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A COX-2 inhibitor nimesulide analog selectively induces apoptosis in Her2 overexpressing breast cancer cells via cytochrome *c* dependent mechanisms

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ABSTRACT

Epidemiological and animal model studies have suggested that non-steroidal anti-inflammatory drugs (NSAIDs) can act as chemopreventive agents. The cyclooxygenase-2 (COX-2) inhibitor nimesulide shows anti-cancer effect in different type of cancers. In the current study, five breast carcinoma cell lines were used to explore the anti-cancer mechanisms of a nimesulide derivative compound 76. The compound dose dependently suppressed SKBR-3, BT474 and MDA-MB-453 breast cancer cell proliferation with IC_{50} of 0.9 μM, 2.2 μM and 4.0 μM, respectively. However, it needs much higher concentrations to inhibit MCF-7 and MDA-MB-231 breast cancer cell growth with IC_{50} at 22.1 μM and 19.6 μM, respectively. Further investigation reveals that compound 76 induced apoptosis in SKBR-3 and BT474 cells. Since these cells are Her2 overexpressing cells, the Her2 intracellular signaling pathways were examined after the treatment. There was no significant changing of kinase activity. However, the cytochrome *c* release assay indicated that the apoptosis induced by the compound was mediated by the mitochondria. These results suggest that compound 76 selectively induce apoptosis in Her2 overexpressing breast cancer cells through the mitochondria, and could be used as a lead to design more potent derivatives.

1. Introduction

Adenocarcinoma of the breast is the most common cancer in women in the United States and ranks second only to lung cancer as a cause of cancer-related mortality. About 182,460 women in the nation were found to have invasive breast cancer in 2008. About 40,480 women will die from the disease this year [1]. Currently over 2 million women living in the United States have been treated for breast cancer. About 25–30% of breast cancer patients have human epidermal growth factor 2 (Her2) over-expressed tumors, which means that their cancer cells depend on the Her2 pathway to proliferate [2]. Her2, also referred as ErbB2, is a member of the ErbB receptor tyrosine kinases family. The ErbB family consists of four members: epidermal growth factor (EGF) receptor/ErbB1, ErbB2 (Her2), ErbB3, and ErbB4. They can form both homo- or heterodimers, which then stimulates the intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of specific tyrosine residues within cytoplasmic domain and drives cancer cell proliferation, invasion and differentiation [3–7]. Her2 appears to be the preferred partner of the other ligand-bound ErbBs [8]. The high level of Her2 protein is generally due to amplification of the Her2 gene in tumors and results in constitutive activation of the receptor. Several studies have shown that patients with breast cancer that overexpressed Her2 are associated with increased disease recurrence, worse prognosis and lower survival [2,9]. It has been observed that targeting Her2 results in efficient inhibition of breast cancer cell proliferation, which proceeds via inhibition of intracellular signaling pathways and directly targets various members of the cell growth machinery [10]. Currently, there are two types of drugs that target Her2. The first one is Her2 monoclonal antibody such as trastuzumab approved by FDA in 1998; the second type is intracellular tyrosine kinase inhibitors such as lapatinib approved by FDA in 2007. In addition, other approaches are also being investigated to develop agents that are able to selectively inhibit Her2 breast cancer cell growth [11].

A growing body of experimental and epidemiological evidence suggests that the use of NSAIDs (non-steroidal anti-inflammatory drugs) may decrease the incidence of mammary cancer, tumor burden, and tumor volume [12–15]. Celecoxib, a COX-2 (cyclooxygenase 2) selective inhibitor, has strong chemopreventive activity against mammary carcinoma in rats in some studies [16]. In

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Abbreviations: PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; PDK, phosphoinositide-dependent kinase; MAPK, mitogen activated protein kinase; COX-2, cyclooxygenase 2; NSAIDs, non-steroidal anti-inflammatory drugs; PGE2, prostaglandin E2; Her2, human epidermal growth factor receptor.

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addition to COX inhibition, these small molecules also target other molecular pathways. For example, celecoxib is able to block phosphoinositide 3-kinase (PI3K)/phosphoinositide-dependent kinase (PDK)/Akt pathway to induce apoptosis in prostate cancer cells [17,18]. Nimesulide (4-nitro-2-phenoxymethane-sulfoanilide) is a non-steroidal anti-inflammatory drug with a preferential cyclooxygenase-2 inhibitory activity and is available in some Asian and European countries since 1985. In fact, the anti-inflammatory activity of nimesulide is almost the same as that of indomethacin, but its alcerogenic potential is much weaker. Studies suggest that nimesulide can induce apoptosis in liver and lung cancer cells; it also suppressed the development of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP)-induced mammary gland carcinogenesis in rats [19–23]. Furthermore, derivatives of nimesulide have been found to be much more active in suppressing SKBR-3 breast cancer cell growth than nimesulide [24].

Our goal is to identify new agents that would selectively inhibit the growth of Her2 overexpressing breast cancer cells, and then investigate the mechanism of the pharmacological activity. In this paper, one lead compound (compound 76) from the nimesulide derivative pool was synthesized (Fig. 1) according to previous procedure [24], and their biological effect on breast cancer cell growth was investigated in a panel of breast cancer cell lines which include SKBR-3, BT474, MDA-MB-453, MCF-7 and MDA-MB-231. Compound 76 selectively inhibited SKBR-3, BT474 and MDA-MB-453 Her2 overexpressing breast cancer cell proliferation, but it was not effective on the proliferation of MCF-7 and MDA-MB-231 Her2 negative cells. Because of the typical apoptotic morphological changes which includes cell shrinkage and condensation of the nucleus after the treatment of compound 76, several assays were performed to determine the possibility of potential apoptosis-inducing effects of compound 76 in SKBR-3 and BT474 cancer cells. The results indicate that compound 76 significantly induced apoptosis mediated by the mitochondria in the cells. Since synthesis of compound 76 derivatives has been well established, new analogs could be generated to further optimize the structure and achieve more active agents.

