Inhibition of Breast Cancer Metastases by a Novel Inhibitor of TGFβ Receptor 1

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Background
Transforming growth factor beta (TGFβ), which is implicated in metastasis to various organs in breast cancer, is a potential target for new antitumor metastasis drugs.

Methods
To identify specific inhibitors of TGFβ receptor 1 (TGFβR1) in breast cancer metastasis, a virtual library of more than 400,000 different compounds was screened by molecular docking modeling and confirmed with Smad-binding element luciferase and TGFβR1 kinase assays. Affymetrix GeneChip expression analysis of mRNA levels and real-time polymerase chain reaction were performed to determine expression changes of TGFβ-mediated, metastasis-associated genes in breast cancer cells after treatment with the small-molecule inhibitor YR-290. YR-290 was also examined for its effects on breast cancer migration, invasion, and metastasis using transwell and epithelial-to-mesenchymal transition (EMT) assays in vitro and three different mouse (BALB/c and NU/NU nude) models (n = 10 per group). Kaplan–Meier analyses were used to assess survival. All statistical tests were two-sided.

Results
YR-290 interacted with the kinase domain of TGFβR1, abrogated kinase activity (half maximal inhibitory concentration = 137 nM, 95% confidence interval = 126.4 to 147.6 nM) and inhibited the TGFβ-mediated downstream signaling pathway and metastasis-associated genes in breast cancer cells. YR-290 inhibited TGFβ-mediated breast cancer cell migration and invasion. In tumor metastasis mouse models, YR-290 almost completely blocked cancer metastasis. Numbers of lung tumor nodules of mice treated with 1 mg/kg and 5 mg/kg YR-290 were reduced by 74.93% (95% confidence interval = 61.45% to 88.41%) and 94.93% (95% confidence interval = 82.13% to 100%), respectively, compared with control mice. Treatment with YR-290 also statistically significantly prolonged the survival of tumor-bearing mice.

Conclusions
YR-290 is a novel inhibitor of tumor metastasis that works by blocking TGFβ signaling pathways.


Breast cancer is one of the most frequently diagnosed cancers and the leading cause of cancer death in women worldwide. Approximately 23% (1.38 million) of total new cancer cases and 14% (458,400) of total cancer deaths in 2008 were from breast cancer (1). More than 90% of lethality in cancer patients is caused by metastasis, and the occurrence of distant metastases severely limits the prognosis of breast cancer patients (2). Although nearly any tissue in the body can be colonized by metastatic breast cancer, lungs and bone are the most common sites for breast cancer metastasis (3). Despite recent improvements in earlier detection and better treatments, the majority of patients with cancer metastasis are still incurable.

Transforming growth factor beta (TGFβ) signaling is involved in many biological functions, including growth, differentiation, motility, adhesion, and programmed cell death (4). TGFβ signaling has two distinct and opposite roles in cancer progression and metastasis. During early stages of carcinogenesis, TGFβ strongly suppresses cell growth and acts as a tumor suppressor (5–11). In contrast, in late stages of cancer progression, primary tumor cells can reprogram their response to TGFβ and ultimately confer this cytokine with tumor-promoting activity, including stimulation of cancer invasion, metastasis, angiogenesis, and evasion from the immune system (4,12–17). This dramatic change in TGFβ function reflects a variety of dynamic alterations that occur not only within tumor cells but also within the cellular and structural composition of the tumor microenvironment. Recent studies have shown that the migratory and invasive properties of epithelial carcinoma could be acquired from TGFβ-induced EMT (18–25). Blocking TGFβ signaling has been shown to be a proven therapeutic method in the treatment of metastatic progression in breast cancer (26–28), melanoma (29,30), gastric cancer (31), pancreatic cancer (32), and ovarian cancer (33). Therefore, there is considerable interest in finding safe and effective pharmacological agents for the treatment of cancer metastasis by targeting TGFβ signaling pathways.
TGFβ transduces its signal through the canonical TGFβ/Smad signaling pathway. Upon binding of TGFβ to its receptor TGFβ receptor 2 (TGFβR2), the activated TGFβR2 makes close contact with and phosphorylates TGFβ receptor 1 (TGFβR1), and the phosphorylated TGFβR1 then recruits and forms a complex with receptor-associated Smads, including Smad2 and Smad3 (34,35). Subsequently, TGFβR1 phosphorylates the receptor-associated Smads at their carboxy-terminal ends to propagate canonical TGFβ signaling. Phosphorylated receptor-associated Smads associate with Smad4 and translocate into the nucleus to modulate the expression of hundreds of downstream genes (11,36).

