SUCI02 inhibits the erbB-2 tyrosine kinase receptor signaling pathway and arrests the cell cycle in G_1 phase in breast cancer cells

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(Received July 24, 2005/Revised August 25, 2005/2nd revised October 26, 2005/Accepted October 28, 2005/Online publication December 18, 2005)

The *erbB-2* gene encodes tyrosine kinase receptor $p185^{neu}$. Overexpression of *erbB-2* plays a key role in tumorigenesis and the progression of tumors such as breast cancer and ovarian cancer. Our investigation suggests that the anti-inflammatory agent *N*-(4-ethoxyphenol)-2-hydroxy-acid amide (SUCI02) reversibly represses tyrosine phosphorylation of erbB-2 in a dose-dependent manner, with half maximal inhibition occurring at a concentration of 21.05 μmol/L without reduced erbB-2 receptor expression. Activation of mitogen-activated protein kinase and protein kinase B, downstream molecules of the erbB-2-mediated signal transduction pathway, was inhibited following exposure to SUCI02. In contrast, tyrosine phosphorylation of epidermal growth factor receptor (EGFR) was relatively unaffected by SUCI02. Proliferation of erbB-2-overexpressing BT474 cells was inhibited to a greater extent than proliferation of EGFR-overexpressing A431 cells following exposure to SUCI02. SUCI02 induced cell cycle arrest in G_1 phase with upregulation of p27 and downregulation of pRb phosphorylation. Systemic administration of SUCI02 in nude mice resulted in inhibition of erbB-2 tyrosine kinase phosphorylation of subcutaneous human breast cancer BT474 xenografts. We conclude that SUCI02 inhibits erbB-2 tyrosine kinase phosphorylation *in vitro* and *in vivo*, shuts down the erbB-2 downstream pathway and induces cell cycle arrest in G_1 phase. These results suggest that SUCI02 is a potential novel anticancer agent that deserves further investigation. (*Cancer Sci* 2006; 97: 84–89)

ErbB-2 is a 185-kDa protein composed of an extracellular ligand-binding domain, a short transmembrane domain and an intracellular domain, which has tyrosine kinase activity.[1] It has been reported that overexpression of the erbB-2 receptor plays a key role in pathological processes such as tumorigenesis and progression, especially in breast and ovarian cancers, and in other cancers such as gastrointestinal, pulmonary and genitourinary neoplasms.[2–6] Breast cancer is the leading cause of death from cancer among women in the USA. Amplification of the human proto-oncogene *erbB-2* reportedly occurs in approximately 30% of breast carcinomas and measurement of the amplification has been proposed as a useful predictor of disease prognosis.[7] Gene amplification and the resulting overexpression of the *erbB-2* proto-oncogene-encoded $p185^{neu}$ may transform cells by chronically stimulating the signal transduction pathway.[8] Overexpression of *erbB-2* has also been shown to correlate with tumor grade, tumor size, lymph node metastasis, survival and resistance to hormonal therapy and chemotherapy in breast cancer patients.[9] Epidermal growth factor (EGF) or heregulin facilitates erbB-2 heterodimerization and homodimerization with itself or other members of erbB family, resulting in activation and autophosphorylation of the protein tyrosine kinase domain.[10] An intact kinase domain is essential for activation of numerous downstream effectors, including phospholipase Cγ, phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK), with the ultimate cellular response being DNA synthesis and cell proliferation.[11,12] The known oncogenic potential mediated by erbB-2 and its high-level expression in tumor tissues compared with normal cells make this oncoprotein an ideal target for antitumor therapeutic approaches. Therapy targeted against erbB-2 has already had proven clinical outcomes. Genetech developed the humanized monoclonal antibody Herceptin, which is targeted against the extracellular domain of erbB-2. The phase III clinical trials of Herceptin provide evidence that systemic administration of Herceptin, alone and in combination with cytotoxic chemotherapy in patients with erbB-2-overexpressing primary tumors, can increase the time to recurrence and overall response rates in metastatic breast cancer.[13,14] Also, Gefitinib (ZD1839),[15] is a low molecular weight, synthetic anilinoquinazoline that selectively inhibits the tyrosine kinase activity of epidermal growth factor receptor (EGFR). In patients with advanced non-small cell lung cancer (NSCLC) who had failed one or two prior chemotherapies, Gefitinib administered at 250 or 500 mg once daily induced an objective response in 19% of patients in a double-blind trial ($n = 210$).[16] Gefitinib was approved by the Food and Drug Administration for use as monotherapy for the treatment of patients with locally advanced or metastatic NSCLC after failure of both platinum-based and docetaxol chemotherapies. The success of Herceptin and Gefitinib clearly shows the therapeutic value of a molecular therapy targeted at erbB-2 and provides much encouragement for the design and development of other therapies targeted at erbB-2.