2. Materials and methods

2.1. Reagents

Trypsin and all enzymes were obtained from Invitrogen (Carlsbad, CA). All the chemical reagents and solvent were from Sigma–Aldrich (St. Louis, MO). For in vitro experiments, compound 76 at various concentrations were dissolved in DMSO. All antibodies were from Cell Signaling (Danvers, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). All the synthesis reagents were from Aldrich (Aldrich, Milwaukee, WI).

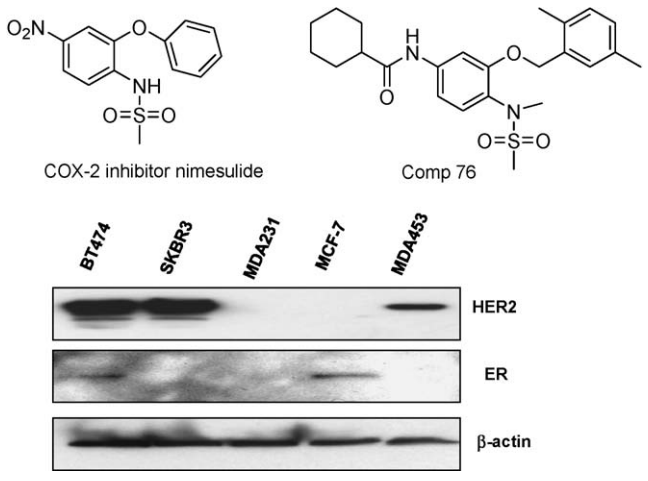

Fig. 1. Structures of nimesulide, compound 76 (top panel); Her2 and ER status in five breast cancer cell lines (bottom panel). Cells were harvested and Her2 and ER proteins were analyzed by Western blotting of cell extracts with specific antibodies as described in Section 2.

2.2. Synthesis of compound 76

Compound 76 was synthesized according to the published procedure [24], and the structure and purity were confirmed by NMR. Cyclohexanecarboxylic acid [3-(2,5-dimethyl-benzyloxy)-4-(methanesulfonyl-methyl-amino)-phenyl]-amide: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (1H, s), 7.29 (2H, m), 7.10 (3H, m), 6.66 (1H, d, J = 8.5 Hz), 5.07 (2H, s), 3.19 (3H, s), 2.71 (3H, s), 2.35 (3H, s), 2.34 (3H, s), 2.24 (1H, m), 1.96 (2H, m), 1.86 (2H, m), 1.73 (1H, m), 1.54 (3H, m), 1.27 (2H, m). Purity (97%) is measured by HPLC analysis. A 1.00 mg/mL stock solution of compound 76 was prepared in acetonitrile. HPLC analysis was performed on a HP1100 system (Hewlett–Packard, Palo Alto, CA), which consists of a vacuum degasser, binary pumps, column compartment, and a UV detector. Reversed phase HPLC was carried out on a C18 column (3.0 mm × 150 mm, 5 μm) from Beckman (Beckman Instruments, Fullenton, CA) at room temperature with a flow rate of 0.5 mL/min. Gradient mobile phase (mobile phase A: water; mobile phase B: acetonitrile) was employed to run 20 min. An injection volume of 25 μL was used. The UV detector was set up at 254 nm and 330 nm.

2.3. Cell culture

SKBR-3, MDA-MB-231, MCF-7, BT474 and MDA-MD-453 cells were obtained from ATCC (Rockville, MD). MDA-MD-453 cells were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin–streptomycin. All the other cell lines were cultured in MEM, supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 100 U/mL penicillin–streptomycin. Cell cultures were grown at 37 °C, in a humidified atmosphere of 5% CO_2 in a Hereaus CO_2 incubator.

2.4. Cell proliferation

The effect of compound 76 on breast cancer cell proliferation was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay (MTT) in six replicates. Cells were grown in culture medium in 96-well, flat-bottomed plates for 24 h, and were exposed to various concentrations of compound 76 dissolved in DMSO (final concentration ≤0.1%) in media at different time intervals. Controls received DMSO vehicle at a concentration equal to that in drug-treated cells. The medium was removed, replaced by 200 μL of 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide in fresh medium, and cells were incubated in the CO_2 incubator at 37 °C for 2 h. Supernatants were removed from the wells, and the reduced 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide dye was solubilized in 200 μL/well DMSO. Absorbance at 570 nm was determined on a plate reader.

2.5. Morphological examination of apoptotic changes

Cells were treated with compound 76 at various concentration for 48 h, and then stained with Hoechst 33342 (5 μg/mL) at 37 °C for 30 min. The stained cells were examined by fluorescence microscopy using a microscope (Nikon, Tokyo) equipped with an epi-illuminator and appropriate filters.

2.6. Apoptosis detection by enzyme-linked immunosorbent assay (ELISA)

Drug-induced apoptotic cell death was assessed using the Cell Death Detection ELISA kit (Roche Diagnostics), which quantitates cytoplasmic histone-associated DNA fragments in the form of mononucleosomes or oligonucleosomes. Cells were seeded and incubated at 10,000 cells per well in 12-well flat-bottomed plates in 10% FBS-supplemented MEM medium. After 24 h, cells were treated with compound 76 for 48 h at the indicated concentrations. Both floating and adherent cells were collected and the assay was done according to the manufacturer's instructions.

2.7. Flow cytometry analysis

For all the assays, cells were treated for 48 h. To analyze the cell cycle profile, treated cells were fixed overnight with 70% EtOH at −20 °C and stained with propidium iodide buffer [38 mM sodium citrate (pH 7.5), 69 μM propidium iodide, and 120 μg/mL RNase A]. For assessment of apoptosis, treated cells were labeled with 5 μL Annexin V-FITC (Invitrogen) and 0.1 μg propidium iodide (Sigma–Aldrich) in 100 μL binding buffer [10 mmol/L HEPES, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$ (pH 7.4)] containing 5×10^5 cells. Samples were mixed gently and incubated at room temperature in the dark for 15 min. Immediately before analysis by flow cytometry, 400 μL binding buffer was added to each sample. Two-color analysis of apoptosis was done using a BD FACSCalibur System (BD Biosciences). Fluorescence compensation on the flow cytometer was adjusted to minimize overlap of the FITC and propidium iodide signals. A total of 1.2×10^4 cells were acquired for each sample and a maximum of 1×10^4 cells within the gated region were analyzed.

