

Determination of the migration effect and molecular docking of verteporfin in different subtypes of breast cancer cells

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Abstract. Breast cancer is one of the most aggressive malignant tumors in women. According to the expression differences of estrogen receptor, progesterone receptor, human epidermal growth factor receptor-2 (HER-2) and cell proliferation antigen Ki-67, breast cancer can be divided into four molecular subtypes: Luminal A, Luminal B, HER-2 overexpression and Basal-like. Yes-associated protein (YAP), a downstream effector of the Hippo pathway, is overexpressed in human cancers and is associated with proliferation, apoptosis, migration, invasion and resistance to chemotherapy drugs in breast cancer cells. Verteporfin (VP) is used as a photosensitizer in the treatment of neovascular macular degeneration. VP is also identified as an inhibitor of YAP/TEA domain transcription factor (TEAD) interaction in the absence of light activation. However, detailed structural information about VP and YAP interactions is relatively scarce and VP research targeting YAP in different molecular subtypes of breast cancer cells is also rare. The aims of the present study were to structurally describe the VP binding site in the YAP crystal structure and to verify the non-photoreactive VP effect targeting YAP on the migration of different molecular subtypes of breast cancer cells. The crystal structure of VP and YAP was calculated by AutoDock 4.2 and the result was illustrated using PyMOL. The non-photoactivated VP effect on the migration of Luminal A MCF-7, Luminal B BT-474 and triple-negative breast cancer BT-549 breast cancer cells was evaluated by wound healing and Transwell migration experiments. Results from molecular docking experiments demonstrated that VP could interact through hydrogen bonds and hydrophobic interactions with important YAP residues

involved in TEADs binding (Gln82, Val84, Met86 and Arg89). Migration experiments revealed that the non-photoinduced VP could inhibit the migration of different molecular subtypes of breast cancer cells. The results of the present study indicated that VP may be a novel repositioned drug for breast cancer treatment in the future.

Introduction

The Hippo signaling pathway serves an important role in the occurrence and progression of breast cancer (1). As a terminal effector of a central kinase cascade of the Hippo signaling pathway, dephosphorylation and nuclear localization of yes-associated protein (YAP) regulates the activity of many YAP downstream compounds, including connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer (CYR61), AXL receptor tyrosine kinase (AXL) and integrin, when combined with the DNA-binding transcription factors of TEA domain transcription factor (TEAD) (2). CYR61, CTGF and integrin serve an important role in cell adhesion, migration, proliferation and angiogenesis (3). Overexpression of YAP in breast cancer promotes the occurrence and development of breast cancer, while YAP knockdown can reduce cell proliferation, migration and increase the radiotherapy sensitivity of triple-negative breast cancer (TNBC) 231 cells (4,5). Activated YAP is a key regulator of tumor cell proliferation, apoptosis, migration, invasion and chemotherapy resistance, leading to cell malignant transformation (6). Taken together, these studies indicate that YAP may be a potential drug target for breast cancer.

Verteporfin (VP) was originally designed as a photosensitizer for macular degeneration (7). VP can produce singlet oxygen and eliminate abnormal hyperplastic blood vessels under the activation of a 693 nm laser (8). In recent years, VP has lost its importance owing to the limited efficacy of its original indications (9). However, VP also serves a key role in the absence of light, VP can inhibit YAP-TEAD binding without photoactivation and markedly prevent liver hepatomegaly/tumorigenesis induced by YAP overexpression (10). YAP is closely associated with the occurrence and development of breast, colon, lung and liver cancer in addition to mesothelioma, as it is the core effector of the Hippo signaling pathway (11). Therefore, targeting YAP is may be

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an attractive therapeutic strategy. VP was rapidly repositioned as a YAP inhibitor for several types of cancer, including liver, esophageal, lung and pancreatic cancer (12).

