Regular Article

Synergistic Inhibition of Breast Cancer Cell Growth by an Epigenome-Targeting Drug and a Tyrosine Kinase Inhibitor

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Background: Epigenome-targeting drugs, for example, histone deacetylases (HDACs) inhibitors, have been recently shown to induce apoptosis in a variety of cancer cells, which could potentially be used as anti-cancer therapy. Tyrosine kinase inhibitors (TKIs) have been widely used in clinical trials of various cancers. HDAC inhibitor vorinostat, TKIs dasatinib have been tested in pivotal phase 2 clinical trials in patients with breast cancer. The combination treatment of vorinostat with dasatinib is expected to have synergistic effect on inhibiting breast cancer cell growth. **Materials and Methods:** Antiproliferation effects of the combined drugs on MCF-7 cells were designed according to Chou–Talalay method and analyzed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell-cycle perturbation and cell apoptosis induction of the combination drugs were examined by Flow cytometry. The generation of reactive oxygen species (ROS), loss of mitochondrial membrane potential, and the expression of Bcl-2 were determined by Western blot. **Results:** Our results revealed that the combination treatment had synergistic effects on anti-MCF7 cells, enhanced G₂/M cell arrest, the generation of ROS, the loss of mitochondrial membrane potential, and cell apoptosis in MCF-7 cells in synergy. Moreover, the combination treatment decreased Bcl-2 expression. **Conclusion:** Our results demonstrated that the combination of vorinostat with dasatinib exerted synergistic anticancer effects on MCF-7 cells by inducing cell cycle arrest, ROS production, and apoptosis through the mitochondria-mediated intrinsic pathway.

Key words vorinostat; dasatinib; breast cancer; apoptosis; combination therapy

Breast cancer is one of the most common malignancies in women and has become a major cause of women's death worldwide.¹⁾ Breast cancer occurs in the mammary epithelium or ductal epithelium. In recent years, the pathogenesis of breast cancer has been continuously investigated by medical researchers.²⁾ As a result, many drugs for treatment of breast cancer have been marketed, including natural ingredients (such as paclitaxel and vincristine), chemical drugs (such as capecitabine and tamoxifen) and targeted drugs (such as Herceptin and lapatinib). Although these drugs have been widely used in the treatment of breast cancer, the serious side effects (especially for chemical drugs) and drug resistance in patients (especially for targeted drugs) also limited their clinical use.^{3–5)}

It is well known that most human complex diseases, especially cancer, are not the result of a single disease gene, but are caused by complex biological networks.⁶⁾ As a result, it is reasonable to speculate that targeting multiple disorder factors of disease will have higher medicinal potential than single-targeted agents. In the context of cancer, drug combinations may target multiple pathways and significantly inhibit the growth of tumor cells, and will be more conducive to treating cancer than single therapeutic agents.⁷⁾ As for breast cancer, drug combinations (such as vinorelbine-cisplatin and capecitabine-vinorelbine) are indeed commonly used in clinical treatment and have shown excellent results.^{8,9)}

With the development of next-generation sequencing technology in the past decade, epigenetic processes have been found to play a key role in the causation, progression and treatment of cancer.¹⁰⁾ The synthesis of small molecules that can target the mutated or dysregulated epigenetic modifiers and alter epigenomes in cancers represents one of novel strate-

gies for cancer therapy.¹¹⁾ For example, histone deacetylase (HDAC) can “erase” acetylation marks on histones, which is crucial in epigenetic processes of cancer.^{12,13)} Previous studies have found that in a variety of cancers, HDACs have abnormal activity and expression.¹²⁾ And epigenome-targeting drug vorinostat (also known as suberoylanilide hydroxamic acid) is a direct HDAC inhibitor that has been approved for clinical trials of various cancers, such as T cell lymphomas.¹¹⁾ In addition, vorinostat has been tested in a pivotal phase 2 clinical trial in patients with breast cancer (<https://clinicaltrials.gov/>).