In this study, a small-molecular-weight compound, YR-290, was identified as a specific inhibitor of TGFβR1 from an in silico screen of a virtual library of more than 400,000 different compounds and was confirmed through TGFβR1 kinase assays. We further examined the effects of YR-290 on breast cancer cell migration and metastasis in vitro and in tumor metastasis models.

**Materials and Methods**

**Cell Cultures and Reagents**

The luciferase-labeled human mammary adenocarcinoma MDA-MB-231 cell line was purchased from Caliper Life Sciences, Inc (Hopkinton, MA). Mouse breast tumor 4T1 cell line was transfected with pGL4 vector (Promega Madison, WI, USA) and selected in G418 for stable 4T1-luc cells (37). Human breast carcinoma cells Hs578T and BT-549 and human keratinocyte cell line HaCaT were obtained from the Chinese Academy of Sciences Committee Type Culture Collection Cell Bank (Shanghai, China). The cell lines were authenticated by the Chinese Academy of Sciences Committee Type Culture Collection Cell Bank. Expression vectors carrying human Flag-tagged Smad2 and the SBE-specific reporter plasmid SBE-luc have been described previously (25,38). TGFβ1 was purchased from PeproTech, Inc (Rocky Hill, NJ).

**Molecular Docking Modeling Assay**

The x-ray crystal structure of TGFβR1 (Protein Data Bank code: 1VJY) was obtained from the Protein Data Bank website (www.rcsb.org/). The structures of the ligands were built and energy was minimized using the Chemoffice software package (Cambridge, MA, USA). We used Autodock version 4.2 freeware developed by the Scripps Research Institute and Olson lab La Jolla, CA, USA for docking experiments. All of the water molecules were removed before the experiments so that our experiments were performed under nonaqueous conditions. The primary ligand bound to the binding pocket was the chosen conformation for the active site. The picture was prepared using Pymol version 1.2R2. DeLano Scientific LLC, South San Francisco, CA, USA.

**In Vitro TGFβR1 Kinase Assay**

Half maximal inhibitory concentration (IC₅₀) values of YR-290 and positive control SB-431542 against TGFβR1 protein kinases were calculated using the Promega Kinase-Glo kit (Promega, Mannheim, Germany) according to the manufacturer’s protocol in the presence of 600 μM ATP. Data are presented as means and 95% confidence intervals (CIs) from three independent experiments.

**Antibodies, Coimmunoprecipitation Assay, Immunofluorescence, and Luciferase Screening for TGFβ Signaling Inhibitors**

Monoclonal and polyclonal antibodies are described in the Supplementary Materials and Methods (available online). The coimmunoprecipitation assay (5), the immunofluorescence assay (39), and the luciferase assay (6,40) were performed as previously described. Brief descriptions are also in the Supplementary Materials and Methods (available online).

**Quantitative Real-Time Polymerase Chain Reaction Analyses and Microarray Analyses**

The RNA isolation and quantitative real-time polymerase chain reaction were performed as described previously (40). A brief description is in the Supplementary Materials and Methods (available online). Primer sequences are available in Supplementary Table 1 (available online).

**Microarray Hybridization**

MDA-MB-231 cells (2 × 10⁶) were plated in 100-mm tissue culture dishes for 12 hours and then were incubated for 4 hours in serum-free medium before the addition of TGFβ (5 ng/mL) and three different TGFβ concentrations (0, 0.5, and 1 μM). Cells incubated in serum-free medium with neither TGFβ nor YR-290 acted as a negative control. After 4 hours, cells were collected in Trizol Reagent (Invitrogen Carlsbad, CA, USA), and mRNA microarray analyses using Affymetrix GeneChip U133 plus2.0 Array (Affymetrix, Santa Clara, CA) were performed according to manufacturer’s instructions. Fluorescent signals were scanned using an Affymetrix GeneChip Scanner 3000, and data were analyzed in R software Institute for Statistics and Mathematics, Wien, Austria.

**Transwell Chamber Invasion Assay**

The cell invasion analysis of MDA-MB-231, Hs578T, and BT-549 cells was performed with Transwell chamber assays as previously described (6,27). A brief description is given in the Supplementary Materials and Methods (available online).