Non-photoactivated VP can disrupt the interaction and transcriptional activity of YAP/TEAD and thus inhibit YAP-mediated tumor proliferation, induce tumor cell apoptosis and restore sensitivity to chemotherapy drugs (13). However, although VP has been identified as a YAP/TEAD inhibitor, a detailed structural analysis of YAP and VP interactions has yet to be presented. Therefore, the present study analyzed the possible binding sites of VP and YAP by molecular simulation. In addition, it evaluated the VP effect on the migration of three breast cancer cell subtypes and on the expression of YAP downstream cell migration-related genes.

Materials and methods

Compound preparation. VP (MedChemExpress) was dissolved in DMSO as a stock solution at a concentration of 10 mM, stored at -20°C and protected from light.

Breast cancer cell culture. Luminal A MCF-7, Luminal B BT-474 and TNBC BT-549 cells (14) were purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. The cells were cultured with RPMI-1640 medium (HyClone; Cytiva) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cells were protected from light at all times.

Molecular docking studies. All molecular docking studies were performed using AutoDock 4.2.6 software package (15). The crystal structure of YAP (PDB code 3KYS) was retrieved from the RCSB Protein Data Bank (<https://www.rcsb.org/>) (16). The structure was prepared by adding hydrogen atoms, removing waters and adding Gasteiger charge. The three-dimensional structure of VP was retrieved from Drugbank 5.1.5 (<https://www.drugbank.ca/>) and was prepared by adding hydrogen atoms and Gasteiger charge. The protonation states of both receptor protein and VP were determined at pH 7 using Propka 3.1 (17). Then the ligand was docked into the binding sites of YAP with default parameters and 20 docking poses were exported for further visual analysis. The docking score of each pose was calculated with AutoDock 4.2. The binding free energy was of best-scored pose calculated using the Molecular Mechanics/Poisson-Boltzmann Surface Area (MM-PBSA) method (13). Fig. 1 presents the best-scored binding pose of VP with YAP docking result, which was constructed with PyMOL 2.4.0 (<https://pymol.org/2/>).

Wound healing assay. Three parallel lines were drawn in advance on the bottom of a 24-well plate. MCF-7, BT-474 and BT-549 cells were placed in the plates at 1.2x10⁵ cells per well. After the cells reached 85-90% confluence, a 10 µl pipette tip was used to draw a light, straight line. The floating cells in the wells were carefully washed off with PBS buffer and images captured under the light microscope at the 0 h time point. The blank control group cells were cultured in fresh medium without FBS and the treatment group was treated with 8 µM VP. After 24 h of culture, the cells were washed with PBS

buffer three times and images captured under the microscope. Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) was used to quantify the migration distance of cells before and after scratches and to calculate the wound healing distance.

Transwell assay. For these experiments, the cells were cultured at a density of 3x10⁵ in FBS-free medium. In the control group, 10% FBS medium was dripped along the side wall of the lower Transwell chamber. In the treatment group, 10% FBS medium containing 8 µM VP was added to the lower Transwell chamber. The 3x10⁴ cell suspensions were carefully added to the upper Transwell chamber and then cultured at 37°C in the incubator for 24 h. The medium was then removed, and the cells were washed twice with PBS and fixed for 20 min with 4% paraformaldehyde at room temperature. The sample were stained with DAPI for 15 min in the dark and images captured under a fluorescence microscope (magnification, x100). A total of five visual fields were randomly selected and the results were quantified by Image Pro Plus 6.0 software (Media Cybernetics, Inc.).

