Solamargine induces apoptosis and enhances susceptibility to trastuzumab and epirubicin in breast cancer cells with low or high expression levels of HER2/neu

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Synopsis

Trastuzumab is used for breast cancer patients with high expression levels of HER2 (human epidermal growth factor receptor 2)/neu; however, it has no effect on cancers with low levels of HER2/neu. SM (solamargine), a major steroidal alkaloid glycoside purified from Solanum incanum, triggered apoptosis of breast cancer cells (MCF-7 and SK-BR-3 cells) and non-cancerous breast epithelial cells (HBL-100 cells) within 3 h. To extend the application of trastuzumab in breast cancer patients, the regulation of HER2/neu expression by SM was investigated. SM significantly up-regulates HER2/neu expression in breast cancer cells with low and high expression levels of HER2/neu, and synergistically enhanced the effect of trastuzumab in inhibiting cell proliferation. Additionally, HER2/neu and TOP2A [TopoII (topoisomerase II) α] genes share the same amplicon on an identical chromosome. Notably, SM co-regulates HER2/neu and TopoIIα expression markedly, and enhances TopoII inhibitor–EPI (epirubicin)-induced cytotoxicity to breast cancer cells.

Key words: epirubicin (EPI), HER2/neu, solamargine (SM), topoisomerase IIα (TopoIIα), trastuzumab

INTRODUCTION

The HER2 [human EGFR (epidermal growth factor receptor) 2]neu oncogene is located on chromosome 17 (17q21), and the gene product is a 185 kDa transmembrane oncoprotein (p185HER2) that has intrinsic tyrosine kinase activity [1]. On activation, HER2/neu promotes cell proliferation in a manner similar to that of a growth factor. In normal cells, the HER2/neu receptor triggers signal-transduction pathways that control normal cell growth, differentiation, motility and adhesion in several cell lines [2]. In tumour cells, HER2/neu gene expression is uncontrolled. Amplification of the HER2/neu gene and protein overexpression has been demonstrated in a variety of human carcinomas, including ovarian and breast cancers [3]. High levels of HER2/neu can be an indicator and a predictive marker of drug sensitivity in association with a survival benefit in breast cancer patients [4–6]. However, HER2/neu gene amplification and protein overexpression is not significantly correlated with patient survival, metastases, recurrence, overall histological grade or drug resistance [7].

Trastuzumab (Herceptin™; Genentech), which recognizes epitopes on the extracellular domain of p185HER2, is a humanized monoclonal antibody that has been approved for treating women with metastatic breast cancers [8]. Several cellular effects have been determined in vitro and in vivo, including internalization and degradation of the HER2 receptor, PI3K (phosphoinositide 3-kinase) and MAPK (mitogen-activated protein kinase) cascades, cell-cycle arrest, the inhibition of DNA repair, triggering of apoptosis and tumour size reduction, the inhibition of angiogenesis and a reduction in microvessel density [9]. An unaccountable cardiotoxicity may develop when trastuzumab is administered independently or combined with other therapeutic drugs [10,11]. Although trastuzumab has marked effects in...
HER2/neu-overexpressing breast cancer cells, only 15% of breast cancer patients with HER2/neu overexpression respond to trastuzumab therapy [12]. Furthermore, trastuzumab has no effect on 70% of breast cancer patients displaying low levels of HER2/neu expression. In vitro studies have indicated that co-treatment with trastuzumab and anthracycline or taxane increases drug sensitivity in an additive or synergistic manner [5,6,13]. Previous investigations have indicated that amplification of the HER2 gene and overexpression of its product in breast cancer cells may be associated with responsiveness to anthracycline [14] or doxorubicin [15] [the TopII (topoisomerase II)α inhibitors]-containing chemotherapy regimens. These experimental findings suggest that high expression of HER2/neu is an indicator and a predictive marker of drug sensitivity in association with the survival of breast cancer patients. Additionally, overexpression of HER2/neu may not induce drug resistance independently, but other factors influencing drug sensitivity must be involved.

SM (solamargin), a common steroidal alkaloid glycoside, inhibits the growth of human colon, prostate and hepatoma cells [16]. Notably, SM enhances TNF (tumour necrosis factor)-induced apoptosis by increasing TNF receptor expression in human lung cancer cells [17,18]. The present study further demonstrates that SM induces apoptosis of HBL-100, MCF-7 and SK-BR-3 cells, and enhances the expression of p185HER2 in these cells with low or high expression levels of HER2/neu. Moreover, these effects probably promote the cytotoxicities of trastuzumab and EPI (epirubicin) in human breast cancer cells.