**In Vivo Mouse Experiments and Analyses**

BALB/c or nu/nu nude mice were purchased from Sino-British Sippr/BK Lab Animal Co, Ltd (Shanghai, China). The mice were randomly divided into different groups (n = 10 mice in each group). All mouse studies were performed in accordance with animal protocol procedures approved by the Institutional Animal Care and Use Committee of East China Normal University. All mice were killed with excess amounts of anesthetic. For detailed mouse analyses and assays, please see Supplementary Materials and Methods (available online).

**Statistical Analyses**

The data are expressed as means and 95% confidence intervals. P values less than 0.05 were considered statistically significant. The differences between control and experimental groups were determined by the two-sided, unpaired Student’s t test. Survival curves were compared using the Kaplan–Meier test, and the statistical significance was ascertained with the two-sided log-rank test. All statistical tests were two-sided.
Results

Screening for TGFβ/Smad Signaling Inhibitors

To screen for TGFβ/Smad signaling inhibitors of cancer metastasis, we used the molecular docking modeling assay to screen a virtual library of more than 400,000 different compounds for the potential inhibitors of kinase activity of TGFβR1. A collection of 119 small molecules was obtained by this assay. To confirm this bioinformatics analysis, luciferase reporter assays were employed in mammalian cells using the 119 small molecules (Supplementary Table 2, available online). Two compounds, YR-290 and YR-306 (Supplementary Figure 1, A and B, available online), differing from the well-known TGFβR1 inhibitor SB-431542 (Supplementary Figure 1, C, available online), were identified with excellent inhibitory effect in SBE reporter assays (Supplementary Table 2, available online) and low cytotoxicity in HaCaT cells (Supplementary Table 3, available online [IC_{50} > 10 μM]. We chose the compound YR-290 (N-phenylacetlyl-1,3,4,9-tetrahydro-1H-beta-carboline; molecular weight = 290.36), which had a better inhibitory effect on SBE reporter gene expression, for further investigation. To confirm the inhibitory effects of YR-290 on the TGFβ pathway, we performed TGFβ-stimulated SBE reporter gene assays and demonstrated that YR-290 inhibited the expression of the TGFβ-mediated SBE reporter gene in a dose-dependent manner, with a half maximal inhibitory concentration of approximately 0.5 μM (Figure 1, A), suggesting that YR-290 is an effective inhibitor of the TGFβ/Smad signaling pathway.

Analysis of YR-290 Binding With TGFβR1 Kinase Domain in the Docking Model

To confirm whether YR-290 directly inhibits the kinase activity of TGFβR1, we performed a molecular docking modeling with the x-ray crystal structure of TGFβR1 kinase domain and YR-290. Our results showed that YR-290 could bind with the kinase domain of TGFβR1 (Supplementary Figure 1, D, left panel, available online). The key interactions for the common binding mode observed with YR-290 include warhead beta-carboline bonding with the two goalkeeper residues His283 and Tyr282 between the lactam moiety and the kinase hinge region in the ATP binding pockets of TGFβR1 (Supplementary Figure 1, D, right panel, available online).

Effects of YR-290 on TGFβR1 Kinase Activity

To further confirm that YR-290 has an inhibitory effect on the kinase activity of TGFβR1, kinase assays were carried out in the presence of YR-290 or the known TGFβR1 inhibitor SB-431542. The half maximal inhibitory concentration for the inhibition of kinase activity for SB-431542 was 92 nM (95% CI = 84 to 100 nM) (Figure 1, B), which is consistent with a previous report (41). The half maximal inhibitory concentration for YR-290 inhibition was 137 nM (95% CI = 126.4 to 147.6 nM) (Figure 1, B), suggesting that YR-290 directly inhibited the kinase activity of TGFβR1 at concentrations similar to SB-431542.