Statistical analysis. Data are presented as the mean ± standard deviation. Comparisons between VP-treated and -untreated control groups were made by unpaired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Molecular docking analysis. The crystal structure of YAP (PDB code 3KYS) was used as docking receptor for VP in docking molecular experiments and was prepared by adding hydrogen atoms, removing waters and adding Gasteiger charge. The best-scored pose of VP was selected as the possible bind conformation for the docking analysis (Fig. 1) and the docking score was -9.73. VP can form a hydrogen bond with the side chain of Gln82 and the distance was 2.1 Å (O...H-N-Gln82). In addition, VP could have a hydrogen bond with the main chain of Val84 at the distance of 1.8 Å (O...H-N-Val84). VP can also form a hydrogen bond with the main chain of Met86 at the distance of 2.0 Å (O...H-N- Met86). VP can establish two hydrogen bonds with the positively charged Arg89 side chain at distances of 1.8 Å (O...H-N-Arg89) and 1.7 Å (O...H-N-Arg89), respectively. In addition, VP established hydrophobic interactions with the surrounding residues Leu68, Ala71, Val72, Pro75, Val80, Pro81, Val84, Pro85 and Met86. These interactions tightly bound to YAP and inhibited the interaction of YAP and TEAD. The binding free energy was further investigate using MM-PBSA and the binding free energy was -32.16 kcal/mol, which was favorable for the binding of VP. This result suggested that VP was a good inhibitor for the YAP/TEAD complex.

VP effect on the migration of different breast cancer cell subtypes. The expression of YAP target proteins in MCF-7, BT-474 and BT-549 cells was evaluated. Western blotting demonstrated that 8 µM VP treatment could notably downregulate the protein expression levels of YAP, TEAD, and CYR61. The expression of CTGF were differential in three subtypes of breast cancer and no CTGF expression was detected in MCF-7 cells (Fig. 2).

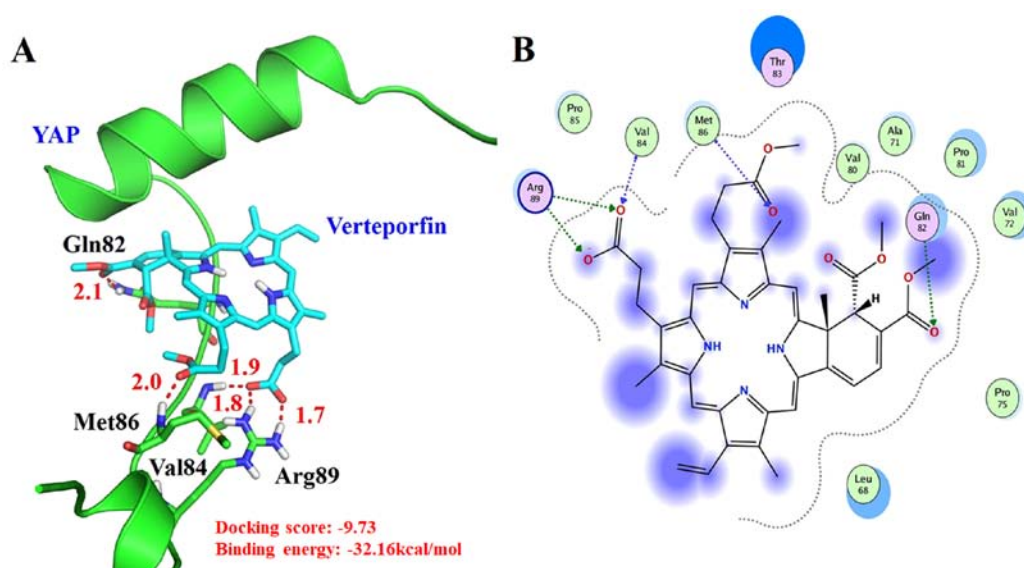


Figure 1. Molecular docking analysis (A) Docking results of verteporfin interaction with protein surface and (B) surrounding residues of YAP (PDB code 3KYS). VP is shown in green stick model and key residues of YAP were shown in cyan stick models. The nitrogen, oxygen and sulfur atoms were shown in blue, red and yellow, respectively. The hydrogen bonds are represented by red dashed lines. YAP, yes-associated protein.