MATERIALS AND METHODS

Drugs
SM (CAS number 20311-51-7) was purified from Solanum in- canum according to procedures described previously [19]. SM was diluted to a concentration of 12 mM in 100% DMSO as stock solution. DMSO concentrations used throughout the present study were less than 1% (v/v), and the cells were not affected by these DMSO concentrations. EPI (CAS number 56420-45-2; Genentech) were diluted in 1× PBS. The drugs were stocked at 4°C and freshly diluted to the concentrations required with culture medium.

Cell culture
The human breast cancer cell lines MCF-7 and SK-BR-3 and the non-cancerous breast epithelial cell line HBL-100 were obtained from the A.T.C.C. SK-BR-3 cells overexpress HER2/neu, whereas MCF-7 and HBL-100 cells express low levels of HER2/neu. All cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hazleton Products, Denver, PA, U.S.A.), 100 μg/ml streptomycin and 100 units/ml penicillin (Life Technologies). Cells were grown in a humidified incubator at 37°C in 5% CO₂/95% air.

Measurement of cell proliferation
For the determination of cell proliferation inhibition, 10⁴ cells were seeded into a 96-well plate (Corning) and cultured for 16 h. They were then washed twice with 1× PBS, followed by incubation with 100 μl/well of medium containing serial concentrations of SM (0, 1.2, 3, 6, 9 and 12 μM) or co-treated with SM and 50 and 200 μg/ml trastuzumab for 16 h, followed by a 4 h incubation with [Me-³H]thymidine (0.1 μCi/well; Amershams Biosciences). Cells were then washed twice with ice-cold 1× PBS and lysed with lysis buffer (1% SDS and 0.3 M NaOH). The cell lysates were harvested into Unifilter GF/C 96-well microfilter plates (Packard BioScience) and radioactivity was quantified using a gamma counter (Packard BioScience). Cell proliferation was expressed as a percentage of treated and untreated values, and IC₂₅ (concentration giving 25% inhibition), IC₅₀ and IC₇₅ (concentration giving 75% inhibition) values were calculated. All results are means ± S.D. (n = 3). The IC₂₅, IC₅₀ and IC₇₅ values of SM that caused 25%, 50% and 75% inhibition of proliferation were approx. 0.91, 1.81 and 3.00 μM for HBL-100 cells; 1.08, 1.98 and 2.38 μM for MCF-7 cells; and 1.40, 3.13 and 4.38 μM for SK-BR-3 cells.

Measurement of cytotoxicity
MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96® aqueous non-radioactive cell proliferation assay; Promega) was used for co-treatment of SM and EPI. The MTS test is a colorimetric method based on the capacity of viable cells. It is composed of solutions of a yellow tetrazolium salt (MTS) in the presence of PMS (phenazine methosulfate) acting as an electron-coupling agent to a purple formazan, which is directly soluble in tissue-culture medium. Breast cancer cells (10⁴/well) were seeded and cultured in a 96-well plate (Corning) for 16 h. To detect the effect of SM and EPI on cell survival, breast cancer cells were treated with IC₂₅ and IC₅₀ concentrations of SM (0.91 and 1.81 μM for HBL-100 cells; 1.08 and 1.98 μM for MCF-7 cells; and 1.40 and 3.13 μM for SK-BR-3 cells) and EPI (1.84, 5.25 and 9.20 μM) for 16 h. The culture medium was removed before cell harvesting, 20 μl of MTS/PMS reagent mixed with 100 μl of culture medium was added to each well, and cells were then incubated at 37°C for 2 h. Absorbance was recorded at 490 nm with an automated plate reader (Dynatech).

Measurement of cell apoptosis
Breast cancer cells (10⁴/well) were seeded in 24-well multi-dishes (Corning) and cultured for 16 h. After treatment with IC₅₀ concentrations of SM (1.81 μM for HBL-100 cells; 1.98 μM for MCF-7 cells and 3.13 μM for SK-BR-3 cells) for 16 h, cells were fixed by incubation with 100% ice-cold methanol for 30 min at −20°C and then stained by incubation with Hoechst dye (1:100 dilution; Molecular Probes) for 20 min at 4°C. The morphological changes in the nuclei were observed by fluorescence microscopy (Olympus BX-50 microscope with a 3CCD colour camera). For analysis of cell-cycle distribution, breast cancer cells were incubated with IC₂₅ and IC₅₀ concentrations of SM (0.91 and 1.81 μM for HBL-100 cells; 1.08 and
1.98 μM for MCF-7 cells; and 1.40 and 3.13 μM for SK-BR-3 cells) for 16 h, and fixed by incubation with ice-cold 4% (w/v) paraformaldehyde in 1 × PBS (pH 7.4) for 30 min at 4°C. After centrifugation at 300 g for 10 min, the cells were permeabilized by incubation with 0.1% Triton X-100/0.1% sodium citrate for 2 min at 4°C. PI (propidium iodide) in 1 × PBS (10 μg/ml) was added for 30 min at 4°C to stain the cells. The intensity of red fluorescence was measured by a FACScan flow cytometer (Becton Dickinson). A minimum of 5000 cells were collected for analysis by WinMDI software (http://facs.scripps.edu).