Tyrosine kinase is also a hot spot of cancer therapeutic target. For instance, the second-generation c-abl oncogene (ABL) tyrosine kinase inhibitors (TKIs) (e.g., imatinib, ponatinib and dasatinib) are Food and Drug Administration (FDA)-approved drugs for chronic myelogenous leukemia treatment (DrugBank). Combination of ABL TKIs and vorinostat has shown potential for treating leukemia.¹⁴⁾ Considering the fact that the dasatinib have phase 2 clinical records of anti-breast cancer in clinical trials, we speculated that combining dasatinib with vorinostat may have synergistic effects for breast cancer. This stimulated our interest to test this hypothesis by experiments.

MATERIALS AND METHODS

Cells and Reagents The human breast cancer cell line MCF-7 was obtained from China Center for Type Culture Collection (Wuhan, Hubei, China). The cells were maintained in an RPMI-1640 medium (Gibco, U.S.A.) supplemented with 10% newborn bovine serum in a humidified 5% CO₂ incubator at 37°C. Vorinostat and dasatinib (purity>98%) were

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Fig. 1. Synergistic Anticancer Effects of the Combination of Vorinostat with Dasatinib in MCF-7 Cells

Cells were treated with vorinostat (A) and dasatinib (B), alone and in combination (C) for 48 h. The viabilities of cells were qualified by MTT assay, and reported as fraction affected (fraction of cells affected by treatment), and the data represented the mean $\pm$ S.D. from five replicate experiments. The combination indexes (CIs) (D) and dose-reduction indexes (DRIs) (E) of the combinations of vorinostat with dasatinib were calculated as described. The data points below CI values of 1, denoted by horizontal dotted lines in (D), are indicative of synergistic effects.

purchased from Haoyuan Chemexpress Co., Ltd. (Shanghai, China).

Cell Viability Assay Cell viability was determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, U.S.A.).¹⁵⁾ The cells were seeded onto 96-well plates (6×10^3 cell/well), and then incubated overnight. The cells were then treated with vorinostat and dasatinib alone or in combination for 48 h, with 0.1% dimethyl sulfoxide (DMSO) as negative control. Approximately 10 μL of the 5 mg/mL MTT solution was added to each well, and the plates were incubated for more 4 h at 37°C. Finally, the absorbance was detected at 492 nm using a Thermo Multiskan MK3 (Thermo Fisher, U.S.A.). IC_{50} was determined based on absorbance readings. The ratios of drug combination were based on the ratios of IC_{50} . All samples were tested in five replicates, and the experiments were repeated at least three times.

The interaction between two drugs was analyzed according to the median-effect principle proposed by Chou and Talalay.^{16,17)} The median effect dose of the drug combinations equation, Dm, (analogous to the IC_{50}) and combination index (CI) were calculated by CompuSyn software and CI value below 1 represents synergism. Dose-reduction index (DRI) was used to measure the number of reduction folds in the dose may be produced by the synergistic combination of the two drugs at a given effect

level compared with the doses of each drug alone.¹⁷⁾

Cell Cycle and Cell Apoptosis by Flow Cytometry

Cell cycle and apoptosis were detected by flow cytometry. The cells were seeded onto 6-well plates and cultured with vorinostat (1.5 μM) and dasatinib (4.5 μM) alone and in combination for 48 h. Then, the detached/dead and viable cells were collected. Apoptosis was determined by staining with Annexin V-fluorescein isothiocyanate (FITC) according to the kit instruction. Cells for cell cycle analysis were washed with cold phosphate buffered saline (PBS), fixed with 70% ethanol, incubated with RNAase and stained with propidium iodide (PI). Finally, the cells were analyzed by a FACSCalibur flow cytometer equipped with the CellQuest and Modifit software (Becton Dickinson, U.S.A.).