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doi: 10.1111/j.1349-7006.2006.00143.x

Certain limitations are associated with large molecule strategies, including poor delivery, poor *in vivo* stability and high cost.[17] To overcome these limitations, we have been actively pursuing small molecule therapeutic strategies targeted at the erbB-2 receptor-mediated signal transduction pathway. *N*-(4-ethoxyphenol)-2-hydroxy-acid amide (SUCI02), which is as an anti-inflammatory agent, has been used clinically in China. In the present study, we found that SUCI02 could inhibit tyrosine phosphorylation of erbB-2 through comprehensive screening, block the erbB-2 signaling pathway and induce G_1 arrest in erbB-2-overexpressing breast cancer cells.

Materials and methods

Cell culture and reagents

The human breast cancer cell lines BT474 and A431 were obtained from the American Type culture collection (Rockville, MD, USA). BT474 was grown in Dulbecco's modified Eagle's medium (DMEM) medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. MDA-MB-453 and A431 cells were cultivated in RMPI-1640 medium (Life Technologies). Cells were grown in an incubator at 37°C under 5% CO_2 in air. The erbB-2 antibody was obtained from Calbiochem (San Diego, CA, USA). Anti-Akt, phospho-Akt, phospho-erbB-2, phospho-EGFR, MAPK and phospho-MAPK antibodies were purchased from Cell Signaling (Beverly, MA, USA). The synthesis of SUCI02 has been described previously.[18] Its structure is shown in Fig. 1. Drug stock solution was prepared by dissolving SUCI02 in dimethylsulfoxide (DMSO). The final concentration of DMSO in SUCI02-treated cultures was always less than 0.2% (v/v) and did not contribute to toxicity.

Western blot analysis

Cells treated with SUCI02 were washed twice with phosphate-buffered saline (PBS) and lysed in 100 μL of lysis buffer (20 mM Na_2PO_4 [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenymethysulfonyl fluoride, 10 mg/mL leupeptin, 100 mM NaF and 2 mM Na_3VO_4). Lysates were centrifuged at 13 400 *g* for 10 min and the supernatant collected. The protein content was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). A total of 40 μg of protein was resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–20% gel and transferred electrophoretically to a nitrocellulose membrane (Amersham, Piscataway, NJ, USA). The membranes were blotted for 30 min at 25°C with non-fat dry milk (5%) in Tris-buffered saline-Tween (TBST) (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween), and then incubated with primary antibodies for 2 h at 25°C. The membranes were washed with TBST and incubated with horseradish peroxidase-labeled antimouse or rabbit secondary antibodies for 1 h at 25°C. After washing with TBST, the bound antibody complex was detected using an ECL chemiluminescence reagent (Amersham) and XAR film (Kodak, Pittsburgh, PA, USA) as described by the manufacturers.

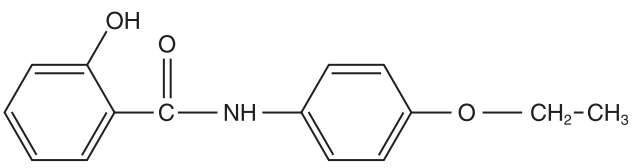

Fig. 1. The structure of SUCI02.