**Determination of gene expression by RT (reverse transcription)-PCR and Southern hybridization**

After breast cancer cells (10^6 cells) were treated with IC_{50} concentrations of SM (1.81 μM for HBL-100 cells, 1.98 μM for MCF-7 cells and 3.13 μM for SK-BR-3 cells), total RNA was isolated using the RDiact™ RNA/DNA isolation kit (Maxim Biotech). Total RNA yield and purity were assessed by spectrophotometry (DU® 640B, Beckman Coulter). RNA (3 μg) from each sample was subjected to RT with a random hexamer primer, dNTPs and M-MLV reverse transcriptase (Reverse Transcription system, Promega) in a total reaction volume of 20 μl. Thirty-five cycles of PCR were performed on cDNA using Taq DNA polymerase, dNTPs, MgCl₂, 10 x reaction buffer and the corresponding primers. Specific PCR primers were used for detecting HER2/neu, TOP2A (TopoIIα gene) and TOP2B (TopoIIβ gene). RT-PCR of human β-actin using the same conditions was carried as a control. The primers were as follows: HER2/neu (420 bp), 5′-TCGGGCTCTGATACAGGGACTT-3′ and 5′-TCGGAGAATTCAGACACCACT-3′; TOP2A (170 bp), 5′-GCCTCTGTCACTACATTCC-3′ and 5′-AAACACTTGGGCTTTACCTCACTT-3′; and TOP2B (180 bp), 5′-ATCAAAGCCACTCCAGAATACT-3′ and 5′-AGAAGGTGGTCCTAGGAGTC-3′. The PCR products were analysed by agarose-gel electrophoresis [2% (w/v) gels] and visualized using ethidium bromide. Polaroid photographs were taken and quantification was performed by densitometric analysis (Bio-Light software; Vilber Lourmat).

The PCR products of HER2/neu and TOP2A were further confirmed by Southern hybridization. The amplified products were resolved by agarose-gel electrophoresis [1.5% (w/v) gels] at 100 V for 30 min. After electrophoresis, the gel was processed using a denaturing buffer (1.5 M NaCl and 0.5 M NaOH), neutralized with 0.5 M Tris/HCl (pH 7.4), 1.5 M NaCl and 1 mM EDTA and transferred on to a Hybond-N membrane (Amersham Biosciences) by capillary blotting. The HER2/neu- and TOP2A-specific oligonucleotides were synthesized as a probe and end-labelled with [35S]dCTP. Hybridization was carried out using Rapid-Hyb buffer (Amersham Biosciences) at 42°C for 1 h. The membrane was washed once at room temperature (25°C) with 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) and 0.1% SDS, and washed twice at 42°C with 0.5 × SSC and 0.1% SDS. Autoradiography was performed using Kodak X-Omat AR film (Kodak) and quantification was performed by densitometric analysis (Bio-Light software, Vilber Lourmat).

**Analysis of expression of p185HER2**

[125I]-labelled antibodies were prepared using the chloramine-T method published previously [20]. To determine the expression of p185HER2 on the membranes of breast cancer cells after 16 h of SM treatment (0.91 and 1.81 μM for HBL-100 cells; 1.08 and 1.98 μM for MCF-7 cells; and 1.40 and 3.13 μM for SK-BR-3 cells), 10^7 trypsinized cells in 200 μl of 1 × PBS/0.1% BSA were incubated with 80 pM of [125I]-labelled anti-p185HER2 antibody for 4 h. The cells were washed and centrifuged three times with 3 ml of ice-cold PBS/0.1% BSA buffer. The radioactivity was determined by using a gamma counter (Packard Bioscience). All results are means ± S.D. (n = 3).