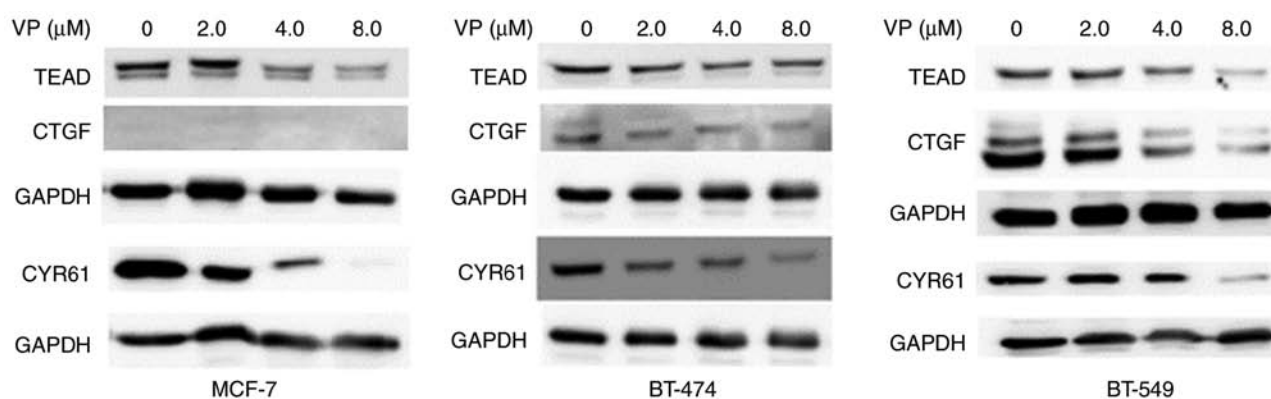


Figure 2. Effects of VP on the protein expression levels of downstream YAP targets. CTGF, connective tissue growth factor; CYR61, cysteine-rich angiogenic inducer; TEAD, TEA domain transcription factor; YAP, yes-associated protein; VP, verteporfin.

Transwell migration assays revealed that 8 μ M VP could inhibit the number of migratory cells of Luminal A MCF-7, Luminal B BT-474 and TNBC BT-549 cells ($P < 0.01$; Fig. 3). Wound healing assay results demonstrated that 8 μ M VP decreased the migratory ability of the three different breast cancer cell subtypes (Fig. 4).

Discussion

The core components of the Hippo signaling pathway are involved in the regulation of proliferation, migration, invasion and chemoresistance of breast cancer cells (11). YAP is the main effector of the Hippo signaling pathway (1). The core kinases Mst1/2 of the Hippo signaling pathway and the activation complex formed by the interaction of their regulatory protein, protein salvador homolog 1, can directly phosphorylate large tumor suppressor homolog 1 (LATS1) and LATS2, which interact with Mob kinase activator 1 (Mob1). The activity of LATS1/LATS2 and Mob1 can be inhibited by YAP phosphorylation, which is degraded by proteasome

or ubiquitination in the cytoplasm (4). By contrast, when the upstream kinase signal of the Hippo pathway is inhibited, the unphosphorylated Yap is transferred to the nucleus (12). Owing to the lack of a DNA-binding domain, YAP must be combined with DNA-binding transcription factors to serve the role of transcription coactivators (18). YAP overexpression can induce epithelial-mesenchymal transition in the MCF-10A breast cell line and overexpression of YAP promotes the formation and growth of tumor in breast cancer cells in a mouse xenograft tumor model (19). The activation of YAP in tumor cells can ultimately promote the occurrence of breast cancer bone metastasis (20). These data therefore suggest that YAP is closely associated with the occurrence and development of breast cancer. Since it was identified as an inhibitor of the YAP/TEAD complex, several studies have reported the therapeutic potential of VP in different types of cancer (21-23). However, detailed structural information on the interaction between VP and YAP remains lacking. The N-terminal YAP region contains a TEAD-binding domain and 14-3-3-binding domain site (HXRXXS motif-containing Ser127 residue) (24).