Reversibility and irreversibility of erbB-2 tyrosine kinase phosphorylation inhibition

MDA-MB-453 cells overexpressing erbB-2 were plated at a density of 5×10^5 cells/well in 12-well plates. On the following day, the regulatory medium was changed to serum-free medium. Drug stock was added. Following treatment with 77.8 μM SUCI02 for 30 min, cells were washed twice with serum-free medium. Serum-free medium (1 mL) was added and incubated in an incubator at 37°C under 5% CO_2 in air for different periods of time. Cells were collected, lysed and western blot assays were carried out to determine erbB-2 phosphorylation.

MTT assay

Cells were plated at a density of 2000 cells/well in 96-well plates. The stock of SUCI02 was diluted with medium, then added to the wells for the desired final assay concentrations. After 3 days of exposure to SUCI02, 10 μL of 5 mg/mL MTT was added to each well and incubated for ≥ 4 h, and the liquid in the wells was aspirated. DMSO (100 μL) was added to each well to dissolve the formazam. The absorbance was detected in a microplate reader at 565 nm wavelength. Cell viability rates were expressed as a percentage of absorbance detected in the control wells that were treated with 0.1% DMSO alone. DMSO controls were not different from cells in the regulatory growth medium. The IC_{50} value was calculated using Bliss software.

Cell cycle analysis

Cells were harvested by trypsinization, washed twice with ice-cold PBS, resuspended in cold PBS, and fixed with 70% ethanol. After overnight refrigeration at −20°C and subsequent rehydration in PBS for 30 min at 4°C, the cell nuclei were stained for 30 min in the dark with 50 μg/mL propidium iodide (Sigma, St Louis, MO, USA) containing 125 IU/mL protease-free RNase, both diluted in PBS. Cells were filtered through a 95-μm pore size nylon mesh, and a total of 10 000 stained nuclei were analyzed with a fluorescence-assisted cell sorting (FACS) machine.

Inhibitory effect of SUCI02 on erbB-2 tyrosine kinase phosphorylation *in vivo* in nude mice

Human tumor xenografts were established by 1×10^6 BT474 cells injected into nude mice subcutaneously. When the volume of the tumor reached ~200 mm^3, 20 mg/kg SUCI02 was injected intraperitonealy into mice bearing transplanted BT474 cells to determine the inhibitory effect of erbB-2 tyrosine kinase phosphorylation *in vivo*. The mice were killed, and the tumor tissues were excised, homogenated and lysed for determination of erbB-2 phosphorylation using western blot analysis.