For immunocytochemical determinations, the breast cancer cells were fixed by incubation with 4% (w/v) paraformaldehyde in 1 × PBS (pH 7.4) for 10 min at 4°C. The expression of HER2/neu protein was detected by incubation with anti-p185HER2 antibody (1:300 dilution, Dako) for 24 h. The samples were then incubated with Dako LSAB® 2 system and HRP (horseradish peroxidase) for 1 h. Staining was then developed in DAB (3,3′-diaminobenzidine) solution (Sigma). Mayer’s haematoxylin was used as a counterstain. The positive and negative controls were supplied with the Dako HercepTest™ kit (Dako). Three formalin-fixed pellets containing paraffin-embedded human breast cancer cell lines with different staining intensities served as positive controls. The negative control slides were incubated with a negative control reagent. The expression of p185HER2 was categorized into three groups: negative (0 and 1+), positive (2+) and strongly positive (3+) (0, no staining at all, or membrane staining in less than 10% of the tumour cells; 1+, barely perceptible partial membrane staining in more than 10% of the tumour cells; 2+, weak to moderate staining of the entire membrane in more than 10% of the tumour cells; and 3+, strong staining of the entire membrane in more than 10% of the tumour cells).

**Immunofluorescence and flow cytometry**

Breast cancer cells (2 × 10^6) were seeded in 24-well multi-dishes (Corning) and cultured for 16 h. After treatment with IC_{50} concentrations of SM (1.81 μM for HBL-100 cells, 1.98 μM for MCF-7 cells and 3.13 μM for SK-BR-3 cells) for 16 h, adherent cells were fixed by incubation with 100% ice-cold methanol for 30 min at −20°C and exposed to 3% (v/v) H₂O₂ to inactivate endogenous peroxidase. Rabbit anti-human p185HER2 antibody (1:100 dilution; Dako) was used as a primary antibody to detect the expression of p185HER2 in breast cancer cells. The cells were washed twice with ice-cold 1 × PBS containing 0.2% BSA. FITC-conjugated anti-rabbit IgG (to detect the anti-p185HER2 antibody) (1:100 dilution, Dako) was used as the secondary antibody. Hoechst dye (1:100 dilution) was included with the secondary antibody to visualize nuclei. The FITC-stained cells were viewed and photographed by fluorescence microscopy (Olympus BX-50 microscope with a 3CCD colour camera).

For flow cytometry analysis, after treatment with IC_{50} concentrations of SM (1.81 μM for HBL-100 cells, 1.98 μM for MCF-7 cells and 3.13 μM for SK-BR-3 cells) for 16 h, the cell suspension was centrifuged at 300 g for 5 min. The supernatant was removed, and cells were fixed by incubation with ice-cold...
4% (w/v) paraformaldehyde in 1 × PBS (pH 7.4) for 30 min at 4°C. After centrifugation at 300 g for 5 min at 4°C, cells were resuspended in 200 μl of 1 × PBS. Rabbit anti-human p185HER2 antibody (1:100 dilution, Dako) was used as a primary antibody to detect the expression of p185HER2. The cells were washed twice with ice-cold 1 × PBS containing 0.2% BSA and FITC-conjugated anti-rabbit IgG (1:100 dilution, Dako) was used as the secondary antibody to detect anti-p185HER2 antibody. The green fluorescence was measured by a FACScan flow cytometer (Becton Dickinson) and a minimum of 5000 cells were collected for analysis by WinMDI software.

Combination data analysis by the isobologram method

The experimental results obtained from the growth inhibition assays in response to the combination of SM with trastuzumab and EPI were subjected to isobologram analysis. The isobologram method relies on the calculation of the combined concentrations of SM, trastuzumab and EPI that cause a given effect, such as 25% or 50% growth inhibition [21]. For the combination of drug concentrations (D_{SM} X, D_{agent} X), producing in combination the effect (X), the CI (combination index) was calculated as follows:

\[ CI = \frac{D_{SM} X}{IC_{X,SM}} + \frac{D_{agent} X}{IC_{X,agent}} \]

where IC_{X,SM} and IC_{X,agent} are the inhibitory concentrations of each individual drug that would produce X if given alone. CI values < 1 indicates a synergistic effect, CI = 1 indicates an additive effect and CI > 1 indicates an antagonistic effect.

Statistical analysis

The experimental results are expressed as means ± S.D. Statistical differences were determined by independent and paired Student’s t tests in unpaired and paired samples respectively. Whenever a control group was compared with more than one treated group, the one-way or two-way repeated measures ANOVA was used. When the ANOVA manifested a statistical difference, the Dunnett’s or Student–Newman–Keuls test was applied. \( P < 0.05 \) was considered to be significant in all experiments. Analysis of the data and plotting of the Figures were done using SigmaStat and SigmaPlot (Version 8.0) software (SyStat).