2.8. Western blot

Cells were cultured in 60-mm culture dishes and incubated with DMSO or drugs for 48 h and then lysed with CelLytic M (Sigma–Aldrich) supplemented with protease inhibitor tablets (Roche, Indianapolis, IN). Cell lysates were sonicated briefly to reduce viscosity. Protein concentration was determined and samples were stored at −70 °C until use. Sixty micrograms of proteins for each sample were boiled with 1× loading buffer (100 mmol/L DTT plus bromophenol blue) for 5 min. Samples were then electrophoresed in 10% SDS-polyacrylamide gel and transferred to a Trans-Blot nitrocellulose membrane (Bio-Rad) using a Trans-Blot SD semidry transfer cell (Bio-Rad) at 100 mA for 90 min. The membrane was blocked for 2 h with 5% nonfat milk in 1× TBS-T at room temperature to reduce background and then incubated with primary antibody, in 1% bovine serum albumin at 4 °C overnight. After the membrane was incubated with the primary antibody and washed four times with 1× TBS-T for 5 min each wash, it was incubated with the secondary antibody in 5% nonfat milk for 60 min at room temperature. The membrane was washed four times again for 5 min each time with 1× TBS-T and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the protocol of the manufacturer. The membrane blot was exposed to Basic Autorad Film (ISC Bioexpress, Kaysville, UT) and developed using a Konica SRX-101A (Konica, Tokyo, Japan). The blot was reprobed with anti-actin (Santa Cruz Biotechnology) at 1:2000 dilution as a loading control. Anti-pAKt, AKt, p-ERK, ERK, pBAD112, pBAD136, BAD, BAX, BCL-2, BCL-XL were diluted (1:1000).

2.9. Cytochrome c release

Drug-treated cells were collected and lysed with 100 μL of chilled hypotonic lysis solution [220 mmol/L mannitol, 68 mmol/L sucrose, 50 mmol/L KCl, 5 mmol/L EDTA, 2 mmol/L $MgCl_2$, and 1 mmol/L DTT in 50 mmol/L PIPES-KOH (pH 7.4)] for 45 min. The solution was centrifuged at $600 \times g$ for 10 min to collect the supernatant. The supernatant was further centrifuged at 14,000 rpm for 30 min, and equal amounts of proteins (50 μg) from the supernatant were resolved in 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and analyzed by immunoblotting with anti-cytochrome *c* antibodies [25].

2.10. Statistical analysis

Statistical and graphical information was determined using GraphPad Prism software (GraphPad Software Incorporated) and Microsoft Excel (Microsoft Corporation). Determination of IC_{50} values were performed using nonlinear regression analysis. Statistically significant differences were calculated with the two-tailed unpaired Student's *t*-test and *P*-values reported at 95% confidence intervals.

3. Results

3.1. Characterization of five breast cancer cell lines

Five breast cancer cell lines from ATCC were determined for their Her2 and estrogen receptor (ER) status by Western blot. The results indicate that SKBR-3 and BT474 have the highest Her2 expression. MDA-MB-453 has relatively lower Her2 expression, and MCF-7 and MDA-MB-231 do not have any detectable Her2 protein (Fig. 1 and Table 1). BT474 and MCF-7 cells have similar level of ER expression, and the rest of them do not show any ER band.

3.2. Anti-proliferation activity of compound 76 on five breast cancer cell lines

Compound 76 has been found to be effective in suppressing SKBR-3 breast cancer cells proliferation with an IC_{50} about 1.0 μM [24]. However, it is unclear whether this compound is generally cytotoxic to other breast cancer cells as well. To investigate the selectivity of compound 76 on inhibiting breast cancer cell growth, five breast cancer cell lines which include both Her2 positive and negative cells were used to explore the anti-cancer mechanisms of compound 76. The compound dose dependently suppressed SKBR-3, BT474 and MDA-MB-453 breast cancer cell proliferation with IC_{50} at 0.9 μM, 2.2 μM and 4.0 μM, respectively. However, they were not effective in suppressing MCF-7 and MDA-MB-231 breast cancer cell growth, with IC_{50} at 22.1 μM and 19.6 μM, respectively (Fig. 2A and Table 2). To further measure the biological activity of the compound, it was also tested in a time course study with SKBR-3 and BT474 cells. The results revealed that the compound did not cause acute cytotoxicity effect to the cells. The cell viability dropped after 48 h of the compound treatment (Fig. 2B and C). Within the five breast cancer cell lines, compound 76 was more active in SKBR-3, BT474 and MDA-MB-453 which are all Her2 overexpressing cells. It appears that compound 76 was able to selectively inhibit Her2 overexpressing breast cancer cell growth.

Table 1
Her2 and ER expression levels in five breast cancer cell lines.