Effects of YR-290 on the TGFβ/Smad Signaling Pathway

To investigate whether YR-290 inhibits the signaling pathway downstream of TGFβR1 in breast cancer cells, we first examined the effect of YR-290 on TGFβ-induced Smad2/3 phosphorylation. TGFβ strongly stimulated the phosphorylation of Smad2/3 protein in three breast cancer cell lines (MDA-MB-231, Hs578T, and BT-549), whereas YR-290 inhibited TGFβ-induced Smad2/3 phosphorylation in a dose-dependent manner in these breast cancer cell lines (Figure 1, C). Phosphorylated Smad2/3 can form a complex with Smad4 (42). To determine whether YR-290 affects TGFβ-induced Smad2/4 complex formation, we performed a coimmunoprecipitation assay between Smad2 and Smad4 in MDA-MB-231 breast cancer cells. Our results show that TGFβ strikingly enhanced the interaction between Smad2 and Smad4, whereas YR-290 inhibited the interaction in a concentration-dependent manner (Supplementary Figure 2, A, available online). Because the formed Smad2/3/4 complex can translocate into the nucleus (38), we then examined the effect of YR-290 on Smad2/3 nuclear translocation in breast cancer cells by immunofluorescence staining. Our data demonstrate that YR-290 suppressed TGFβ-induced Smad2 nuclear translocation in a concentration-dependent manner (Figure 1, D). At 5 μM, YR-290 abolished the TGFβ-induced Smad2 nuclear translocation (Figure 1, D). To confirm the data generated by immunofluorescence staining, we performed Western blot analysis of phospho-Smad2/3 in nuclear and cytoplasmic extracts. As shown in Supplementary Figure 2, B (available online), phospho-Smad2 and phospho-Smad3 accumulated in nuclear extracts when stimulated by TGFβ. Similar to the positive control SB431542, YR-290 inhibited TGFβ-induced Smad2/3 phosphorylation and nuclear translocation induced by TGFβ at 5 μM. Taken together, all of our data indicate that YR-290 suppresses the TGFβ/Smad signaling pathway in breast cancer cells.

Regulation of YR-290 on TGFβ-Mediated Transcription of Metastasis-Associated Genes

To confirm that YR-290 inhibits the TGFβ signaling pathway in breast cancer, we also investigated whether YR-290 regulates TGFβ-mediated gene expression by genome-wide expression microarray analysis using an Affymetrix human U133plus 2.0 array. Our data show that TGFβ upregulated the expression of 387 genes more than 1.5-fold. However, induction of 287 genes (74%) was blocked by YR-290 in a concentration-dependent manner (Supplementary Table 4, available online). On the other hand, TGFβ downregulated the expression of 202 genes more than 1.5-fold, and YR-290 recovered 146 of TGFβ downregulated genes (72%) in a concentration-dependent manner (Supplementary Table 4, available online), suggesting that YR-290 regulates TGFβ-mediated gene transcription. Specifically, we examined 15 lung metastasis–related genes (43) and 64 bone metastasis–related genes (26) induced by TGFβ and found that most of them were reversed by the treatment of YR-290 (Figure 2, A). To further confirm whether YR-290 inhibits TGFβ-mediated transcription of metastasis-associated genes in breast cancer, five well-known TGFβ-mediated, metastasis-associated genes (CXCR4, IL-11, ANGPTL4, CTGF, and VEGF) were chosen to confirm the microarray analysis (Figure 2, B). Our results indicated that TGFβ markedly stimulated the expression of these five genes, whereas
YR-290 inhibited the expression in a dose-dependent manner, with a half maximal inhibitory concentration of approximately 0.5 µM (Figure 2, C).

Effects of YR-290 on TGFβ-Induced Breast Cancer Cell Migration and Invasion

We next examined whether YR-290 suppresses breast cancer cell migration and invasion induced by TGFβ using wound-healing and transwell invasion assays. As shown in Figure 3, A, exogenous TGFβ increased the migration capacity of three breast cancer cell lines and YR-290 reduced TGFβ-induced cell migration in a dose-dependent manner. Similar results were obtained from the transwell invasion assays, in which YR-290 repressed the TGFβ-induced invasion in a concentration-dependent manner (Figure 3, B and C). The half maximal inhibitory concentration for YR-290 inhibition of breast cancer cell migration and invasion was approximately 0.5 µM, which is consistent with the half maximal inhibitory concentration of YR-290 inhibition of TGFβ-induced phosphorylation of Smad2/3, SBE reporter gene expression, and TGFβ-induced expression of metastasis-associated genes.
suggesting that YR-290 inhibits cell migration and invasion by suppressing the TGFβ signaling pathway.