RESULTS

Effects of SM on cell proliferation and breast cancer cell apoptosis

In studies published previously, SM was shown to induce apoptosis rapidly and irreversibly in human lung cancer cells [18]. The present study investigates the inhibition of proliferation of SM using an [3H]thymidine incorporation assay in breast cancer cells. After 16 h treatment with SM in HBL-100, MCF-7 and SK-BR-3 cells, 0–12 μM SM significantly promoted the inhibition of proliferation in a dose-dependent manner (Figure 1A). The IC_{25}, IC_{50} and IC_{75} values of SM that caused 25%, 50% and 75% inhibition of proliferation were approx. 0.91, 1.81 and 3.00 μM for HBL-100 cells; 1.08, 1.98 and 2.38 μM for MCF-7 cells; and 1.40, 3.13 and 4.38 μM for SK-BR-3 cells. The time course of SM-induced inhibition of proliferation was determined. Maximum inhibition of these cells was detected within 3 h of incubation with constant concentrations of SM (IC_{25}, IC_{50} and IC_{75} concentrations); no further inhibition of cell proliferation was observed after extended incubation with SM for 16 h (Figure 1B).

When cells undergo apoptosis, many characteristics can be observed, including nucleus condensation, apoptotic body formation and sub-G1 phase enhancement. The SM-induced morphological changes were visualized. After SM treatment (IC_{50} concentration), nuclear condensation and apoptotic bodies appeared when the exposure time was extended to 16 h (Figure 1C). After SM treatment (IC_{25} and IC_{50} concentrations), cells were stained with PI and the cell-cycle distribution was determined. Table 1 shows the changes in cell-cycle distribution; the cell population in the G2/M phase was reduced with a concurrent increase in the sub-G1-phase population after 16 h incubation with SM. This experimental finding suggests that SM may stimulate cell apoptosis in the G2/M phase predominantly.

Up-regulation of HER-2/neu expression by SM

The proto-oncogene HER-2/neu is an important proliferation regulator. After SM treatment (IC_{50} concentration) for 0, 0.5, 1.2 and 3 h, HER-2/neu expression in cells was analysed by RT-PCR. SM elevated HER2/neu mRNA expression in HBL-100, MCF-7 and SK-BR-3 cells (Figure 2A). The statistical significance was obtained by comparing the means of the densitometric values of HER2/neu in SM-treated cells with untreated cells (taken to be 100%) (\( P < 0.05, n = 3 \)). These experimental results were further confirmed by Southern hybridization (Figure 2B) (\( P < 0.05, n = 3 \)). Additionally, HER2/neu and TOP2A are at the same amplicon in chromosome 17q12–q21, which results in co-amplification and co-regulation. The mRNA expression of HER2/neu and TOP2A were strongly correlated. Conversely, the TOP2B gene is located on a different chromosome (3p) from TOP2A, and functions as a negative control [22]. To further confirm the up-regulation of HER2/neu by SM, TOP2A expression was used as a positive control and TOP2B as a negative control. All cells were treated with IC_{50} concentrations of SM under the same conditions as used for HER2/neu determination. Similar to HER2/neu modulation, SM significantly elevated the mRNA expression of TOP2A in HBL-100, MCF-7 and SK-BR-3 cells (\( P < 0.05, n = 3 \)), whereas SM had no effect on TOP2B expression (Figure 3). These experimental results demonstrate that SM may up-regulate HER2/neu and TOP2A mRNA expression in the breast cancer cells.

The HER2/neu gene encodes a p185HER2 protein which is localized to the cell membrane. Detection of this protein on cell membranes was performed by radioimmunoassay, immunocytochemistry, immunofluorescence and flow cytometry with [125I]-biotin- and FITC-labelled p185HER2 antibodies. After treatment with IC_{25} and IC_{50} concentrations of SM for 16 h, total-cell extracts were collected and analysed by radioimmunoassay. Expression of p185HER2 was significantly increased in HBL-100, MCF-7 and SK-BR-3 cells, and these regulated effects were dose-dependent (Figure 4A). The intensity of p185HER2 expression...
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Table 1  Cell-cycle distribution of HBL-100, MCF-7 and SK-BR-3 cells after SM treatment

