. Proteins were detected by using enhanced chemiluminescence reagents (Thermo). The primary antibodies include anti-ERα (1:500 dilution; Epitomics, Inc.), anti-EGFR, anti-p-EGFR (1:500 dilution; Cell...
Figure 1. HMQ1611 reduced proliferation of human breast cancer cells. A, diagram of the HMQ1611 synthesis. B, breast cancer cells (MDA-MB-231, SK-BR-3, MCF-7, and ZR-75-30) were treated with HMQ1611 at 0, 2, 10, and 50 μmol/L concentrations. Cell viability was measured after 48 hours using WST-1. Data are represented as means ± SEM from 3 repeated experiments.

C, ZR-75-30 cells were treated with HMQ1611 at indicated concentration for 48 hours. Cells viability was measured after 24, 48, and 72 hours using WST-1. Data are represented as the means ± SEM from 3 repeated experiments.

D, HMQ1611 blocked ZR-75-30 cell cycle at the S-phase. Cells were treated with HMQ1611 for 48 hours and stained with RNase and propidium iodide. The cells were subjected to flow cytometric analysis to detect the DNA content. Data are represented as percentage of cell population in G0–G1, S, and G2–M phases of the cell cycle. Representative images are shown from 3 separated experiments.
Signaling Technology), anti-MEK1/2, anti-p-MEK1/2, anti-ERK1/2, anti-p-ERK1/2, anti-AKT, anti-p-AKT (1:1,000 dilution; Cell Signaling Technology), anti-PLCγ, anti-p-PLCγ (1:1,000 dilution; Upstate), and anti-GAPDH (1:1,000 dilution; Proteintech) antibodies.

**Reverse transcriptase PCR and quantitative real-time PCR**

Total RNA was extracted by the RNAfast2000 kit (Fastagen) according to the manufacturer’s protocol. The reverse transcriptase PCR (RT-PCR) was carried out by PrimeScript RT Master Mix Perfect Real Time Kit (TaKaRa) and a Thermal Cycle Dice Real Time System (TaKaRa). The result was analyzed with the manufacturer’s program (Thermal Cycler Dice Real Time System). The primer sequences were as follows:

GAPDH forward primer: 5’-GCACCGTCAAGGCTGAAAG-3’

GAPDH reverse primer: 5’-TGGTGAAGACGCCAAGCTGA-3’

ERα forward primer: 5’-TGTGCCTGGCTAGAGATCCTGA-3’

ERα reverse primer: 5’-AGCCAGCAGCATGTCGAAGA-3’

Melt curve analysis was conducted at the end of each PCR to confirm the specificity of the PCR product. Threshold cycle (Ct) values of ERα in each sample were normalized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

**In vivo tumor model and immunohistochemistry**

All animal experiments were carried out according to the guidelines and approval of the Institutional Animal Care and Use Committee of Xi’an Jiaotong University. Six-week-old immunodeficient female BALB/c mice were purchased from Shanghai Laboratory Animal center of the Chinese Academy of Sciences and housed under aseptic and ventilated condition. The mice were inoculated by subcutaneous injection into the mammary fat pad with ZR-75-30 cells expressing, real-time PCR and immunoblotting assay were carried out using SYBR Premix Ex Taq II and a Thermal Cycle Dice Real Time System (TaKaRa). The result was analyzed with the manufacturer’s program (Thermal Cycler Dice Real Time System). The primer sequences were as follows:

ERα forward primer: 5’-TGTGCCTGGCTAGAGATCCTGA-3’

ERα reverse primer: 5’-AGCCAGCAGCATGTCGAAGA-3’

Melt curve analysis was conducted at the end of each PCR to confirm the specificity of the PCR product. Threshold cycle (Ct) values of ERα in each sample were normalized with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

To assess the effects of HMQ1611 on breast cancer cell growth, breast cancer cells (MDA-MB-231, SK-BR-3, MCF-7, and ZR-75-30) were treated with HMQ1611 at 0, 2, 10, 50 μmol/L concentrations. Cell viability was measured using WST-1, an antiproliferative assay. MCF-7 and ZR-75-30 cells are positively expressed ERα whereas MDA-MB-231 and SK-BR-3 cell are not (38–43). HMQ1611 decreased the cell viabilities of the 4 types of cancer cells (Fig. 1B). HMQ1611 showed more suppressive effect in MCF-7 and ZR-75-30 cell growth than in MDA-MB-231 and SK-BR-3 cells. Furthermore, HMQ1611 inhibited ERα-positive cells, ZR-75-30 in a dose- and time-dependent manner (Fig. 1C). The 50% growth inhibitory concentrations (IC50) of HMQ1611 at 24, 48, and 72 hours were 29.31, 15.75, and 6.13 μmol/L, respectively.