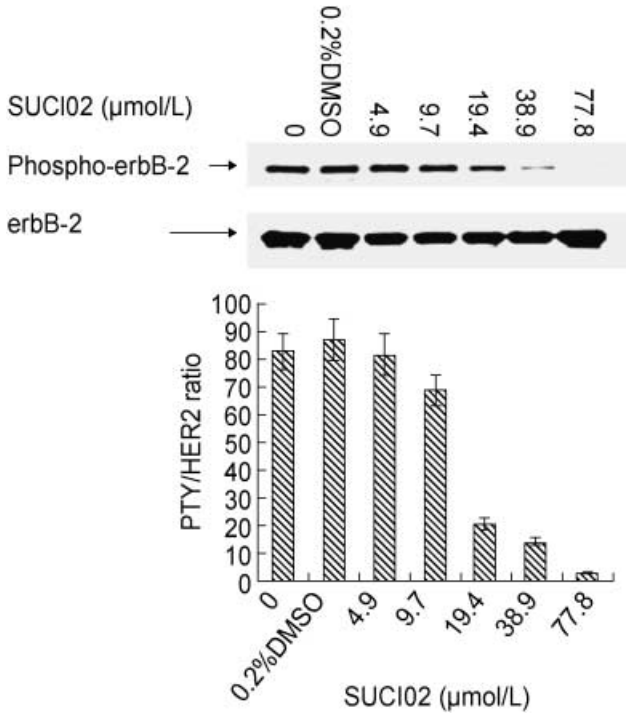

Fig. 2. Inhibition of erbB-2 phosphorylation by SUCI02 in BT474 cells. BT474 cells were treated with different concentrations of SUCI02 for 1 h. Cells were collected and lysed. Western blot analysis was conducted using antiphospho-erbB-2 and anti-erbB-2 antibodies. The lower panel shows the ratios of phospho-erbB-2 protein relative to erbB-2 protein. This experiment is representative of three experiments.

Results

Effect of SUCI02 on tyrosine phosphorylation of erbB-2

To examine the effects of potential of erbB-2 tyrosine kinase inhibitors, we carried out comprehensive phosphorylation inhibition. This is because autophosphorylation of erbB-2 increases the velocity of the kinase reaction and only phosphorylated erbB-2 can provide binding sites for its substrates (such as phospholipase Cγ, Grb-2 and Shc), allowing these substrates to bind to the activated receptor. After comprehensive screening, SUCI02 was found to inhibit tyrosine phosphorylation of erbB-2. To examine the dose–effect relationship of inhibition, BT474 cells, which overexpress $p185^{neu}$, were treated in serum-free medium with indicated concentrations of SUCI02 for 1 h. Cells were then lysed and resolved by SDS-PAGE. The results from western blot analysis of $p185^{neu}$ protein levels and phosphorylation of erbB-2 suggested that SUCI02 inhibited phosphorylation of $p185^{neu}$ significantly in a dose-dependent manner, with an IC_{50} value of 21.05 μM in BT474 (Fig. 2). To study the inhibition of erbB-2 tyrosine phosphorylation by SUCI02 but not protein expression of erbB-2, protein expression levels of erbB-2 were detected using the same lysates. The results showed that the levels of erbB-2 protein were unaffected following SUCI02 treatment.

Effect of SUCI02 on activation of MAPK and AKT, downstream targets of the erbB-2 signaling pathway

Activation of erbB-2 leads to breast cancer cell proliferation, presumably by inducing activation of the MAPK and PI3K/AKT pathways. To examine whether SUCI02 can inhibit activation of MAPK and AKT, BT474 cells were treated with varying concentrations of SUCI02 for 1 h, and phospho-MAPK and MAPK were detected with antiphospho-MAPK and anti-MAPK antibodies. Phospho-Akt and Akt were detected with antiphospho-AKT and anti-AKT antibodies. Phospho-MAPK and phospho-Akt are the active forms of MAPK and Akt, respectively. The results showed that phospho-MAPK and phospho-Akt levels decreased significantly after treatment of BT474 cells with SUCI02 (Fig. 3) without reducing total MAPK and Akt. This suggests that SUCI02 has a potent inhibitory effect on activation of MAPK and Akt in a dose-dependent manner in erbB-2-overexpressing cancer cells following SUCI02 treatment.

Selectivity of inhibition of erbB-2 phosphorylation by SUCI02

SUCI02 can inhibit tyrosine phosphorylation of erbB-2. It is of interest to investigate the selectivity of SUCI02 toward erbB-2. To examine this issue, human epidermal carcinoma A431 cell line, which overexpresses EGFR, was treated with 77.8 or 155.6 μM of SUCI02 for 1 h, then analyzed for the levels of EGFR and phosphorylation of EGFR. As shown in Fig. 4, SUCI02 had no inhibitory effect on tyrosine phosphorylation of EGFR and activation of MAPK and AKT following up to 155.6 μM SUCI02 treatment for 1 h in A431 cells.