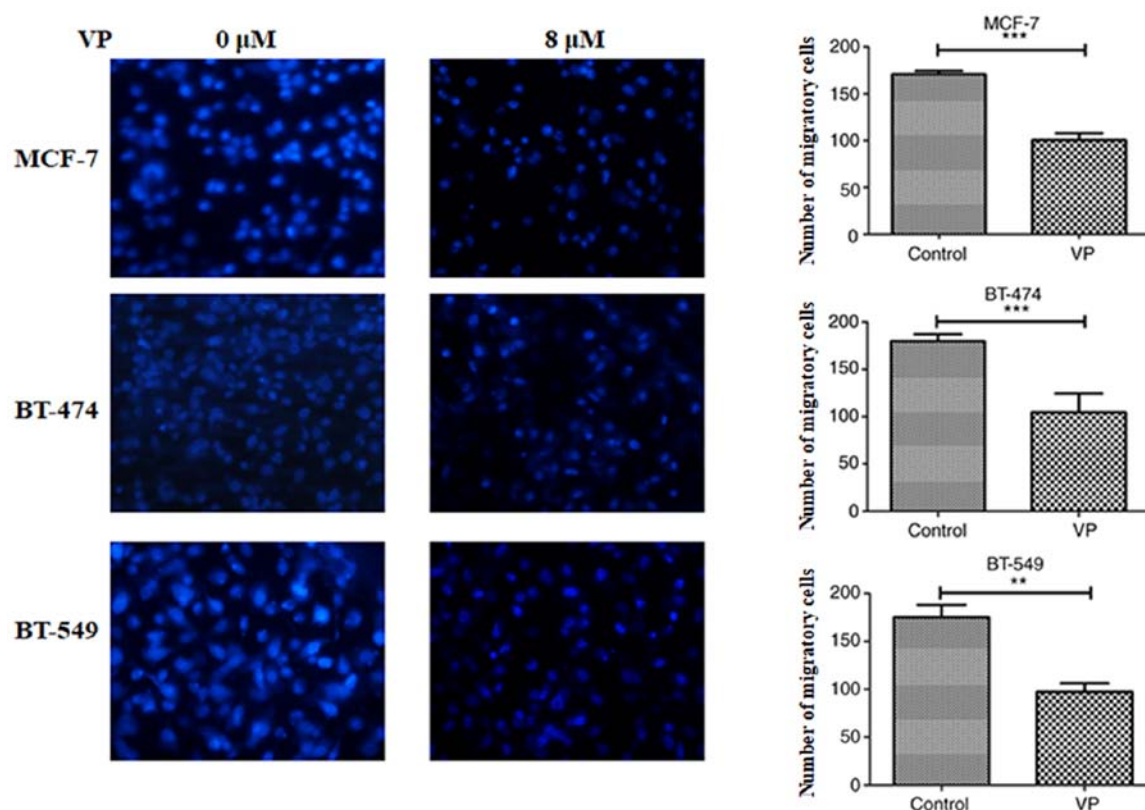


Figure 3. VP affects the migratory ability of three breast cancer cell subtypes: MCF-7, BT-474 and BT-549. ** $P < 0.01$, *** $P < 0.001$ vs. control group. VP, verteporfin.

YAP binding to 14-3-3 proteins depends on Ser127 phosphorylation, which also serves a key role in determining the cytoplasmic localization and inactivation of YAP (25,26). YAP-TEAD interaction is important for the YAP-mediated transcription activation and the disruption of this interaction is being considered as a strategy in cancer therapy (27). Liu-Chittenden *et al* found that VP selectively binds to YAP and abrogates its interaction with TEAD (10). In recent years, some crystal structures have been presented, including YAP (residues 47-85)-TEAD4 (residues 210-427) complex from *Mus musculus* (PDB code 3JUA), YAP (residues 50-171)-TEAD1 (residues 209-426) from *Homo sapiens* (PDB code 4RE1) and YAP (residues 60-100)-TEAD4 (residues 217-434) complexes from *Homo sapiens* (PDB code 3KYS) (28). Structure analysis has shown that the 61-100 region of human YAP serves an essential role in TEAD-binding domain, it is observed that the hydrophobic interaction of the YAP Ω -ring formed by Met86, Arg87, Leu91, Phe95 and Phe96 residues causes the Arg89 and Ser94 side chains to form hydrogen bonds with Glu255, Asp264 and Tyr421 residues of TEAD1 (28-30).