	BT474	SKBR-3	MDA-453	MCF-7	MDA-231
HER2	+++	+++	+	–	–
ER	+	–	–	+	–

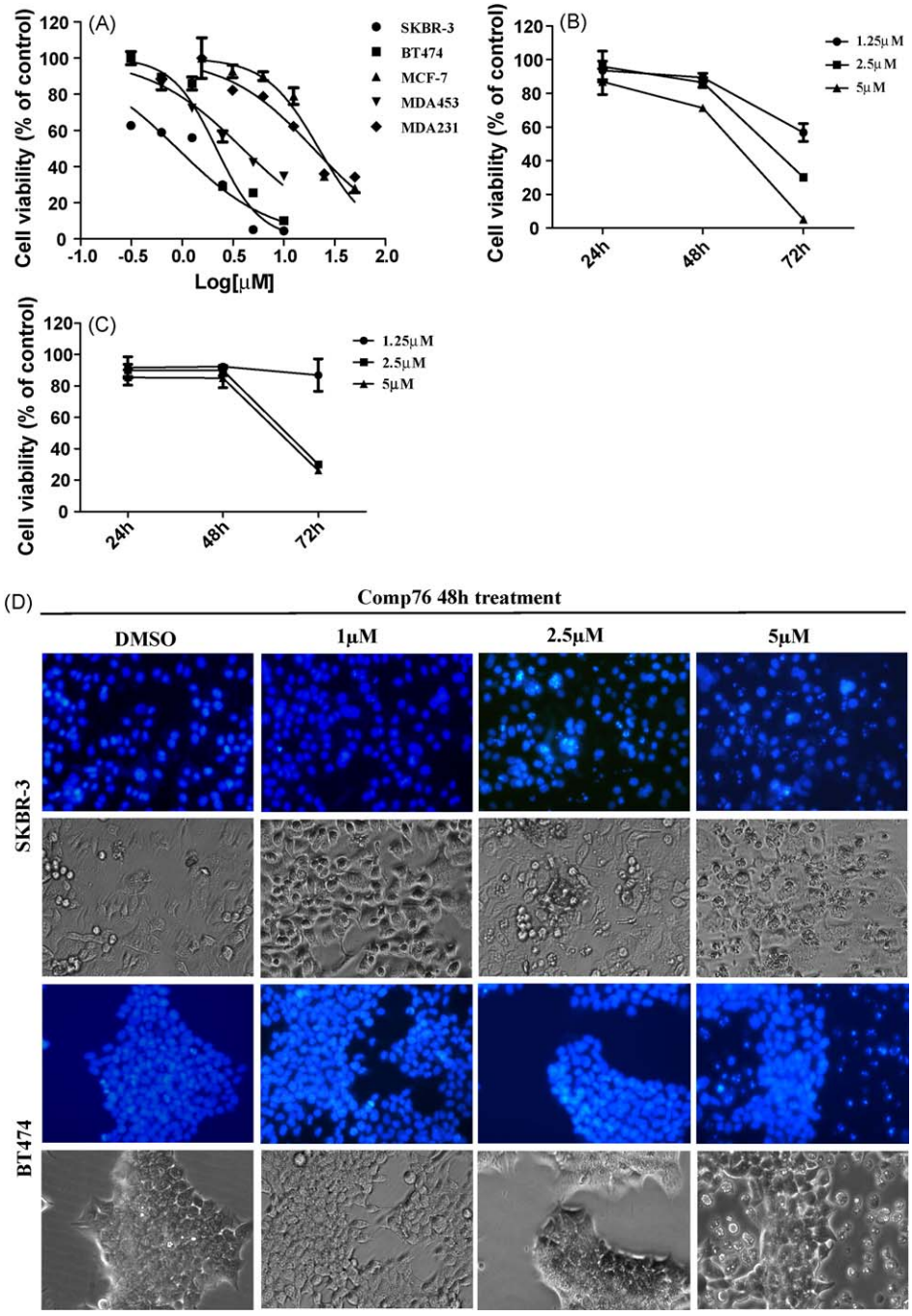

Fig. 2. Compound 76 dose and time dependently inhibited breast cancer cell growth. Cells were exposed to compound 76 with various concentration and time intervals. Cell viability was then measured by MTT assay as described in Section 2. The results were normalized against a control treatment with vehicle. Each data point represents the mean results of six independent determinations ± SD. (A) Compound 76 dose-dependently inhibited breast cancer cell growth. (B) Compound 76 time-dependently inhibited SKBR-3 cell growth. (C) Compound 76 time-dependently inhibited BT474 cell growth. (D) Compound 76 induced cellular and nuclear morphology changes. The effects of compound 76 on cell morphology were examined with the fluorescent chromatin dye Hoechst 33342 in SKBR-3 and BT474 cells. Cells were treated with compound 76 for 48 h and then stained with the fluorescent chromatin dye for 30 min. Up two panels, SKBR-3 cell; lower two panels, BT474 cells.

Table 2
IC_{50} of compound 76 on inhibition of breast cancer cell growth. Cells exposed to compound 76 with various concentrations for 72 h. Cell viability was then measured by MTT assay as described in Section 2. The results were normalized against a control treatment with vehicle. Each data points represent the mean results of six independent determinations ± SD. IC_{50} values were calculated by a nonlinear regression analysis (GraphPad Prism).

	SKBR-3	BT474	MCF-7	MD453	MD231
IC_{50} (μM)	0.89 ± 0.14	2.17 ± 0.24	22.10 ± 3.76	3.93 ± 0.53	19.58 ± 2.16

3.3. Compound 76 induced cellular and nuclear morphology changes

Based on the cell proliferation studies, continuous exposure to 2.5 μM compound 76 for 48 h significantly reduced cell survival. Cells undergoing apoptosis show typical morphological characteristics, including cell shrinkage, condensation of the nucleus, and formation of apoptotic bodies. The effects of compound 76 on cell morphology were examined with the fluorescent chromatin dye Hoechst 33342 (Fig. 2D). A large portion of the SKBR-3 cells treated with compound 76 at 2.5 μM and 5 μM for 48 h stained with

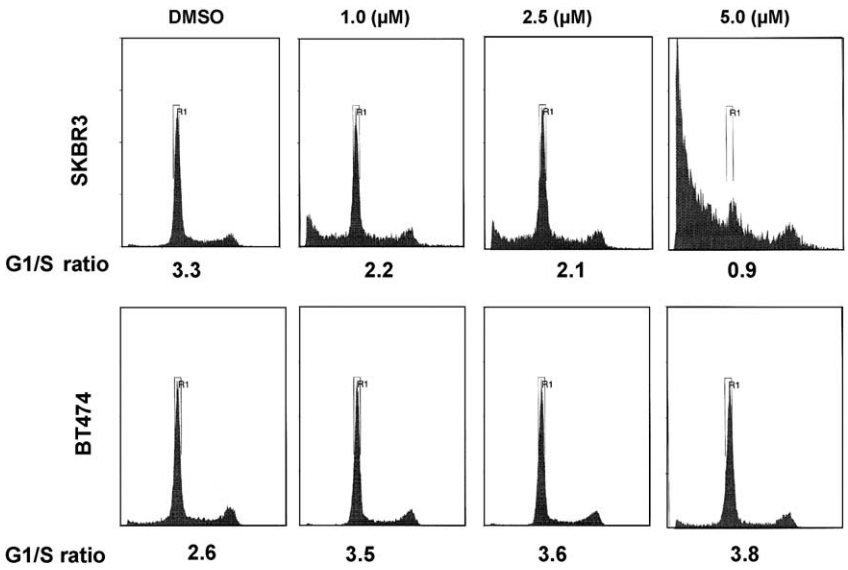

Fig. 3. Apoptosis induced by compound 76 confirmed in cell cycle study. SKRB-3 and BT474 cells were treated for 48 h in the presence of the indicated concentrations of compound 76. Cells were processed for FACS using propidium iodide as described in Section 2.