To further explore the effects of HMQ1611 on the cell cycle, the cell-cycle profiles of ZR-75-30 were assessed with flow cytometric analysis. Cells were treated with HMQ1611 at 2.5, 5, 10 μmol/L concentrations for 48 hours and stained with propidium iodide. The cells were subjected to flow cytometric analysis to detect the DNA content. HMQ1611 treatment caused a significant increase of cells in the S-phase and a corresponding decreased proportion of cells in the G0–G1 and G2–M phases (Fig. 1D). We suggest that S-phase arrest may be due to the G1–S transition increasing in the stage of HMQ1611 treatment.

To further investigate the effect of HMQ1611 on ERα expression, real-time PCR and immunoblotting assay were used to examine mRNA and protein expression after the cells were treated with HMQ1611 for 48 hours. HMQ1611 decreased ERα expression at both the mRNA and the protein levels in a dose-dependent manner in ZR-75-30 cells (Fig. 2A–C). Knockdown of ERα by siRNA in ZR-75-30 significantly attenuated the inhibitory effects of HMQ1611 on proliferation (Fig. 2D–F). The IC50 value of HMQ1611 in ZR-75-30 cells transfected with siRNA targeting ERα was 46.96 μmol/L whereas the IC50 value in cells transfected with control siRNA was only 15.75 μmol/L. In contrast, overexpression of ERα in MDA-MB-231 (ERα-negative) cells strikingly enhanced the inhibitory effects of HMQ1611 on proliferation (Fig. 2G and H) compared with empty vector control cells with the IC50 value at 18.52 and 42.33 μmol/L, respectively.

To further investigate the effect of HMQ1611 on ERα signaling pathway, we examined MAPK activity induced by estrogen in ZR-75-30 cells in which ERα was selectively

**Statistical analysis**

All values are expressed as means ± SEM. Statistics was determined with ANOVA. Results were considered statistically significant if the P value was less than 0.05.

**Results**

**HMQ1611 suppresses breast cancer cell growth**

HMQ1611 was synthesized as described previously (37). To assess the effects of HMQ1611 on breast cancer cell growth, breast cancer cells (MDA-MB-231, SK-BR-3, MCF-7, and ZR-75-30) were treated with HMQ1611 at 0, 2, 10, 50 μmol/L concentrations. Cell viability was measured using WST-1, an antiproliferative assay. MCF-7 and ZR-75-30 cells are positively expressed ERα whereas MDA-MB-231 and SK-BR-3 cell are not (38–43). HMQ1611 decreased the cell viabilities of the 4 types of cancer cells (Fig. 1B). HMQ1611 showed more suppressive effect in MCF-7 and ZR-75-30 cell growth than in MDA-MB-231 and SK-BR-3 cells. Furthermore, HMQ1611 inhibited ERα-positive cells, ZR-75-30 in a dose- and time-dependent manner (Fig. 1C). The 50% growth inhibitory concentrations (IC50) of HMQ1611 at 24, 48, and 72 hours were 29.31, 15.75, and 6.13 μmol/L, respectively.

To further explore the effects of HMQ1611 on the cell cycle, the cell-cycle profiles of ZR-75-30 were assessed with flow cytometric analysis. Cells were treated with HMQ1611 at 2.5, 5, 10 μmol/L concentrations for 48 hours and stained with propidium iodide. The cells were subjected to flow cytometric analysis to detect the DNA content. HMQ1611 treatment caused a significant increase of cells in the S-phase and a corresponding decreased proportion of cells in the G0–G1 and G2–M phases (Fig. 1D). We suggest that S-phase arrest may be due to the G1–S transition increasing in the stage of HMQ1611 treatment.