YAP and TEAD proteins are the best molecular target candidates to regulate the Hippo pathway, as the formation and activation of the YAP/TEAD complex is the last step of the Hippo-YAP pathway. VP is a compound that directly destroys the formation of YAP/TEAD complexes and inhibits the most essential part of the Hippo pathway (10). The molecular docking analysis of the present study found that VP could form hydrogen bonds and establish hydrophobic interactions with residues from the YAP Ω -ring that directly interacted with TEAD1 (Met86 and Arg89). Our molecular docking results demonstrated that VP could also interact with several

surrounding residues (Leu68, Ala71, Val72, Pro75, Val80, Pro81, Gln82, Val84 and Pro85), particularly by hydrophobic interactions, highlighting that it occupies the TEAD binding site and inhibits the YAP-TEAD complex formation.

YAP is highly expressed in many solid tumors and its carcinogenic function is mediated by its nuclear localization and interaction with TEAD transcription factors (31,32). By interfering with YAP/TEAD, non-photoactivated VP can inhibit the proliferation of esophageal (33), lung (34) and pancreatic cancer (21), in addition to malignant mesothelioma cells (35) and bladder cancer cells (22), inducing cell apoptosis. VP can significantly inhibit the transcriptional activity of TEAD in MDA-MB-231 cells, reduce the protein expression level of YAP target genes AXL and CTGF, inhibit the migration of paclitaxel resistant cells and induce apoptosis *in vitro*. VP reduces tumor volume and Ki67 expression in paclitaxel-resistant mice and reverses the resistance of TNBC tumor cells to paclitaxel therapy *in vivo* (23). The overexpression of YAP promotes resistance to chemotherapy, radiotherapy and targeted treatment of various types of cancer (13). Non-photoactivated VP can also increase the sensitivity of HER-2 positive breast cancer cells to lapatinib (36) and increase the sensitivity of TNBC MDA-MB-231 cells (37), urothelial cells (38), esophageal cancer cells (30) and colon cancer cells (39) to chemotherapy drugs. Therefore, non-photoactivated VP may provide a new choice for drug-targeted YAP intervention in breast cancer.

Abnormal expression of CTGF and Cyr61 proteins is associated with the progression of breast cancer, prostate cancer and malignant melanoma (3,40,41). The present study found that CTGF were expressed in Luminal B BT-474 and TNBC BT-549 breast cancer cells and no CTGF expression