Hoechst 33342 had apoptotic bodies and/or irregularly punctate nuclei, characteristic of cells undergoing programmed cell death (Fig. 2D top panel). For BT474 cells (Fig. 2D bottom panel), this effect was not very clear. The apoptosis bodies were clearly observed only at 5 μM treatment.

3.4. Compound 76 caused cell population concentrated at sub-G1 phase

Since compound 76 dramatically inhibited SKBR-3 and BT474 breast cancer cell growth, we hypothesized that it might cause cell cycle arrest. However, in the SKBR-3 cell cycle study, a significant amount of cells concentrated at the sub-G1 phase (Fig. 3 and Table 3) after compound 76 treatment even at 1 μM, which indicated cell apoptosis. The G1/S ratio did not change significantly. Only after 5 μM treatment, G1/S was dramatically changed, mainly due to cell death. This suggests that the compound did not cause cell cycle arrest. It mainly induced apoptosis in SKBR-3 cells. However, this effect was not significant in BT474 cells. It is possible that BT474 cells have both Her2 and ER expression and depend on both pathways for proliferation. These multiple growth pathways make cells easy to survive under single treatment targeting only one pathway.

3.5. Measure apoptosis by using cell death detection ELISA

Induction of apoptosis was also assessed by using a Cell Death Detection ELISA (Roche Diagnostics, Mannheim, Germany) after compound 76 treatments in SKBR-3 and BT474 cells. This test is based on the quantitative determination of cytoplasmic histone-associated DNA fragments in the form of mononucleosomes and oligonucleosomes after induced apoptotic death (Fig. 4A and B). Significant DNA fragments were shown in SKBR-3 cells after compound 76 treatments at 2.5 μM and 5 μM for 48 h. In BT474 cells, only at 5 μM was this effect observed.

Table 3
Summary of altered cell cycle distribution in response to treatment with compound 76. SKBR-3 and BT474 cells were treated for 48 h with the indicated concentrations of compound. Cells were processed for FACS using propidium iodide and AnnexinV/7-AAD staining as described. Percent distribution of cells in each cell cycle phase was displayed.

Cell lines	Compound 76	Sub-G1%	G1%	S %	G2/M %	Dead cells
SKBR-3	DMSO	3.04	65.79	20.19	9.01	1.97
	1 (μM)	19.69	42.65	19.83	7.22	10.61
	2.5 (μM)	18.77	44.15	20.8	8.17	8.11
	5 (μM)	42.35	12.12	13.39	6.9	25.24
BT474	DMSO	9.13	58.12	21.96	7.63	3.16
	1 (μM)	2.48	68.17	19.39	8.53	1.43
	2.5 (μM)	2.31	69.1	18.99	8.04	1.56
	5 (μM)	9.56	61.33	16.24	8.66	4.21

3.6. Analysis apoptosis by cell flow cytometry and Western blot

Apoptosis was also detected by Annexin V analysis of phosphatidylserine externalization as shown in Fig. 5. Normalization to the DMSO-treated controls revealed that compound 76 at 2.5 μM and 5 μM induced 18% and 66% apoptotic death compared with 8% by DMSO in SKBR-3 cells after 48 h treatment, respectively (Fig. 5A top panel). In BT474 cells, apoptotic cells were not clearly shown in the panel at 5 μM treatment of compound 76 (Fig. 5A bottom panel). Furthermore, apoptosis was also characterized by Western blot analysis of PARP cleavage to the apoptosis-specific 85-kDa fragment and cytochrome *c* release (Fig. 5B). After 48 h treatment of compound 76 at 5 μM or even lower concentration, SKBR-3 cells showed clear 85-kDa fragment and released cytochrome *c* band; for BT474 cells, both marks were not very clear. All the results suggest that compound 76 dramatically induced apoptosis in SKBR-3 cells, but was not very effective in BT474 cells compared with SKBR-3 cells.

3.7. Compound 76 did not affect Her2 downstream kinase activity, nor the BCL family and BAD protein levels

To further explore the mechanisms of compound 76 inducing apoptosis in Her2 over expressing breast cancer cells, PI3K/Akt and MAPK kinases which are downstream of Her2 pathway were determined after compound 76 treatments. The results exhibit that pAkt, total Akt, pERK and total ERK remained the same in both cell lines (Fig. 6). This suggests that the compound did not affect the Her2 signal transduction. Previous cytochrome *c* release assay results suggest that the apoptosis induced by compound 76 was mediated from the mitochondria. Therefore, BCL-2, BCL-XL, BAX, BAD, and pBAD were also checked after compound 76 treatment. The results reveal that compound 76 did not alter the concentration of any of those apoptosis controlling proteins. It has been well documented that the dimerization of those proteins is also the key

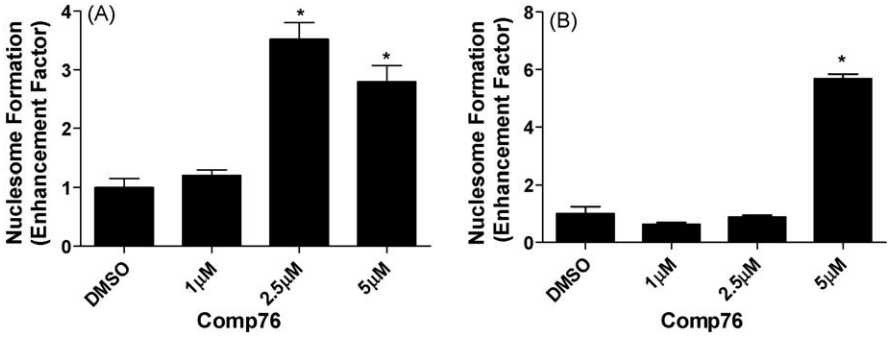

Fig. 4. Cell death ELISA analysis of apoptosis in SKBR-3 and BT474 cells receiving compound 76 treatments. Effect of compound 76 treatment on cell nucleosomal fragmentation was evaluated. Cells were treated for 48 h in the presence of the indicated concentrations of compound 76. DNA fragmentation was quantitatively measured by a cell death detection ELISA kit. Columns, mean; bars, ±SD ($n = 3$). (A) SKBR-3 cells, (B) BT474 cells. $^*P < 0.05$ vs. DMSO by unpaired t-test.