**ERα signaling pathway partially mediates HMQ1611-suppressing breast cancer cell growth**

To further investigate the effect of HMQ1611 on ERα expression, real-time PCR and immunoblotting assay were used to examine mRNA and protein expression after the cells were treated with HMQ1611 for 48 hours. HMQ1611 decreased ERα expression at both the mRNA and the protein levels in a dose-dependent manner in ZR-75-30 cells (Fig. 2A–C). Knockdown of ERα by siRNA in ZR-75-30 significantly attenuated the inhibitory effects of HMQ1611 on proliferation (Fig. 2D–F). The IC50 value of HMQ1611 in ZR-75-30 cells transfected with siRNA targeting ERα was 46.96 μmol/L whereas the IC50 value in cells transfected with control siRNA was only 15.75 μmol/L. In contrast, overexpression of ERα in MDA-MB-231 (ERα-negative) cells strikingly enhanced the inhibitory effects of HMQ1611 on proliferation (Fig. 2G and H) compared with empty vector control cells with the IC50 value at 18.52 and 42.33 μmol/L, respectively.

To further investigate the effect of HMQ1611 on ERα signaling pathway, we examined MAPK activity induced by estrogen in ZR-75-30 cells in which ERα was selectively
knocked down or the cells were treated with HMQ1611. ZR-75-30 cells were plated in a 6-well plate and the medium was changed into phenol red free RPMI-1640 containing 5% dextran-coated charcoal-stripped FBS. After 24 hours incubation, ZR-75-30 cells were pretreated with 5 μmol/L HMQ1611 for 30 minutes or transfected with siRNA against ERα for 24 hours followed by addition of E2 (0 and 10 nmol/L) for 15 minutes. The cell lysates were subjected to Western blot analysis using antiphosphorylated ERK1/2 and anti-ERK1/2 antibodies. As shown in Fig. 2I and J, treatment with 10 nmol/L E2 for 15 minutes resulted in a 2.5-fold increase of ERK1/2 phosphorylation in ZR-75-30 cells, siRNA targeting ERα, or
treatment of HMQ1611 attenuated E2-induced ERK1/2 phosphorylation.

**EGFR signaling pathway is involved in HMQ1611-suppressing breast cancer cell growth**

We also determined the effect of HMQ1611 on EGFR signaling pathway. ZR-75-30 cells were treated with HMQ1611 for 48 hours followed by 100 nmol/L EGF stimulation for 10 minutes. The cell lysates were subjected to Western blot analysis with various antibodies including anti-EGFR, anti-p-EGFR, anti-MEK1/2, anti-p-MEK1/2, anti-ERK1/2, anti-p-ERK1/2 anti-AKT, anti-p-AKT, anti-PLCγ, anti-p-PLCγ, and anti-GAPDH antibodies. As shown in Fig. 3A, treatment of HMQ1611 significantly decreased EGF-induced EGFR phosphorylation, ERK1/2 phosphorylation, and AKT phosphorylation. There was no change in PLCγ phosphorylation. Moreover, we also found that HMQ1611 reduced expression of p-EGFR by α-screen assay using AlphaScreen SureFire Cellular Kinase Assay Kit (PerkinElmer LAS; data not shown).

Because HMQ1611-induced growth inhibition was observed in MDA-MB-231 cells after HMQ1611 treatment for 48 hours, we investigated the effect of HMQ1611 on EGFR signaling pathway in MDA-MB-231 cells. As shown by immunoblotting, the levels of phosphorylated EGFR, phosphorylated ERK1/2, and phosphorylated AKT proteins were decreased after HMQ1611 treatment for 48 hours (Fig. 3B).

**Figure 3.** EGFR signaling pathway is involved in HMQ1611-inhibitory effect on cancer cell growth. ZR-75-30 cells (A) and MDA-MB-231 cells (B) were treated with HMQ1611 at indicated concentrations for 48 hours. The cells lysates were subjected to Western blot analysis using anti-EGFR, anti-p-EGFR, anti-MEK1/2, anti-p-MEK1/2, anti-ERK1/2, anti-p-ERK1/2 anti-AKT, anti-p-AKT, anti-PLCγ, anti-p-PLCγ, and anti-GAPDH antibodies. Data were represented as means ± SEM. *P < 0.05; **P < 0.01 by ANOVA, compared with the control.
HMQ1611 inhibits tumor growth in mouse model

To determine the effects of HMQ1611 on breast cancer in vivo, we used the ZR-75-30 xenograft mouse tumor model. HMQ1611 treatment was initiated when tumors were palpable and continued 21 days. Table 1 showed that there was significant reduction of tumor weight in the mice treated with HMQ1611 compared with the vehicle control tumor-bearing mice. No body weight loss and any other abnormalities were observed in the HMQ1611-treated mice indicating there were not toxic effects in our treatment regimen. Moreover, ERα expression levels were significantly reduced in tumor-bearing mice treated with HMQ1611 in a dose-dependent manner (Fig. 4).