Reactive Oxygen Species (ROS) Detection Intracellular ROS generation was evaluated by using the oxidation-sensitive fluorescent probe 2',7'-dichloro-fluorescein diacetate according to the method of Engelmann *et al.*¹⁸⁾ Briefly, the cells ($1 \times 10^5/\text{mL}$) were seeded on cover slips and cultured with vorinostat (1.5 μM) and dasatinib (4.5 μM), alone and in combination, for 12 h. The cells were then washed with PBS and incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) (100 μL , 10 μM) for 30 min in the dark. Fluorescence was detected by a confocal microscope (Leica TCS SP2) with

Fig. 2. Synergistic Induction of G₂/M Arrest and Cell Apoptosis by the Combination of Vorinostat with Dasatinib in MCF-7 Cells

(A and B) Cells were treated with vorinostat (1.5 μ M) and dasatinib (4.5 μ M), alone and in combination for 12h and cell cycle distribution was examined using PI staining followed by flow cytometric analysis. (C and D) Cells were treated with vorinostat (1.5 μ M) and dasatinib (4.5 μ M), alone and in combination for 12h and cell apoptosis was examined using Annexin V/PI staining followed by flow cytometric analysis. The numerical data were presented as mean $\pm$ S.D. ($n=3$). * Indicates a significant difference ($p<0.05$) by one-way ANOVA.

100 $\times$ objective at an excitation wavelength 488nm and an emission wavelength of 530nm. The fluorescence intensity (FI) of 50 cells from each sample was quantified using the Leica software and Image J software.

Mitochondrial Transmembrane Potential Assessment

The changes in the mitochondrial membrane potential were measured with rhodamine 123 by confocal scanning microscopy.¹⁹⁾ The cells (1 $\times 10^5$ /mL) were seeded on cover slips and cultured with vorinostat (1.5 μ M) and dasatinib (4.5 μ M) alone and in combination, for 12h. The cells were then washed with PBS and incubated with rhodamine 123 (100 μ L, 10 μ M) for 30min at 37°C. The fluorescent images were recorded by a confocal microscope (Leica TCS SP2) with 100 $\times$ objective and excitation and emission settings of 505 and 520nm, respectively. The FI of 50 cells from each sample was quantified

with the Leica software and Image J software.

Western Blot The cells (1 $\times 10^5$ /mL) were seeded on 10-cm dishes and cultured with vorinostat (1.5 μ M) and dasatinib (4.5 μ M), alone and in combination, for 12h. Then, the cells were harvested, lysed with the ice-cold RIPA lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30min at 4°C and centrifuged at 12000rpm for 10min at 4°C to collect the liquid supernatant. The protein was quantified using a BCA Kit (Thermo), separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels at 14% polyacrylamide and blotted onto a polyvinylidene difluoride (PVDF) nitrocellulose membrane (Biosharp, China). Non-specific binding sites were blocked in 5% skimmed milk powder in PBST for 1h before probed with primary antibodies overnight at 4°C. Antibodies were used at the following di-

lutions: 1:1000 (Bcl-2 and β -actin), which were purchased from Cell Signaling Technology. They were incubated with horseradish peroxidase-conjugated immunoglobulin Gs (IgGs)

(1:5000) for 1 h at room temperature, and then visualized with enhanced chemiluminescence reagent (Millipore, U.S.A.).

Statistical Analysis All data are presented as means $\pm$

Fig. 3. Synergistic Induction of Intracellular Reactive Oxygen Species (ROS) Generation and Decrease Mitochondrial Membrane Potential ($\Delta\Psi_m$) by the Combination of Vorinostat with Dasatinib in MCF-7 Cells

(A and B) ROS production was monitored in MCF-7 cells with DCF after treatment with vorinostat ($1.5\mu\text{M}$) and dasatinib ($4.5\mu\text{M}$), alone and in combination, for 12h. (C and D) $\Delta\Psi_m$ changes were detected with Rhodamine 123 in MCF-7 cells after treatment with vorinostat ($1.5\mu\text{M}$) and dasatinib ($4.5\mu\text{M}$), alone and in combination, for 12h. FI was measured by laser scanning confocal microscopy. Data were presented as mean $\pm$ S.D. from three independent experiments (B and D). $p < 0.05$. *Significantly different compared with the control group.