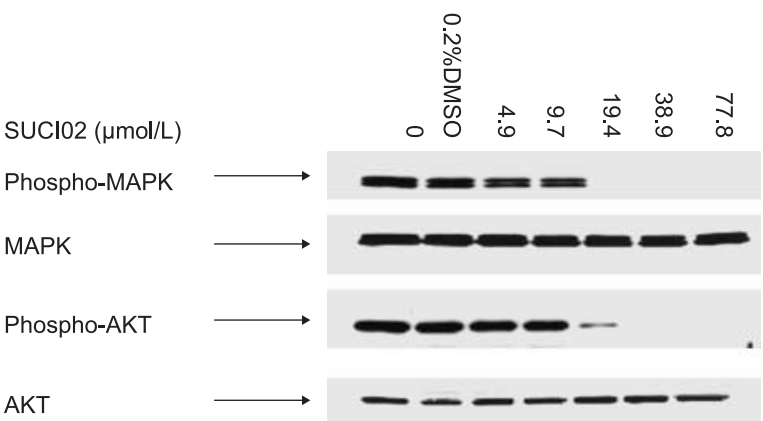

Fig. 3. Inhibition of mitogen-activated protein kinase (MAPK) and AKT phosphorylation by SUCI02 in BT474 cells. BT474 cells were treated with different concentrations of SUCI02 for 1 h. Cells were collected and lysed. Western blot analysis was conducted using antiphospho-MAPK, anti-MAPK, antiphospho-AKT and anti-AKT antibodies. This experiment is a representative of two experiments.

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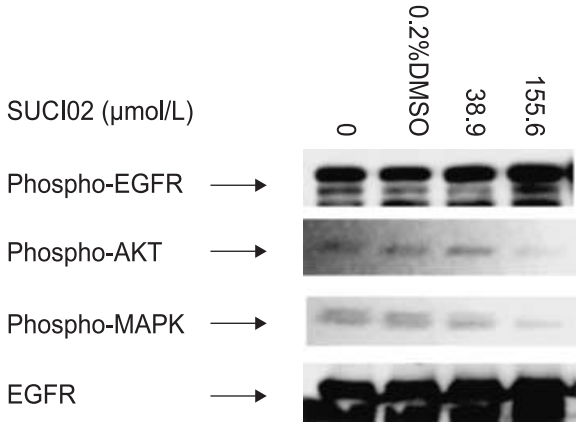

Fig. 4. Effect of SUCI02 on epidermal growth factor receptor (EGFR), protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) phosphorylation. A431 cells were treated with different concentrations of SUCI02 for 1 h. Cells were collected and lysed. Western blot analysis was conducted using anti-EGFR, antiphospho-AKT, antiphospho-MAPK and antiphospho-EGFR antibodies. This experiment is representative of two experiments.

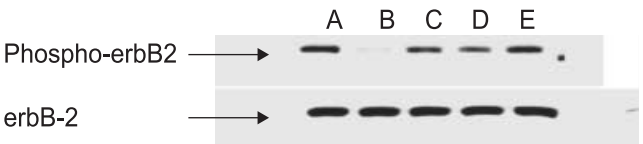

Fig. 5. Reversible inhibition of erbB-2 phosphorylation by SUCI02. MDA-MB-453 cells were treated with 0.2% dimethylsulfoxide (A,E), or 38.9 μmol/L SUCI02 (B,C,D) for 30 min, then washed with medium and incubated for 30 min (C) or 8 h (D). Western blot analysis was carried out using antiphospho-erbB-2 or anti-erbB-2 antibodies. This experiment was repeated twice.

Reversible inhibition of erbB-2 tyrosine kinase phosphorylation by SUCI02

The results shown above indicate that SUCI02 inhibits tyrosine phosphorylation of erbB-2. To investigate whether inhibition of tyrosine phosphorylation of erbB-2 by SUCI02 is reversible, MDA-MB-453 cells treated with 38.9 μmol/L SUCI02 in serum-free medium for 30 min were washed twice with serum-free medium and then incubated for either 30 min or 8 h. Tyrosine phosphorylation of erbB-2 was then detected. The results in Fig. 5 show that inhibition of erbB-2 phosphorylation was recovered 30 min after SUCI02 was washed out. This indicates that inhibition of erbB-2 phosphorylation is reversible.