Discussion

The main findings of this study is that HMQ1611, a novel taspine derivative, can inhibit breast cancer cell growth in cultured cancer cells and tumor-bearing in vivo models by targeting ERα and EGFR. HMQ1611 reduced breast cancer cell proliferation and blocked cancer cells at the S stage. Moreover, HMQ1611 reduced ERα expression and downregulated membrane ERα mediated MAPK signaling in response to E2 and EGFR/ERK1/2, EGFR/Pi3K/AKT pathways. Our findings showed that HMQ1611 can be a potential novel agent for intervention with one compound targeting 2 major pathways involved in breast cancer development.

Breast cancer is a common cancer with high mortality among women. Anti-ER therapy is one of the common used treatments in the cancer clinics. However, long-term anti-ER treatment results in drug resistance that may be likely due to activation EGFR signaling pathways (19–21). Thus, a strategy that dual blockade of EGFR and ER pathways is an important issue in ant-breast cancer drug development. In this study, we found that our newly synthesized compound, HMQ1611, significantly reduced breast cancer growth in 4 breast cancer cell lines including MCF-7, ZR-75-30, MDA-MB-231, and SK-BR-3. Interestingly, we found that HMQ1611 had more potent inhibitory effects in ERα expression–positive cells: MCF-7 and ZR-75-30 than in ERα expression–negative cells: MDA-MB-231 and SK-BR-3, suggesting that ERα is a target of HMQ1611. To further validate this, knockdown of ERα expression in ZR-75-30 cells by siRNA significantly attenuated HMQ1611-inhibitory effects. Moreover, overexpression of ERα in MDA-MB-231 cells significantly enhanced HMQ1611-inhibitory effects. We next investigated the effect of HMQ1611 on ERα signaling pathway. Immunoblotting and real-time PCR analysis showed HMQ1611 downregulated ERα expression.

Table 1. Effect of treatment with taspine derivate HMQ1611 in nude mice bearing ZR-75-30 human breast cancer xenografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial body weight (g)</th>
<th>Initial tumor volume, cm³</th>
<th>Final tumor volume, cm³</th>
<th>Final tumor weight, g</th>
<th>Final body weight, g</th>
<th>Tumor growth inhibition (%)</th>
<th>Ratio of body growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.31 ± 0.98</td>
<td>0.10 ± 0.02</td>
<td>2.71 ± 0.31</td>
<td>3.55 ± 0.43</td>
<td>19.56 ± 1.45</td>
<td>—</td>
<td>1.29</td>
</tr>
<tr>
<td>HMQ1611 (50 mg/kg)</td>
<td>18.44 ± 0.95</td>
<td>0.11 ± 0.02</td>
<td>2.20 ± 0.47*</td>
<td>2.39 ± 0.34*</td>
<td>20.44 ± 1.13</td>
<td>32.74</td>
<td>10.85</td>
</tr>
<tr>
<td>HMQ1611 (100 mg/kg)</td>
<td>19.00 ± 0.75</td>
<td>0.10 ± 0.03</td>
<td>1.54 ± 0.40*</td>
<td>1.77 ± 0.39*</td>
<td>20.86 ± 1.02</td>
<td>50.19</td>
<td>9.79</td>
</tr>
<tr>
<td>HMQ1611 (200 mg/kg)</td>
<td>18.38 ± 1.13</td>
<td>0.10 ± 0.03</td>
<td>0.97 ± 0.58b</td>
<td>1.25 ± 0.52b</td>
<td>20.75 ± 1.19</td>
<td>64.74</td>
<td>12.89</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± SEM.

*P < 0.05.