Fig. 4. Synergistic Decrease Bcl-2 Expression by the Combination of Vorinostat with Dasatinib in MCF-7 Cells

(A and B) The changes of Bcl-2 expression were detected by the western blots in MCF-7 cells after treatment with vorinostat ($1.5\mu\text{M}$) and dasatinib ($4.5\mu\text{M}$), alone and in combination, for 12h. Data were presented as mean $\pm$ S.D. from three independent experiments (B). $p<0.05$. *Significantly different compared with the control group.

standard deviation (S.D.) from more than three independent experiments. SPSS16.0 and one-way ANOVA were used to analyze the data and $p<0.05$ was considered as statistically significant.

RESULTS

Synergistic Inhibition of MCF-7 Cell Proliferation by Vorinostat and Dasatinib We first evaluated the ability of vorinostat or dasatinib alone to inhibit growth of MCF-7 cells. The cells were treated with increasing concentrations of vorinostat or dasatinib and both the drugs showed dose-dependent cytotoxic effects in MCF-7 cells, with Dm (IC_{50}) values of 6.32 and $21.06\mu\text{M}$, respectively. Considering the Chou–Talalay model requires drugs to be used at a fixed dose ratio,¹⁷⁾ we combined these drugs as the following ratios: vorinostat:dasatinib as 1:3 according to the Dm value ratios of 48h. The dose–effect relationship of vorinostat or dasatinib, alone and in combination, were evaluated on the growth of MCF-7 cells after exposure for 48h (Figs. 1A–C). The median-effect analysis was conducted according to Chou's method.¹⁷⁾

CI were calculated to determine the antitumor effects of the combinations; the plots of CI *versus* fraction affected (Fa) were shown in Figs. 1D and E. The combination of vorinostat and dasatinib exhibited a synergistic effect where the combination doses were 1.5 to $24\mu\text{M}$ at 48h (Figs. 1C, D). As shown in Fig. 1E, the DRI values of vorinostat ranged from 2.34 to 4.18, whereas those of dasatinib ranged from 2.17 to 5.42. Overall, the results of the MTT assay suggested that vorinostat and dasatinib could inhibit the proliferation of MCF-7 cells. The combinations of vorinostat with dasatinib (1:3), exhibited synergistic cytotoxic effects in MCF-7 cells.

Modulation of Cell Cycle Distribution and Induction of Apoptosis in MCF-7 Cells by Vorinostat and Dasatinib in Synergy The effects of vorinostat and dasatinib on cell cycle distribution were analyzed by the flow cytometry (Figs. 2A, B). Dasatinib induced a significantly accumulation of G_0/G_1 cells, together with a decrease in S and G_2/M phases in MCF-7 cells ($p<0.05$), compared with the control. In contrast, vorinostat caused a remarkable accumulation in G_2/M phase cells at the expense of G_1 phase cells. The combination of vorinostat with dasatinib, exhibited similar effects to that of vorinostat alone on cell cycle distribution ($p<0.05$).

The apoptosis induced by vorinostat and dasatinib was determined through flow cytometry to further investigate the

decreased viability of MCF-7 cells (Figs. 2C, D). Vorinostat and dasatinib alone can increase the proportion of apoptosis cells after treatment for 48h ($p<0.05$). The combination of vorinostat with dasatinib caused a much higher apoptosis rate than those of drugs alone (36 vs. 16 and 12.57%, $p<0.05$), indicating that the combination of dasatinib enhanced the apoptotic effect, especially early apoptosis-induction of vorinostat significantly.

Synergistic Induction of Intracellular ROS Generation and Mitochondrial Transmembrane Potential ($\Delta\Psi_m$) Loss by Vorinostat and Dasatinib The intracellular ROS generation was investigated using the fluorescent probe DCFH-DA to reveal that whether ROS was involved in mediating apoptosis induced by vorinostat and dasatinib. Treatment with vorinostat or dasatinib alone significantly enhanced the ROS generation in MCF-7 cells compared with the untreated cells ($p<0.05$) (Figs. 3A, B). Interestingly, combined treatment with vorinostat and dasatinib remarkably promoted ROS generation (Figs. 3A, B).