Effect of SUCI02 on proliferation of human breast cancer cells

The results described above suggest that SUCI02 inhibits tyrosine phosphorylation of $p185^{neu}$ in a breast cancer cell line overexpressing erbB-2. However, it was not known whether SUCI02 could inhibit cell growth preferentially in breast cancer cells that overexpress $p185^{neu}$. In order to examine this issue, BT474 and A431 were used. The results shown in Fig. 6 indicate that SUCI02 has a greater inhibitory effect on BT474 cells compared to A431 cells. Their IC_{50} values were 12.8 μM and 43.9 μM, respectively. This indicates that SUCI02 inhibits growth of cells overexpressing erbB-2 preferentially.

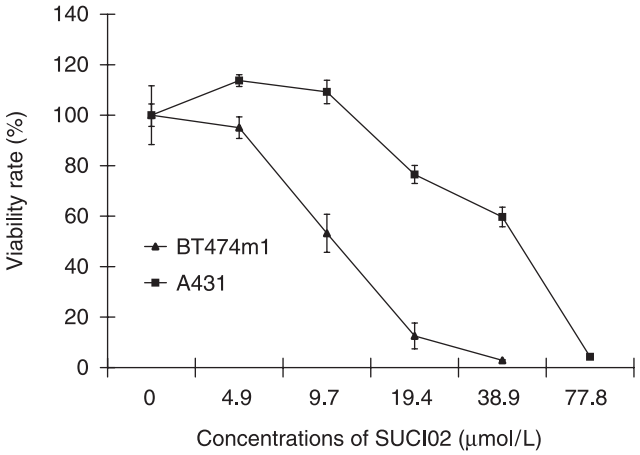

Fig. 6. Growth inhibition of SUCI02 on cancer cell lines. BT474 and A431 cells were plated at a density of 2000 cells/well in 96-well plates. The stock of SUCI02 was added to wells. In the serum-free treatments, the cells were incubated for 3 days. Cell viability was determined using the MTT assay is expressed as cell viability rate ± SE ($n = 4$).

Table 1. Effect of SUCI02 on the cell cycle in BT474 cells

Phase (%)	SUCI02 (μM)				
	0	4.9	9.7	19.4	38.9
G_0/G_1	56.68	61.21	60.81	64.93	78.91
S	41.1	34.67	36.42	30.67	18.38
G_2/M	2.22	4.12	2.77	4.40	2.70

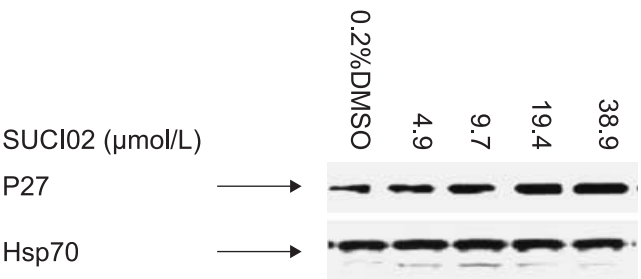

Fig. 7. Effect of SUCI02 on p27 protein levels in BT474 cells. BT474 cells were treated with different concentrations of SUCI02 for 24 h. Cells were collected and lysed. Western blot analysis was conducted using anti-p27 and anti-hsp70 antibodies. This experiment was repeated twice.

SUCI02 halts the cell cycle in BT474 cells

The effects on cell cycle were studied following SUCI02 treatment in BT474 cells. Cell cycle distribution was detected at various concentrations of SUCI02 (0, 4.9, 9.7, 19.4 and 38.9 μM). As shown in Table 1, the percentages of cells in G_1 phase were 56.4, 62.1, 66.3, 71.5 and 78.7%, respectively. Also, the results from western blot analysis showed that p27 protein expression was upregulated and hyperphosphorylated Rb decreased after SUCI02 treatment (Figs 7 and 8).