To evaluate the specificity of YR-290 on TGFβ-induced cell migration and invasion, we examined whether YR-290 has any inhibitory effect on phorbol myristate acetate (PMA)-induced cell invasion. As shown in Figure 3, D, PMA, which regulates cell migration and invasion through the Ras/Raf/MAPKs and PI3K/AKT signaling pathways rather than the TGFβ/Smad signaling pathway (44), substantially stimulated breast cancer cell invasion as measured by the transwell invasion assay. YR-290 had little effect on PMA-induced cell invasion, suggesting that the inhibition of cell migration and invasion by YR-290 is due to the inhibition of the TGFβ signaling pathway. Together, all of our data indicate that YR-290 inhibits breast cancer cell motility and invasiveness by specifically suppressing the TGFβ signaling pathway.

**Regulation of TGFβ-Induced EMT by YR-290**

EMT is strongly induced by TGFβ/Smad signaling (23,25,45). To examine whether YR-290 also has inhibitory effects on TGFβ-induced EMT, we examined the TGFβ-induced expression and distribution patterns of E-cadherin, fibronectin, and vimentin by immunofluorescence staining in HaCaT cells. After being exposed

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**Figure 2. Inhibitory effects of YR-290 on transforming growth factor beta (TGFβ)/Smad signaling in breast cancer cells.**

A) Heat map depicting microarray gene expression profiling in breast cancer cells treated with indicated concentrations of YR-290. TGFβ-induced lung metastasis and bone metastasis-related genes are shown. MDA-MB-231 breast cancer cells were pretreated with different concentrations of YR-290 (0, 0.5, 1 μM), and then incubated with or without 5 ng/mL of TGFβ for 4 hours. Total RNA was isolated and subjected to Affymetrix human U133plus2.0 array assay. The reported 15 lung metastasis-associated genes (43) and 64 bone metastasis-associated genes (26) were selected and aligned by the software.

B) Five representative genes with expression levels from the microarray analysis. ANGPTL4 = angiopoietin-like 4; CTGF = connective tissue growth factor; CXCR4 = chemokine (C-X-C motif) receptor 4; IL-11 = interleukin 11; VEGF = vascular endothelial growth factor A.

C) Effect of YR-290 on transcription of five TGFβ-induced, metastasis-associated genes. MDA-MB-231 breast cancer cells were pretreated with indicated concentrations of YR-290, and then incubated with or without 5 ng/mL of TGFβ for 4 hours. Total RNA was isolated and subjected to real-time polymerase chain reaction. The means and error bars representing 95% confidence intervals from three independent experiments are presented.
to TGFβ, HaCaT cells became polar, decreased the expression of an epithelial cell marker (E-cadherin), and increased the expression of mesenchymal cell markers (vimentin and fibronectin) (Figure 4, A). In contrast, cells exposed to YR-290 had a dose-dependent decrease in the expression of vimentin and fibronectin and a corresponding increase in E-cadherin expression. Western blot and reverse-transcription polymerase chain reaction analyses confirmed that TGFβ-induced suppression of E-cadherin was completely reversed by YR-290, whereas N-cadherin, vimentin, and fibronectin levels were decreased by YR-290 (Figure 4, B, and data