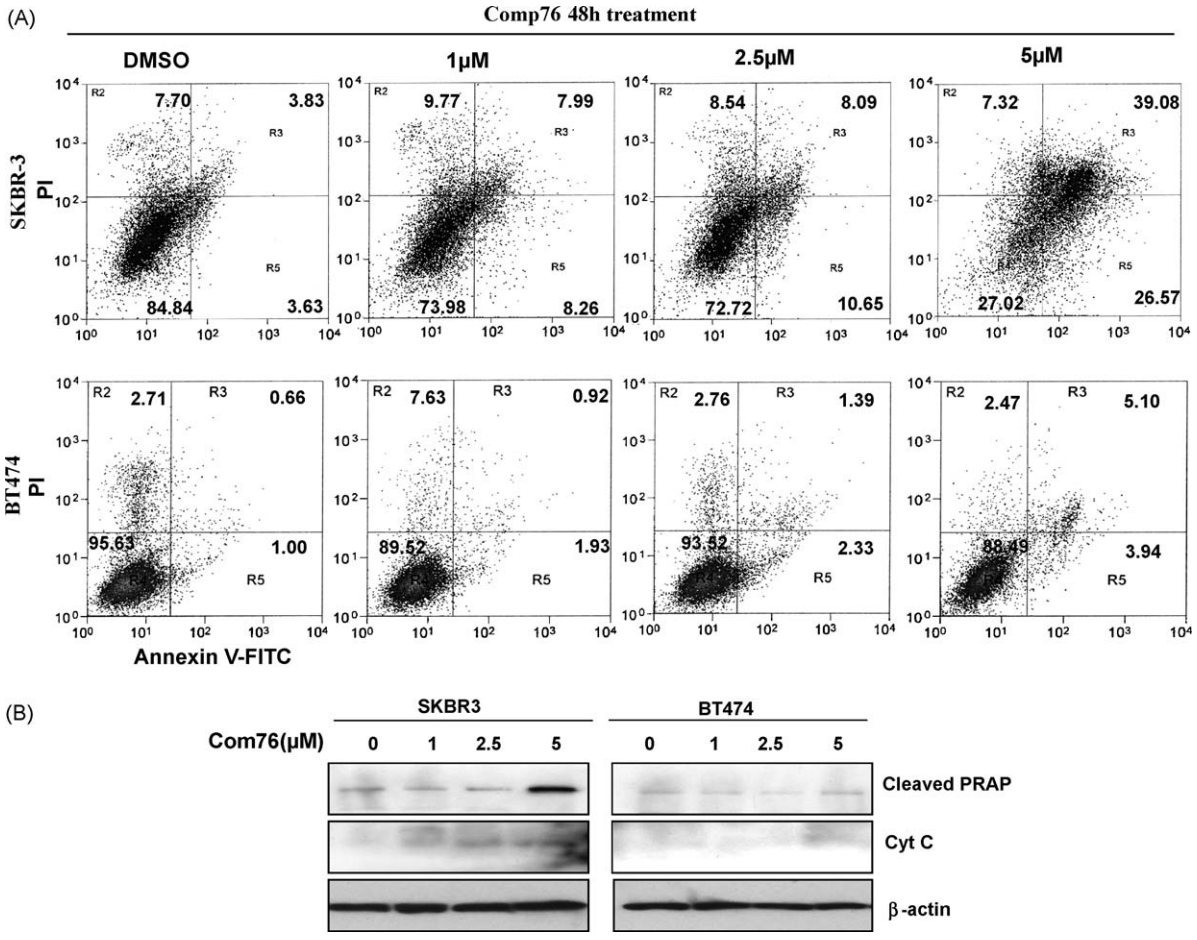

Fig. 5. Annexin V flow cytometric and Western blot analysis of apoptosis in SKBR-3 and BT474 cells receiving compound 76 treatments. (A) Cells were treated for 48 h in the presence of the indicated concentrations of compound 76 and then were processed for FACS by using Annexin V/propidium iodide staining as described in Section 2. Note that the apoptotic population resides in the upper and lower right (UR + LR) panels of the Annexin V stained cells. (B) Compound 76 induce cytochrome *c* release and PARP cleavage in both cell lines. Mitochondria-free lysates and total lysates were prepared as described in Section 2 for the Western blot analysis of cytochrome *c* release and PARP cleavage, respectively.

reason of pro- or anti-apoptosis [26,27]. The compound might interfere with the dimerization process to induce apoptosis. However, the complexity of the dimerization makes it difficult to focus on.

4. Discussion

Her2 gene amplification and resultant protein overexpression in breast cancer are associated with a more aggressive clinical course [2,9]. The Her2 receptor controls multiple pathways associated with epidermal growth factor (EGFR) and Her receptor activation. Furthermore, these pathways regulate proliferation, cell death, angiogenesis and migration [3–8]. Therefore, aberrant expression of Her2 receptors contributes significantly to the progression of cancer. The overexpressed biological marker makes targeted therapy possible in the cancer treatment. Several studies demonstrated that the inhibition of this receptor reduced the proliferative capacity and increased apoptosis of Her2-overexpressing malig-