Inhibition of erbB-2 phosphorylation by SUCI02 in vivo

To investigate the *in vivo* effect of SUCI02 on erbB-2 phosphorylation under systemic administration in animals,

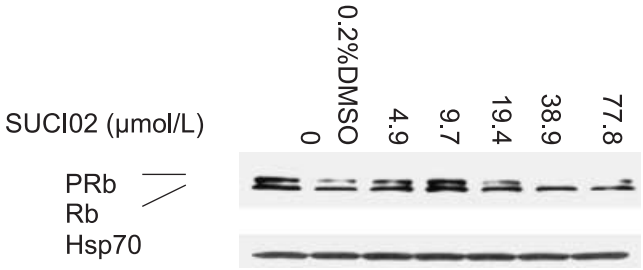

Fig. 8. Effect of SUCI02 on the levels of pRb protein phosphorylation in BT474 cells. BT474 cells were treated with different concentrations of SUCI02 for 24 h. Cells were collected and lysed. Western blot analysis was conducted using anti-pRb and anti-hsp70 antibodies. This experiment is representative of two experiments.

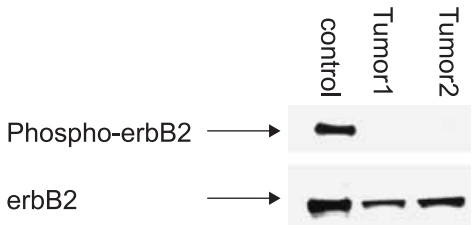

Fig. 9. Inhibition of erbB-2 phosphorylation by SUCI02 *in vivo*. The nude mice bearing BT474 xenografts were treated with 20 mg/kg SUCI02 twice daily for 2 days. The mice were killed, and the tumors were excised and homogenated. Western blot analysis was conducted using anti-erbB-2 and anti-phospho-erbB-2 antibodies.

the BT474 xenograft model, which forms progressive tumors in nude mice, was utilized. The mice bearing BT474 xenografts was treated with SUCI02 at 20 mg/kg, twice daily for 2 days. Tumors were excised at 24 h after treatment, and homogenates were prepared for determining the levels of phosphorylated erbB-2 by western blot analysis. Treatment with SUCI02 suppressed erbB-2 phosphorylation markedly (Fig. 9). It was of note that SUCI02 had no effect on total erbB-2 protein expression.

Discussion

Overexpression of erbB-2, which is a member of the EGFR family, correlates to a poor prognosis and decreased survival rate in patients with breast cancer, ovarian cancer and other cancers.[3] Activation of erbB-2 by EGF-like ligand results in its receptor tyrosine kinase phosphorylation. Phosphorylated erbB-2 receptor provides binding sites for Src homology (SH)-2 domain-containing proteins, including the adaptor proteins Grb-2 and Shc. In addition to the SH-2 domain, Grb-2 binds small nucleotide exchange proteins such as SOS through its SH-3 domain, and then activates Ras through exchanging GTP for GDP on Ras. Activated Ras binds to and facilitates Raf activation. Activated Raf stimulates mitogen-activated protein kinase kinase kinase (MEKK) activity, which, in turn, activates the MAPK pathway. The MAPK pathway is important for cell proliferation, transformation and differentiation. Blocking MAPK activity can inhibit cancer cell growth *in vitro* and *in vivo*.[19,20] The same results could be observed under inhibition of Ras by a farnesyl : protein transferase inhibitor.[21] ErbB-2 is also critical to activation of the PI3K pathway by heterodimerization with erbB-3, which is the only erbB-2 receptor able to recruit the p85 subunits of PI3K.[12]

ErbB-2 is a potent target for cancer treatment. In the present study, we found that SUCI02 inhibited tyrosine phosphorylation of erbB-2. ErbB-2 tyrosine phosphorylation was inhibited by SUCI02 in erbB-2-overexpressing BT474 cells, but expression of the erbB-2 protein was unaffected. This indicates that inhibition of phosphorylation of erbB-2 is not via reduced erbB-2 expression. SUCI02 has no inhibitory effect on tyrosine phosphorylation of EGFR under the same concentrations of SUCI02. This indicates that SUCI02 can selectively inhibit erbB-2 phosphorylation.