Figure 3. YR-290 inhibited transforming growth factor beta (TGFβ)-induced migration and invasion of breast cancer cells. A) Effect of YR-290 on breast cancer cell migration by a wound-healing migration assay. MDA-MB-231, Hs578T, and BT-549 breast cancer cells “healing” back into the scratched surface were counted after 36 hours. The cells were treated with or without TGFβ1 (5 ng/mL) in the presence or absence of the indicated concentrations of YR-290. The means and error bars representing 95% confidence intervals from three independent experiments are presented. B and C) Effect of YR-290 on breast cancer cell invasion by transwell assay. After the breast cancer cells (MDA-MB-231, Hs578T, and BT-549) were serum starved for 12 hours, 2 × 10⁴ cells with the indicated doses of YR-290 were seeded in the top-chamber coated with 50% reconstituted Matrigel. The bottom-chambers were filled with media with or without TGFβ1 (5 ng/mL). The cancer cells were allowed to invade for 16 hours. The stained migrated purple cells in the images were counted (B) and photographed (C). The means and error bars representing 95% confidence intervals from three independent experiments are presented. Scale bar = 100 μm. D) Effect of YR-290 on phorbol myristate acetate (PMA)-induced breast cancer invasion. After the breast cancer cells were serum starved for 12 hours, the cells (2 × 10⁴ cells) were seeded in the top-chamber of a Matrigel-coated transwell insert in the presence of indicated doses of YR-290. The bottom chambers of the transwells were filled with media with or without 100 nM PMA. Cancer cells were allowed to migrate for 12 hours or invade for 16 hours. The stained invaded purple cells with irregular shapes in the images were photographed (left) and counted (right). The means and error bars representing 95% confidence intervals from three independent experiments are presented. Scale bar = 100 μm.
confirm this observation, mouse lungs were removed (Figure 5, C, top panel), and the tumor nodules per lung were counted (Figure 5, D). Our data showed that the number of tumor nodules in the lungs of mice treated with YR-290 at 1 mg/kg and 5 mg/kg was reduced by 74.93% (95% CI = 61.45% to 88.41%; P <.001) and 94.93% (95% CI = 82.13% to 100%; P <.001), respectively, compared with control mice (Figure 5, D). Hematoxylin-eosin staining of the lung sections also confirmed that the lungs of mice treated with 5 mg/kg/day of YR-290 had virtually no tumors (Figure 5, C, bottom panel), suggesting that YR-290 could completely inhibit highly metastatic breast cancer cell lung metastasis at the 5 mg/kg/day concentration. As a result, YR-290 treatment was associated with a significant prolongation in survival for the tumor-bearing mice (Figure 5, E). Furthermore, body weight results showed no evidence of weight loss in YR-290 treated mice (Supplementary Figure 3, available online), suggesting that YR-290 did not cause obvious toxicity in vivo.

To further confirm the mechanism by which YR-290 blocked tumor metastasis, we evaluated the metastasis-related TGFβ/Smad signaling pathway molecules from control mouse tumors and YR-290-treated mouse tumors by Western blot analysis and immunohistochemistry analysis. Compared with control mouse tumors, YR-290 inhibited the phosphorylation of Smad2/3 in a dose-dependent manner in both Western blot and immunohistochemistry analysis (Figure 5, F and G), suggesting that YR-290 suppresses the TGFβ/Smad pathway in vivo. Similar results were obtained from the analysis of CXCR4 expression (Figure 5, F), which is a well-known TGFβ-induced tumor metastasis marker (43). Taken together, these in vivo results demonstrate that YR-290 suppresses breast cancer cell metastasis by suppressing the TGFβ/Smad signaling pathway.

Preventive Effects of YR-290 on Highly Metastatic Mammary Carcinoma Cell Metastasis In Vivo

The mouse tail vein injection tumor metastasis model was used to evaluate the inhibitory effect of YR-290 on metastasis. One week prior to tail vein injection, vehicle control or YR-290 at 1 mg/kg/day or 5 mg/kg/day was administered. Treatment continued for 20 days after luciferase-labeled 4T1 cancer cell injection, and then the preventive effect of YR-290 was examined by bioluminescence imaging. As shown in Figure 6, A and B, pretreatment of YR-290 dramatically reduced the development of lung metastases in a dose- and time-dependent manner. Similar to the previous orthotopic mammary tumor metastasis model, administration of 5 mg/kg/day of YR-290 almost completely blocked 4T1 cell lung metastases, and photon flux in the lungs of mice treated at this dose was dramatically reduced (by 95.89%, 95% CI = 58.97% to 100%; P =.004) compared with control mice (Figure 6, B). To further investigate the metastases of 4T1 mammary carcinoma cells, the mouse organs, including lung, heart, kidney, stomach, spleen, liver, and gut, were removed, and bioluminescence imaging assay was performed. Our results indicated that YR-290 treatment substantially inhibited the metastases of 4T1 mammary carcinoma cells to different organs (Figure 6, C). Furthermore, survival analysis showed that mice receiving a preventive treatment of YR-290 had a statistically significantly longer survival than the mice with vehicle control treatment (Figure 6, D).