<table>
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<tr>
<th>Cell line</th>
<th>Sub-G&lt;sub&gt;1&lt;/sub&gt; phase (%)</th>
<th>S phase (%)</th>
<th>G&lt;sub&gt;2&lt;/sub&gt;/M phase (%)</th>
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<td></td>
<td>0 h</td>
<td>16 h</td>
<td>0 h</td>
</tr>
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<td>HBL-100</td>
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<td>18.4 ± 0.3</td>
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<td>IC&lt;sub&gt;50&lt;/sub&gt; (1.81 μM)</td>
<td>4.0 ± 1.1</td>
<td>20.0 ± 0.7</td>
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<td>MCF-7</td>
<td>IC&lt;sub&gt;25&lt;/sub&gt; (1.08 μM)</td>
<td>8.8 ± 0.3</td>
<td>27.1 ± 0.1</td>
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<td>IC&lt;sub&gt;50&lt;/sub&gt; (1.98 μM)</td>
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<td>SK-BR-3</td>
<td>IC&lt;sub&gt;25&lt;/sub&gt; (1.40 μM)</td>
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<td>IC&lt;sub&gt;50&lt;/sub&gt; (3.13 μM)</td>
<td>7.2 ± 0.6</td>
<td>33.7 ± 0.2</td>
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Figure 1  Effect of SM on cell proliferation and apoptosis of breast cancer cells

(A) Inhibition of proliferation by SM in breast cancer cells. HBL-100 (●), MCF-7 (○) and SK-BR-3 (▼) cells were treated with SM for 16 h. Cell proliferation was measured by [Me<sup>3</sup>H]thymidine incorporation. (B) Time courses of inhibition of proliferation after treatment with constant concentrations of SM [IC<sub>25</sub> (●), IC<sub>50</sub> (○) and IC<sub>75</sub> (▼)] as described in the Materials and methods section for various incubation times. (C) Morphological changes in the nuclear chromatin of breast cancer cells after incubation with the IC<sub>50</sub> concentration of SM (1.81 μM for HBL-100, 1.98 μM for MCF-7 and 3.13 μM for SK-BR-3 cells). Cells were stained with Hoechst and morphological changes were visualized by fluorescence microscopy (×200 magnification). Results are means ± S.D. of the percentage of proliferation of the SM-treated compared with untreated cells (n = 4).
was increased by 153.3 ± 2.3% and 206.9 ± 0.3% for HBL-100; 185.3 ± 0.4% and 212.6 ± 0.1% for MCF-7; and 178.8 ± 7.7% and 215.6 ± 0.2% for SK-BR-3 cells compared with control cells (P < 0.05, n = 3). By immunocytochemistry, p185HER2 staining with the specific antibody was strong in the control SK-BR-3 cells (intensity score of 3+), and minimal in control MCF-7 and HBL-100 cells (intensity score of 1+). However, after 16 h of treatment with SM (IC50 concentration), p185HER2 was upregulated in these cells, and more than 50% of breast cancer cells exhibited p185HER2 staining (Figure 4B). The expression of p185HER2 was determined further by immunofluorescence and flow cytometry. As shown in Figure 4(C), the amount of p185HER2 in SK-BR-3 cells was higher than that of MCF-7 and HBL-100 cells. Following treatment with SM (IC50 concentration), expression of p185HER2 for all cells increased significantly. Flow cytometry is an established approach for measuring p185HER2 using the anti-p185HER2 antibody [23]. After treatment with SM (IC50 concentration) for 16 h, the percentage of p185HER2 expression in SM-treated breast cancer cells was increased (Figure 4D). These experimental results suggest that SM up-regulates HER2/neu expression in breast cancer cells.

Enhanced cytotoxicity of trastuzumab and EPI against breast cancer cells by SM

Trastuzumab (Herceptin™) is a HER2/neu-targeted therapy approved by the FDA (Food and Drug Administration) for treating metastatic breast cancers which overexpress HER2/neu. However, in clinical studies, only approx. 15–26% of women with metastatic breast cancers that overexpress HER2/neu respond to trastuzumab [12,24]. Furthermore, trastuzumab has no effect on patients with low HER2/neu expression levels [25]. SK-BR-3 cells express high levels of p185HER2, whereas HBL-100 and MCF-7 cells express low levels of p185HER2. The dose-response effects of trastuzumab on breast cancer cells were determined by [Me-3H]thymidine incorporation (Figure 5A). After trastuzumab treatment (50 and 200 μg/ml) for 16 h, only
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Figure 3 Changes in TOP2A (TopoIIα) and TOP2B (TopoIIβ) gene expression in breast cancer cells after treatment with SM
Breast cancer cells were treated with IC50 concentrations of SM (1.81 μM for HBL-100, 1.98 μM for MCF-7 and 3.13 μM for SK-BR-3 cells). Total cellular RNA was collected for RT-PCR. The PCR products were detected by 2% (w/v) agarose-gel electrophoresis and quantified using Bio-Light software. Results are means ± S.D. (n = 3). *P < 0.05 compared with control (determined by ANOVA followed by Student–Newman–Keuls test).