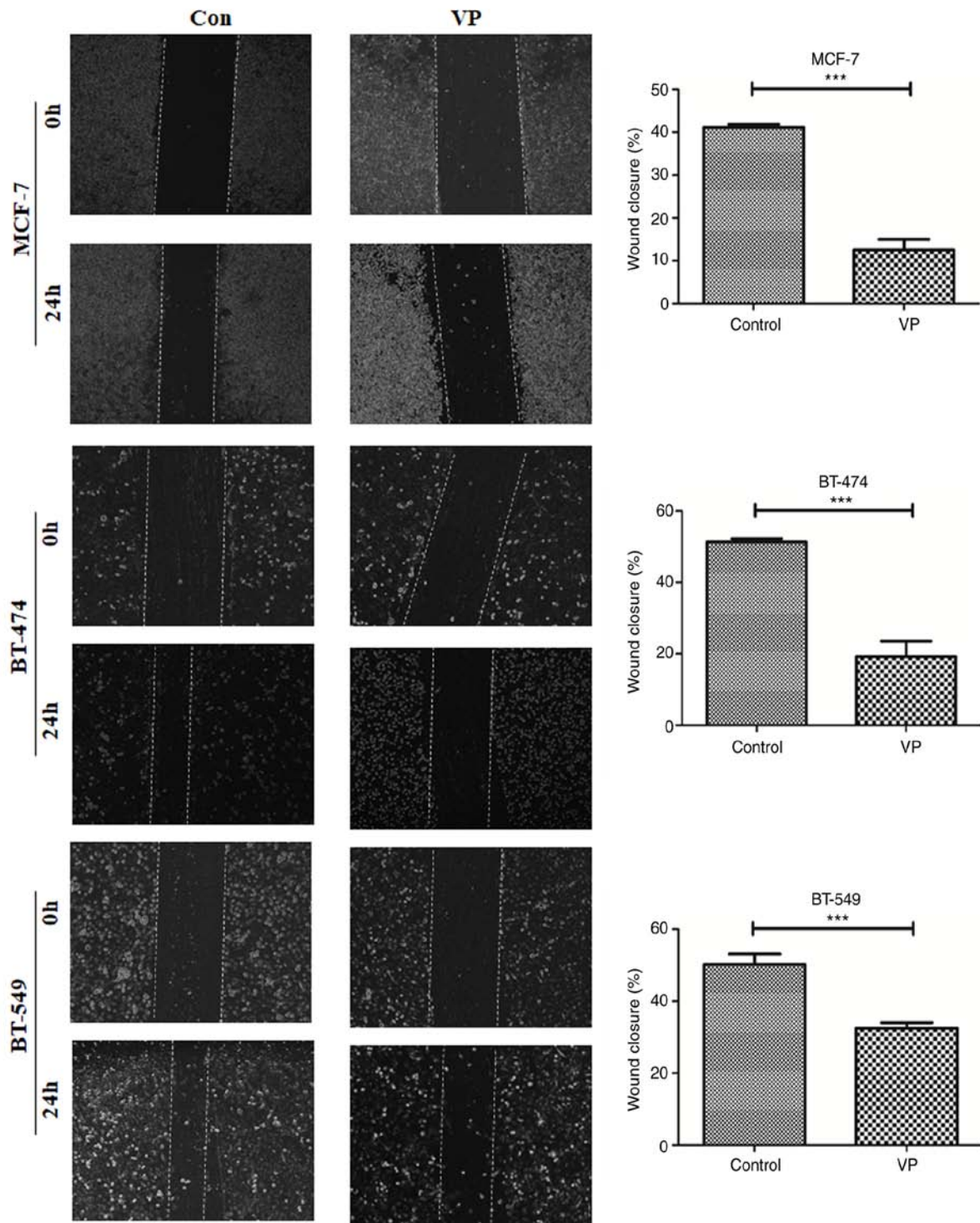


Figure 4. VP effect on the wound healing ability of three breast cancer cell subtypes: MCF-7, BT-474 and BT-549. Magnification, x100. ***P<0.001 vs. control group. VP, verteporfin.

was detected in Luminal A MCF-7 cells, which was in accord with previous data (42). CTGF specific antibody can reduce the bone metastasis incidence in a mouse model of breast cancer (43). The present study found that non-photoactivated VP can inhibit the migration of three breast cancer cell lines *in vitro* by downregulating the expression of YAP/TEAD downstream target genes CYR61, which are closely associated with cell migration and invasion.

In conclusion, VP is an FDA-approved photosensitizer for the treatment of age-related macular degeneration and can inhibit the occurrence of tumor by destroying the formation of YAP/TEAD complex in its non-photoactivated form (14). In the present study, the molecular interface between VP and YAP was simulated and the binding site between VP and YAP was confirmed by molecular docking experiment. VP, a non-photoactive inhibitor of Hippo-YAP signaling, could inhibit the

migration of three molecular subtypes of breast cancer cells by targeting YAP. The potential role of VP in cancer progression, including cell proliferation and apoptosis, without light activation need to be further studied and evaluated in experimental animals and human breast cancer treatment. The results of the present study provided a new approach for targeting the Hippo pathway effector YAP to treat breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CW acquired and analyzed the data, and drafted and revised the manuscript. XL conceptualized the study, acquired and analyzed the data, and drafted and revised the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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