Effects of YR-290 on Lung Metastases in Mouse Orthotopic Mammary Tumor Metastasis Model

To evaluate the potential of YR-290 as a therapeutic agent, we next investigated whether YR-290 inhibits breast cancer metastasis in three mammary tumor metastasis models: the mouse orthotopic mammary tumor metastasis model, the mouse tail vein injection tumor metastasis model, and the mouse left ventricle injection tumor metastasis model. In the mouse orthotopic mammary tumor metastasis model, which is an extensively used model (37), the luciferase-labeled 4T1 mammary tumor cells metastasized to the lung region after 20 days in the control mice. Administration of YR-290 statistically significantly blocked the 4T1 cell lung metastases, with photon flux in the lungs of mice reduced by 74.71% (95% CI = 51.56% to 97.92%; P <.001) and 97.38% (95% CI = 76.83% to 100%; P <.001) in the groups treated with 1 mg/kg/day and 5 mg/kg/day of YR-290, respectively (Figure 5, A and B). To not shown). These results suggest that YR-290 plays an important role in suppressing TGFβ-induced EMT.

**Figure 4.** Effect of YR-290 on transforming growth factor beta (TGFβ)–induced epithelial mesenchymal transition. A) Immunofluorescence staining for E-cadherin, vimentin, and fibronectin in HaCaT cells. Cells were treated with or without 5 ng/ml of TGFβ and indicated concentrations of YR-290 for 48 hours, and then the cells were fixed and stained with indicated antibodies. Dilutions were as follows: E-cadherin: 1:100; vimentin: 1:50; and fibronectin: 1:100. Scale bar = 15 μm. B) Immunoblotting analysis of E-cadherin, N-cadherin, vimentin, and fibronectin in HaCaT cells. Cells were treated with or without 5 ng/ml of TGFβ and indicated concentrations of YR-290 for 48 hours. Cell lysates were subjected to Western blotting analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. Dilutions were as follows: E-cadherin: 1:1000; N-cadherin: 1:1000; vimentin: 1:500; fibronectin: 1:1000; and GAPDH: 1:1000.
Therapeutic Effects of YR-290 on Breast Cancer Cell–Induced Isteolytic Bone Metastases in Mice

Because YR-290 suppresses tumor bone metastasis–related gene expression induced by TGFβ, we next examined whether YR-290 had an inhibitory effect on breast cancer bone metastasis. The left ventricle injection tumor metastasis model is a widely used model for tumor bone metastasis. We planned the experiment as a therapeutic protocol. Three days after luciferase-labeled human mammary carcinoma cells were inoculated into female nude mice by left ventricular injection, mice were treated with YR-290 at 1 mg/kg/
day or 5 mg/kg/day or vehicle control. In the control mice, tumor cells were predominantly detected in the hind legs and head and strongly induced osteolytic bone metastasis, visualized by bioluminescence imaging and x-ray imaging after 35 days (Figure 7, A). Treatment with YR-290 statistically significantly reduced osteolytic bone metastasis and head metastasis (Figure 7, A and B). Similar to the lung metastasis model, administration of 5 mg/kg/day of YR-290 almost completely blocked tumor bone metastasis (by 98.12%, 95% CI = 81.44% to 100%; P < .001) and the formation of osteolytic lesion area (by 93.92%, 95% CI = 68.84% to 100%; P < .001) (Figure 7, B and C). Moreover, consistent with our previous study, no statistically significant difference in mouse body weight was observed among these three groups, suggesting low compound toxicity (Figure 7, D). Together, our data indicate that administration of YR-290 therapeutically blocked breast cancer cell osteolytic bone metastasis.