The changes in $\Delta\Psi_m$ have been linked to apoptosis²⁰⁾; thus, we examined the influence of vorinostat and dasatinib on the $\Delta\Psi_m$ of the MCF-7 cells with rhodamine 123. Rhodamine 123 is a mitochondria-binding specific cationic fluorescent dye that is proportional to the $\Delta\Psi_m$. The results showed that incubation with vorinostat or dasatinib alone for 12h led to a remarkable loss of $\Delta\Psi_m$ in MCF-7 cells, revealed by the reduction of Rhodamine 123 FI compared with the control ($p<0.05$) (Figs. 3C, D). The combination treatment of vorinostat and dasatinib showed a sharp decline of Rhodamine 123 FI to 51.34 compared with the control, vorinostat or dasatinib (96.36) alone after treatment for 12h.

Synergistic Decrease of Bcl-2 Expression by Vorinostat and Dasatinib Bcl-2 is an inner mitochondrial membrane protein with an important role in preventing apoptosis.²¹⁾ Thus, we examined the effects of vorinostat and dasatinib, alone and in combination, on the Bcl-2 protein level. The results indicated that the combination treatment of vorinostat with dasatinib significantly decreased the expression of Bcl-2 after treatment for 12h in the MCF-7 cells ($p<0.05$) (Figs. 4A, B).

DISCUSSION

In this study, we found that the combination of vorinostat (0.38 to $6\mu\text{M}$) with dasatinib (1:3 ratio) exerted a significant

synergistic cytotoxic effects on MCF-7 cells in a dose-dependent manner. Furthermore, the combined treatment of HDAC inhibitor vorinostat and epidermal growth factor receptor (EGFR) inhibitor dasatinib synergistically induced apoptosis in MCF-7 cells.

As a HDAC inhibitor, vorinostat has been reported to induce cell cycle arrest, apoptosis, senescence, and differentiation in cancer cells.²²⁾ It has been reported that vorinostat combined with gefitinib have synergistic cytotoxicity in non-small cell lung cancers and hepatocarcinoma.^{7,23)} Our results showed that the IC_{50} of vorinostat is $6.32\ \mu\text{M}$, dasatinib is $21.06\ \mu\text{M}$, in human breast cancer cells MCF-7 after 48 h. The results suggest that vorinostat sensitizes dasatinib-induced cytotoxicity in human breast cancer cells and reduces the doses of dasatinib by more than 2 times.

The combined beneficial effect can be achieved partly through cell cycle arrest, ROS production, and apoptosis induction in the mitochondria-mediated intrinsic pathway. Mitochondria have been considered as targets of anti-cancer drugs and have a key function in the regulation of apoptosis.²⁴⁾

Our results indicated that vorinostat caused G_2/M arrest, while dasatinib induced G_1 arrest in MCF-7 cells. It is interesting that the combination of vorinostat and dasatinib led to an accumulation of cells in the G_2/M phases at the expense of the G_1 phase. These findings suggested that vorinostat and dasatinib may be useful for controlling cancer cell growth because several lines of cancer cells have defects in the cell cycle checkpoints.

Mitochondria are the main production sites of ROS. The presence of excessive ROS in the mitochondria leads to the opening of the mitochondrial permeability transition pore, decline in $\Delta\Psi_m$, and release of cytochrome *c*, which, in turn, trigger apoptosis.²⁴⁾ Several studies have provided evidence that vorinostat and dasatinib exhibit antitumor activity by inducing ROS generation and apoptosis.^{25–27)} Our results demonstrated that the incubation of MCF-7 cells with vorinostat and dasatinib, alone and in combination, increased the ROS generation, decreased $\Delta\Psi_m$, down-regulated the anti-apoptosis protein Bcl-2 expression, and induced the mitochondria-mediated apoptosis.

In conclusion, the findings presented in this study demonstrated, for the first time, that vorinostat and dasatinib synergistically inhibited the growth of MCF-7 cells *in vitro*. This inhibition can be achieved partly by the generation of ROS and the decrease of Bcl-2 expression. Additional study is needed to elucidate the mechanistic interactions of combinatorial drugs. Moreover, the idea of combining epigenome-targeting drug with tyrosine kinase inhibitor should be given more attention, because it holds great potential in treating tumors with minimal side effects.

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Conflict of Interest The authors declare no conflict of interest.

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