SK-BR-3 cells exhibited a sensitivity towards trastuzumab, with 86.8 ± 3.7 and 82.9 ± 1.6% inhibition of proliferation for 50 and 200 μg/ml treatments respectively. Furthermore, the combination of SM (IC25 and IC50 concentrations) with different doses (50 and 200 μg/ml) of trastuzumab further inhibited cell proliferation in HBL-100, MCF-7 and SK-BR-3 cells. Antagonism and synergism were qualitatively assessed by isobolograms of the combined treatment of cells with SM (IC25 and IC50 concentrations) and trastuzumab (50 and 200 μg/ml) for breast cancer cells. Drug interaction was assessed using the CI value, which was calculated using the concentrations of pairs of drugs which generated 25% or 50% inhibition of cell proliferation. Average CI values of the combinations for breast cancer cells were <1, thereby demonstrating the synergistic effects at all inhibitory concentrations (Table 2). These experimental results suggest that SM may enhance the susceptibility to trastuzumab by up-regulating p185HER2 expression in breast cancer cells with both low and high expression of HER2/neu. Amplification of HER2/neu is typically accompanied by TOP2A amplification. EPI, an anthracycline, is a TopoIIα inhibitor. In clinical trials, EPI-based treatments are employed commonly when treating breast cancer patients with high expression levels of HER2/neu. Therefore high levels of HER2/neu expression may be an important marker for breast

<table>
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<th>Cell line</th>
<th>Drugs</th>
<th>CI (SM IC25)</th>
<th>CI (SM IC50)</th>
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<td>HBL-100</td>
<td>SM + trastuzumab</td>
<td>0.59 ± 0.14</td>
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<td>SM + EPI</td>
<td>0.97 ± 0.01</td>
<td>0.79 ± 0.05</td>
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<td>MCF-7</td>
<td>SM + trastuzumab</td>
<td>0.76 ± 0.08</td>
<td>0.45 ± 0.11</td>
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<td>SM + EPI</td>
<td>0.88 ± 0.02</td>
<td>0.75 ± 0.06</td>
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<tr>
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<td>SM + trastuzumab</td>
<td>0.50 ± 0.22</td>
<td>0.47 ± 0.07</td>
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<td>SM + EPI</td>
<td>0.82 ± 0.07</td>
<td>0.40 ± 0.04</td>
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cancer therapy with EPI. In the present study, SM significantly elevated HER2/neu and TOP2A gene expression in HBL-100, MCF-7 and SK-BR-3 cells (Figures 2 and 3). The effects of combined SM and EPI therapy on cell survival were determined by the MTS assay. Co-treatments at different doses of EPI (0, 1.84, 5.25 and 9.20 μM) and SM (IC25 and IC50 concentrations) significantly enhanced EPI cytotoxicity against breast cancer cells; these effects were dependent on the concentration of SM (Figure 5B). The CI values were calculated using the isobologram method. All CI values at levels of IC25 and IC50 concentrations of SM were <1 (Table 2), demonstrating that synergistic effects were detected in all of these cell lines. These experimental results indicate that SM has the potential to increase cancer cell susceptibility to EPI.

**DISCUSSION**

The proto-oncogene HER2/neu is the most frequently amplified oncogene in breast cancer cells, and its overexpression in 20–25% of breast cancer patients may be associated with poor clinical outcomes. Activation of the HER2/neu receptor tyrosine kinase triggers cell proliferation, differentiation and drug resistance. However, several previous reports have indicated that the therapeutic response for HER2/neu-overexpressing cancers and anti-cancer drugs were not strongly correlated [4,5]. Additionally, HER2/neu overexpression did not affect the response to first-line hormonal therapy in advanced breast cancer [26]. Other clinical studies found that HER2/neu-positive tumours were three
times more sensitive to taxol, and HER2/neu overexpression enhanced the response rates to chemotherapeutic regimens containing adriamycin [7,27]. Immunohistochemical detection of HER2/neu expression was not a reliable prognostic indicator for patients with axillary lymph-node-negative breast carcinoma, and HER2/neu expression was not associated with significant epidemiological risk factors [28]. Transfection of HER2/neu into breast and ovarian cancer cells was not sufficient to induce intrinsic pleomorphic drug resistance in vitro and in vivo [29]. These previous studies demonstrate that HER2/neu overexpression is not the only factor involved in drug resistance in breast cancer cells. In other words, a direct relationship between the up-regulation of HER2/neu expression and drug resistance remains unclear.