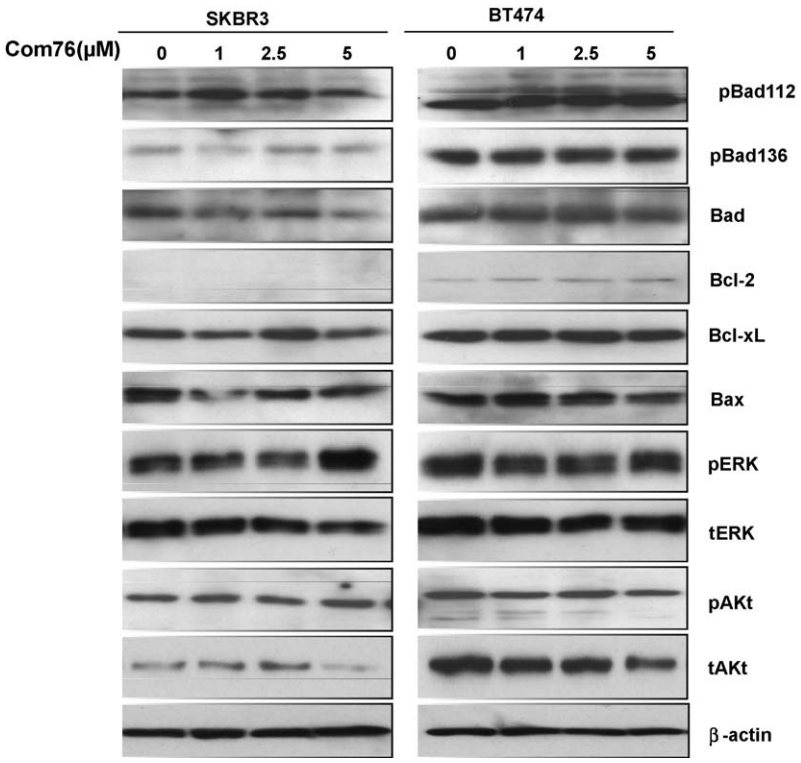

Fig. 6. Effects of compound 76 on the Her2 downstream pAKt, AKT, pERK, ERK expression and also the protein levels of Bad, Bax, BCL-2, BCL-XL in SKBR-3 and BT474 cells. Cells were treated with the indicated concentrations of compound 76 for 48 h, and cell lysates were immunoblotted as described in Section 2.

nant cells [10]. In contrast to conventional anti-cancer treatment which target cellular machinery common to both malignant as well as normal dividing cells, targeted therapy drugs are directed at a more specific target resulting in lower toxicity.

Our goal is to identify new agents that would selectively inhibit the growth of Her2 overexpressing breast cancer cells. Previous studies reveal that nimesulide and its analogs could inhibit breast cancer cell growth [24]. However, the inhibitory mechanism of these agents is not defined. Nimesulide was found to inhibit the proliferation of several breast cancer cell lines (SKBR-3, BT474, MCF-7, MDA-MB-231 cell lines) without any selectivity. The IC_{50} value for nimesulide to achieve this biological activity is around 100–150 μM [24]. It was felt that cell death resulted from general cell toxicity was not worth further investigation. Compound 76, which shares some structure similarity with nimesulide and is the most active one of the compound library, was systematically investigated with several breast cancer cell lines. Although compound 76 is an analog of COX-2 inhibitor nimesulide, it is not a COX-2 inhibitor. For COX-2 inhibition, the sulfonamide group is very critical in nimesulide and its derivatives (Fig. 1). Only when the N–H is available as the ionized form, the compounds inhibit COX-2. Introduction of any group to the sulfonamide, such as a methyl group to eliminate this ionization process, will produce compounds without COX-2 inhibitory activity. Results from previous PGE2 production experiments demonstrate that the N-methyl substituted nimesulide analogs do not exhibit COX-2 inhibitory activity [28]. Compound 76 has an N-methyl substituted sulfonamide group which eliminates it as a COX-2 inhibitor, which also suggests that the anti-breast cancer activity of compound 76 is COX-2 independent. The results presented here show that compound 76 could dramatically inhibited Her2 overexpressing breast cancer cell proliferation at low micromole concentrations. However, it was not very effective in Her2 negative breast cancer cells, and needed much higher concentration to achieve similar biological activity. This indicates that compound 76 might target Her2 related cellular machinery to inhibit cell proliferation. Breast cancer cells that do not depend on Her2 to grow were not sensitive to the compound. Because of the typical apoptotic morphological changes including cell shrinkage and condensation of the nucleus after compound 76 treatment, several assays were performed to determine the possibility of potential apoptosis-inducing effects of compound 76 in Her2 overexpressing breast cancer cells. These assays included cell cycle study, DNA staining, cell death detection ELISA, Annexin V analysis of phosphatidylserine externalization, Western blot analysis of PARP cleavage and cytochrome *c* release. With SKBR-3 cells, compound 76 at 2.5 μM and 5 μM 48 h treatments gave clearly positive results indicating apoptosis. However, for BT474 cells, this effect was not very significant. In the DNA staining, cell death detection ELISA and Annexin V analysis of phosphatidylserine externalization studies, apoptosis was significant after the treatment. The rest of the experiments did not show that compound 76 induced apoptosis in BT474 cells. It is possibly due to the multiple pathways which include Her2 and ER in BT474 cells. Cells could use alternative pathways as compensation after one was blocked by exogenous agents, which has been reported in other studies [29]. SKBR-3 cells mainly depend on Her2 to proliferate and were more sensitive to the treatment.

To further explore why compound 76 selectively induced apoptosis in Her2 overexpressing breast cancer cells, Her2 downstream signals were determined after the treatment. The results exhibited that compound 76 did not affect Akt and MAPK pathways. Cytochrome *c* release assay results suggest that the apoptosis was mediated through the mitochondria. However, compound 76 did not change BCL-2, BCL-XL, BAX, BAD and pBAD protein levels. It is also possible that the compound could interfere with the BAD and BCL-2 family protein dimerization to promote cell apoptosis. However, the dimerization of these proteins was not clearly understood yet, which makes it difficult to further explore the possible molecular target of the compound.

In conclusion, we investigated the mechanism of compound 76 in several breast cancer cell lines. The results demonstrated that the compound could selectively induce apoptosis in Her2 overexpressing cells but was less active in Her2 negative cells. However, it is still not clear the molecular target that the compound was aiming to achieve this biological activity. Her2 overexpression could protect the cells from apoptosis, and multiple downstream cell machinery was involved in this process. Our results indicate that compound 76 promoted cell apoptosis through mitochondria. Although the specific molecular target of the compound is still unclear, more active analogs could be developed from compound 76. Lead optimization of this compound to generate more potent derivatives is undergoing in our laboratory.