Overexpression of erbB-2 activates the MAPK and AKT pathways associated with proliferation and survival of cells overexpressing erbB-2. Both MAPK and AKT are downstream targets of erbB-2. SUCI02 can inhibit tyrosine phosphorylation of erbB-2. It was predicted that MAPK and AKT activation should also be inhibited by SUCI02. Our results indicate that phospho-MAPK and phospho-Akt levels were decreased markedly, but total MAPK and Akt levels were not decreased. These results indicate that SUCI02 can inhibit activation of MAPK and Akt.

SUCI02 inhibits tyrosine phosphorylation of erbB-2, thereby inhibiting the MAPK and PI3K/Akt pathways. Inhibition of BT474 cell and A431 cell proliferation by SUCI02 was investigated. The results show that SUCI02 inhibits BT474 cell growth significantly. By comparison, growth inhibition of A431 cells by SUCI02 was weaker although SUCI02 could inhibit A431 cell growth at high concentrations. It is possible that SUCI02 has other targets or that A431 cells have a low level of erbB-2 expression. SUCI02 did not influence EGFR phosphorylation in A431 cells. Therefore, it might not influence the dissociation of ligands such EGF and transforming growth factor (TGF)-α. However, it could influence the dimerization between human epidermal growth factor receptor (HER)2 and other HER proteins or directly inhibit the tyrosine kinase activity of erbB-2. We did not carry out *in vitro* kinase assays, so it is not known whether SUCI02 directly inhibits the protein tyrosine kinase of erbB-2.

SUCI02 can induce cell cycle arrest in G_1 phase. In order to elucidate the molecules involved in G_1 arrest following SUCI02 treatment in BT474 cells, p27 protein expression and hyperphosphorylated Rb were detected after BT474 cells were treated with various concentrations of SUCI02. The results show that SUCI02 induces p27 upregulation and decreases phosphorylated Rb in a dose-dependent manner. P27 is a member of the KIP family, which was originally discovered as a cyclin-dependent kinase (Cdk) inhibitor induced by extracellular antimitogenic signals.[22,23] It can inhibit cdk4/6 activity and its overexpression halts the cell cycle in G_1 phase. Cdks can phosphorylate Rb, then E2F which is a transcription factor is released. E2F can promote cell cycle progression. Induction of p27 is usually regulated post-transcriptionally. The stabilization of phosphorylated p27 is decreased through the ubiquitin degradation system. Upregulation of p27 following SUCI02 treatment in BT474 cells could cause an increase in p27 stabilization and a decrease in phosphorylation of p27. This may be because inhibition of

doi: 10.1111/j.1349-7006.2006.00143.x

Akt activation by SUCI02 results in a decrease in p27 phosphorylation and accumulation of p27, and eventually arrests the cell cycle in G_1 phase, inhibiting cell growth.

SUCI02 can also inhibit erbB-2 tyrosine kinase phosphorylation *in vivo*. Therefore, SUCI02 should be able to inhibit erbB-2-overexpressing cancer cell growth. Taken together, SUCI02 inhibits tyrosine phosphorylation of erbB-2 selectively, shuts down the erbB-2 signaling pathway, arrests the cell cycle in G_1 phase and inhibits growth of erbB-2-overexpressing cancer cells. Our experiments suggest that SUCI02 is a potential novel anticancer agent that deserves further investigation.

Acknowledgments

This study was supported by Grants from the National Natural Science Foundation of China (Nos 30171092, 30472037) and the Nature Science Foundation of Guangdong Provinces (No. 30126).

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