SM rapidly triggered cell apoptosis and enhanced HER2/neu gene expression in HBL-100, MCF-7 and SK-BR-3 cells within 3 h, suggesting that SM molecules may diffuse easily into cells. This result is consistent with our previous work on hepatoma and lung cancers [17,18,30]. Additionally, SM-induced apoptosis was not affected by HER2/neu high- or low-expressing breast cancer cells, revealing that the level of HER2/neu expression may not correlate with the drug resistance properties of the cells. Furthermore, SM is a steroidal glycoalkaloid that diffuses rapidly into cells and may bind to the receptor specifically. The steroid–receptor complex may function as a key component in the regulation of specific cell functions. Analyses of the receptor for SM may be important to understand the molecular mechanisms of transcriptional control involved. However, the specific receptor for SM is still unknown. In our previous study, the expression of HER2/neu was down-regulated by SM in ZR-75-1 cells [31]. However, in the present study, SM can also up-regulate HER2/neu and TopoIIα expression, and consequently enhance the sensitivity of HBL-100, MCF-7 and SK-BR-3 cells to trastuzumab and EPI. Similar results of up- and down-regulation of HER2/neu were also observed for human lung cancer cells [32,33]. Thus there is, so far, no detailed report of the mechanism of HER2/neu signal transduction regulation by SM. The action mechanism of SM in the regulation of HER2/neu requires further investigation.

Because mRNA decays quickly in the cell, the experiments for detecting changes in mRNA expression after SM treatment had to be performed over short time periods. However, protein
expression needs transcription, translation and protein delivery processes, and so longer time periods need to be examined in order to detect changes in protein levels in the cell. In the present study, SM elevated HER2/neu mRNA expression within 3 h, depending on the cell lines analysed. However, much more time may be needed for HER2/neu receptor formation and transfer. For example, EGFRs are found predominantly at the cell surface, and these receptors undergo constant cycling between the plasma membrane and the endosomal compartment. In the absence of ligand, EGFRs undergo metabolic turnover with a half-life of approx. 10–14 h in both fibroblasts and epithelial cells [34,35] and 20–48 h in transformed cells [36,37]. In our previous study, the time course of HER2/neu protein expression was determined in human lung cancer H661 and H69 cells. It was shown that a significant increase in HER2/neu protein expression was detectable after SM treatment for 5–18 h [32]. Thus in the present study we determined HER2/neu protein expression and analysed the synergistic effect of SM, trastuzumab and EPI on the cells after 16 h of treatment. The results may explain the direct relationship between HER2/neu receptor enhancement and the synergistic effects of the drugs.

Trastuzumab, which is a monoclonal antibody that selectively binds to the HER2/neu protein, is the first biological modifier with significant activity in advanced breast cancer patients with amplified HER2/neu gene expression. In the present study, trastuzumab only caused inhibition of proliferation in SK-BR-3 cells (HER2/neu overexpressing cells), and had no effect on HBL-100 or MCF-7 cells (HER2/neu low-expression level cells). As a result of enhanced p185HER2 expression by SM, an increase in proliferation inhibition by SM and trastuzumab in HBL-100, MCF-7 and SK-BR-3 cells was detected. Notably, SM induces apoptosis and up-regulates HER2/neu gene expression, whereas trastuzumab consequent binds to p185HER2 specifically and therefore markedly expedites the inhibition of proliferation. SM may significantly enhance the effects of trastuzumab in breast cancer cells with low or high expression of HER2/neu. Additionally, the HER2/neu gene is amplified and is present as multiple copies in SK-BR-3 cells; however, it is present as a single copy in MCF-7 cells. Therefore SK-BR-3 cells overexpress HER2/neu and are more sensitive to trastuzumab. In the present study, SM up-regulated HER2/neu mRNA and protein expression in SK-BR-3 and MCF-7 cells. The combination of treatment with SM and trastuzumab seems to be more effective in SK-BR-3 cells than in MCF-7 cells. SM up-regulated HER2/neu expression and enhanced the susceptibility of human lung cancer H661 and H69 cells to trastuzumab, according to our previous study [32]. Hence the up-regulation of HER2/neu by SM may sensitize breast and lung cancer cells to trastuzumab.

TopoIIα, a homodimeric enzyme, is a chemotherapeutic target for a large group of clinical anticancer drugs e.g., anthracyclines (doxorubicin, EPI), epipodophyllotoxins (etoposide, teniposide), actinomycin and mitoxantrone. TopoIIα has two major isoforms in human cells: TopoIIα (170 kDa) and TopoIIβ (180 kDa). These two enzymes are produced by different genes located in chromosomes 17q21 and 3p, and have different functions. For instance, TopoIIα is cell-cycle dependent, whereas TopoIIβ is not [37]. As the TOP2A and HER2/neu genes are near the same ampli-}

**REFERENCES**