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References

[1] American Cancer Society. Cancer facts and figures 2008, Atlanta; 2008.
[2] Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177–82.
[3] Hynes NE. Tyrosine kinase signalling in breast cancer. Breast Cancer Res 2000;2:154–7.
[4] Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 2000; 19:3159–67.
[5] Neve RM, Sutterluty H, Pullen N, Lane HA, Daly JM, Krek W, et al. Effects of oncogenic ErbB2 on G1 cell cycle regulators in breast tumour cells. Oncogene 2000;19:1647–56.
[6] Lane HA, Beuvink I, Motoyama AB, Daly JM, Neve RM, Hynes NE. ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)–Cdk2 complex formation: receptor overexpression does not determine growth dependency. Mol Cell Biol 2000;20:3210–23.
[7] Spencer KS, Graus-Porta D, Leng J, Hynes NE, Klemke RL. ErbB2 is necessary for induction of carcinoma cell invasion by ErbB family receptor tyrosine kinases. J Cell Biol 2000;148:385–97.
[8] Yen L, Benlimame N, Nie ZR, Xiao D, Wang T, Al Moustafa AE, et al. Differential regulation of tumor angiogenesis by distinct ErbB homo- and heterodimers. Mol Biol Cell 2002;13:4029–44.
[9] Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005;353:1659–72.
[10] Piechocki MP, Yoo GH, Dibbley SK, Lonardo F. Breast cancer expressing the activated HER2/neu is sensitive to gefitinib in vitro and in vivo and acquires resistance through a novel point mutation in the HER2/neu. Cancer Res 2007;67:6825–43.
[11] Gundla R, Kazemi R, Sanam R, Muttineni R, Sarma JA, Dayam R, et al. Discovery of novel small-molecule inhibitors of human epidermal growth factor receptor-2: combined ligand and target-based approach. J Med Chem 2008;51: 3367–77.
[12] Harris RE, Namboodiri K, Stellman SD, Wynder EL. Breast cancer and NSAID use: heterogeneity of effect in a case–control study. Prev Med 1995;24:119–20.
[13] Harris RE, Namboodiri KK, Farrar WB. Nonsteroidal antiinflammatory drugs and breast cancer. Epidemiology 1996;7:203–5.
[14] Mazhar D, Ang R, Waxman J. COX inhibitors and breast cancer. Br J Cancer 2006;94:346–50.
[15] Harris RE, Chlebowski RT, Jackson RD, Frid DJ, Ascenseo JL, Anderson G, et al. Breast cancer and nonsteroidal anti-inflammatory drugs: prospective results from the Women's Health Initiative. Cancer Res 2003;63:6096–101.
[16] Harris RE, Alshafie GA, bou-Issa H, Seibert K. Chemoprevention of breast cancer in rats by celecoxib, a cyclooxygenase 2 inhibitor. Cancer Res 2000; 60:2101–3.
[17] Zhu J, Huang JW, Tseng PH, Yang YT, Fowble J, Shiau CW, et al. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. Cancer Res 2004;64:4309–18.
[18] Song X, Lin HP, Johnson AJ, Tseng PH, Yang YT, Kulp SK, et al. Cyclooxygenase-2, player or spectator in cyclooxygenase-2 inhibitor-induced apoptosis in prostate cancer cells. J Natl Cancer Inst 2002;94:585–91.
[19] Bae SH, Jung ES, Park YM, Kim BS, Kim BK, Kim DG, et al. Expression of cyclooxygenase-2 (COX-2) in hepatocellular carcinoma and growth inhibition of hepatoma cell lines by a COX-2 inhibitor, NS-398. Clin Cancer Res 2001;7:1410–8.
[20] Li M, Wu X, Xu XC. Induction of apoptosis in colon cancer cells by cyclooxygenase-2 inhibitor NS398 through a cytochrome *c*-dependent pathway. Clin Cancer Res 2001;7:1010–6.
[21] Sanchez-Alcazar JA, Bradbury DA, Pang L, Knox AJ. Cyclooxygenase (COX) inhibitors induce apoptosis in non-small cell lung cancer through cyclooxygenase independent pathways. Lung Cancer 2003;40:33–44.
[22] Nakatsugi S, Ohta T, Kawamori T, Mutoh M, Tanigawa T, Watanabe K, et al. Chemoprevention by nimesulide, a selective cyclooxygenase-2 inhibitor, of 2-amino-1-methyl-6-phenylimidazo [4, 5-b]pyridine (PhIP)-induced mammary gland carcinogenesis in rats. Jpn J Cancer Res 2000;91:886–92.
[23] Shaik MS, Chatterjee A, Singh M. Effect of a selective cyclooxygenase-2 inhibitor, nimesulide, on the growth of lung tumors and their expression of cyclooxygenase-2 and peroxisome proliferator-activated receptor-gamma. Clin Cancer Res 2004;10:1521–9.
[24] Su B, Darby MV, Brueggemeier RW. Synthesis and biological evaluation of novel sulfonanilide compounds as antiproliferative agents for breast cancer. J Comb Chem 2008;10:475–83.
[25] Shiau CW, Yang CC, Kulp SK, Chen KF, Chen CS, Huang JW, et al. Thiazolidenediones mediate apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 functions independently of PPARgamma. Cancer Res 2005;65:1561–9.
[26] Zhang Z, Lapolla SM, Annis MG, Truscott M, Roberts GJ, Miao Y, et al. Bcl-2 homodimerization involves two distinct binding surfaces, a topographic arrangement that provides an effective mechanism for Bcl-2 to capture activated Bax. J Biol Chem 2004;279:43920–8.
[27] Liu FT, Goff LK, Hao JH, Newland AC, Jia L. Increase in the ratio of mitochondrial Bax/Bcl-XL induces Bax activation in human leukemic K562 cell line. Apoptosis 2004;9:377–84.
[28] Su B, Diaz-Cruz ES, Landini S, Brueggemeier RW. Novel sulfonanilide analogues suppress aromatase expression and activity in breast cancer cells independent of COX-2 inhibition. J Med Chem 2006;49:1413–9.
[29] Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 2004;96:926–35.