**P < 0.01 versus control.

Figure 4. HMQ1611 inhibited breast tumor growth in mice. A, the representative images of breast tumors (top) and immunostaining of ERα expression (bottom) using anti-ERα antibodies from each experimental group at the end point. B, quantification of ERα expression from 6 random fields of each section, 3 sections from each animal. Results were quantified using an image analysis system. Data are represented as means ± SEM. ’’, P < 0.05; ’’’, P < 0.01 by ANOVA compared with the vehicle-treated control group.
mRNA and protein expression. More recently it has been reported that ERα associated with membrane mediated MAPK activation induced by estrogen. Downregulation of ERα with specific small inhibitory RNAs blocked E2-induced MAPK phosphorylation (15, 44, 45). We wondered whether membrane-associated ERα also mediated the effect of HMQ1611 on MAPK signaling. In our experiments, we evaluated MAPK signaling in cells transfected with ERα siRNA or treated with HMQ1611. It was found that decreased ERα caused by specific siRNA led to downregulation of p-ERK1/2, suggesting that HMQ1611 might block MAPK signaling through modulating membrane-associated ERα and finally inhibited cell proliferation. Taken together, our data suggested that HMQ1611 reduced breast cancer cell growth via ERα-linked signaling pathway in ERα expression–positive cells.

Given HMQ1611 also reduced cancer cell growth in ERα expression–negative cells: MDA-MB-231 and SK-BR-3, we thought there must be an alternative target. Indeed, we found that HMQ1611 also reduced EGF-induced phosphorylated EGFR, phosphorylated ERK1/2, and phosphorylated AKT in both ZR-75-30 and MDA-MB-231 cells, suggesting that HMQ1611 downregulates the EGFR/MAPK and EGFR/PI3K/AKT pathways. MAPK and PI3K/AKT are 2 major signaling pathways controlling cell survival and cell proliferation pathways (27–29). Thus, our data indicated that EGFR is an alternative target for HMQ1611 in ERα expression–negative cells.

The inhibitory effect of HMQ1611 on breast cancer can also be monitored in vivo. In vivo experiments showed no adverse effects of HMQ1611 when ingested by mice. In addition, we found that HMQ1611 significantly reduced tumor size and weight in tumor-bearing mice compared with vehicle-treated mice in a dose-dependent manner suggesting that HMQ1611 can be a very good candidate for breast cancer intervention. The decreased tumor size was attributed to the reduced tumor cells through specific downregulating ERα expression, which was measured by immunohistochemical staining of tumor tissues.

In summary, we have characterized a novel taspine derivative, HMQ1611 has an anticancer effects in breast cancer cells and in vivo tumor-bearing mice. As depicted in Fig. 5, the inhibitory effects of HMQ1611 on breast cancer cell are through 2 pathways: (i) ERα pathway: HMQ1611 regulated ERα expression and membrane-associated E2/ERα pathway, membrane-associated E2/ERα pathway cross-talks with the MAPK pathway, in response to reduced membrane-associated ERα, MAPK activity is suppressed. Meanwhile, inhibition of EGF-induced EGFR phosphorylation by HMQ1611 blocks EGFR activation and its downstream pathways such as MAPKs and PI3K/AKT. Combined blockade of ERα and EGFR pathway leads to reduction of cell proliferation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y.Z. Zhan, C.C. Liu, L.C. He
Development of methodology: Y.Z. Zhan, Y.M. Zhang, C.C. Liu, L.C. He
Acquisition of data (proposed animals, acquired and managed patients, provided facilities, etc.): Y.Z. Zhan, Y.M. Zhang, C.C. Liu, N. Wang, Y.N. Chen, L. Zheng, L.C. He
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.Z. Zhan, Y.M. Zhang, J. Zhang, L.C. He
Writing, review, and/or revision of the manuscript: Y.Z. Zhan, W.W. Smith, L.C. He
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.Z. Zhan, Y.M. Zhang, C.C. Liu, L.C. He
Study supervision: L.C. He
Anticancer Effect of Taspine Derivative

Grant Support
This work was supported by National Natural Science Foundation of China (grant no. 30730110 and 81001447).

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Received December 27, 2011; revised March 16, 2012; accepted March 29, 2012; published OnlineFirst April 11, 2012.

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Cancer Prev Res  Published OnlineFirst April 11, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-11-0575

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