Figure 6. Preventive effects of YR-290 on mouse tail vein injection breast tumor metastasis model. Five-week-old BALB/c female mice were randomly allotted to three groups (n = 10 mice per group). Vehicle control or 1 mg/kg/day or 5 mg/kg/day of YR-290 were respectively administered daily for 10 days by intraperitoneal injection before tumor cell implantation. After injection of luciferase-labeled 4T1 tumor cells (2 × 10^6 cells per mouse) through the tail vein, the mice were treated with 1 mg/kg/day or 5 mg/kg/day of YR-290 or vehicle control for another 20 days. A) Tumor metastasis over a 20-day period by bioluminescence analysis. B) Quantitative analysis of metastatic cells in lung bioluminescence analysis. The means and 95% confidence intervals (error bars) are presented; *** P < .001, ** P = .004. P values were calculated using a two-sided Student's t test. p/sec/cm^2/sr = photons/second/cm^2/steradian. C) YR-290 prevents metastasis in multiple organs in vivo. Scale bar = 1 cm. D) Kaplan–Meier analysis of mouse survival after xenograft. P values were calculated using two-sided log-rank test.
YR-290 effects on development and progression of breast cancer osteolytic bone metastasis in vivo. Luciferase-labeled MDA-MB-231 cells (1 × 10⁵ cells) were injected directly into the left ventricle of the heart in 4- to 5-week-old female nu/nu mice. Three days later, the mice were divided into three groups (n = 10 mice per group): vehicle control group, 1 mg/kg of YR-290 treatment group, and 5 mg/kg of YR-290 treatment group. YR-290 or vehicle control was given every day by intraperitoneal injection for another 35 days. After 35 days, bioluminescent (A, B), and radiographic (A, C) analyses of bone lesions were performed. A) Osteolytic bone metastasis by bioluminescent (top) and radiographic (bottom) analyses of treatment with YR-290. Red arrows indicate osteolytic lesions. B) Quantitative analysis of metastatic cells in bone by bioluminescence analysis. The means and 95% confidence intervals (error bars) are presented; *** P < .001. P values were calculated using a two-sided Student’s t test. p/sec/cm²/sr = photons/second/cm²/steradian. C) Quantitative analysis of lesion area in bone. The means and 95% confidence intervals (error bars) are presented; *** P < .001. P values were calculated using a two-sided Student’s t test. D) Effect of YR-290 on mouse body weight.

Toxicity of YR-290 In Vivo

To confirm the limited toxicity of YR-290 in vivo, we used the same administration route (intraperitoneal injection), maximal dose (5 mg/kg/day), and maximal time period (30 days) of YR-290 treatment in female BALB/c mice (n = 6). Our results showed that behavior (data not shown), coat appearance (Supplementary Figure 4, A, available online), and body weight of mice (Supplementary Figure 4, B, available online) showed no statistically significant change during the 30 days of treatment. To further confirm the limited toxicity of YR-290, all of the mice were killed, and selected organs, including lung, heart, liver, spleen, and kidney, were harvested (Supplementary Figure 4, C, available online), and their pathology sections were stained by hematoxylin-eosin staining (Supplementary Figure 4, D, available online). Compared with untreated and vehicle-treated mice, mice treated with YR-290 showed no statistically significant change in size and cell morphology. In addition, YR-290 had little cytotoxicity on human nontumorigenic mammary and pulmonary epithelial cells and on human umbilical vein endothelial cells in our toxicity assays at the concentrations effective for tumor cells (Supplementary Figure 5, available online). All of our data demonstrate that YR-290 does not cause general toxicity to normal cells and mice at the tested concentrations.

Discussion

The TGFβ signaling pathway has been shown to promote metastatic progression in many types of human cancers and mouse models. In this study, we identified a small molecule, YR-290, which can inhibit the kinase activity of TGFβR1 and TGFβ-mediated, metastasis-associated gene expression. Moreover, YR-290 inhibited breast cancer cell migration, invasion, and EMT induced by TGFβ in a dose-dependent manner. In three different mouse tumor metastasis models, we also found that YR-290 preventively and therapeutically blocked breast cancer pulmonary and skeletal metastasis by suppressing the TGFβ pathway. In addition, the effective concentration of YR-290 (5 mg/kg/day) was lower than the reported TGFβ receptor antagonists (30,32). Notably, YR-290 almost fully blocked cancer metastasis and did not induce apparent toxicity at the test concentration (5 mg/kg/day). Therefore, our data suggest that YR-290 can safely suppress breast cancer metastasis by blocking TGFβ signaling pathways.

To evaluate the specificity of YR-290, we also examined the inhibitory effect of this compound on PMA-induced cell invasion, which regulates cell migration and invasion through the Ras/Raf/MAPKs and PI3K/AKT signaling pathways. Our data showed that YR-290 had little effect on PMA-induced cell migration and invasion, suggesting that YR-290 inhibits cancer cell migration by specifically inhibiting the TGFβ signaling pathway. Although we have not screened YR-290 against panels of kinases, this chemotype has no effect on cyclin-dependent kinase 4 (CDK4) (46).

Several small-molecular-weight compounds and antibodies inhibiting the TGFβ signaling pathway are now under clinical trials (13). LY-573636, the TGFβR1 inhibitor, is in phase II clinical trials for metastatic breast cancer, metastatic non-small-cell lung cancer, and malignant melanoma (13). Lerdelimumab (CAT-152), the TGFβ antibody, is in phase III clinical trials for some metastatic tumors (13). SB-431542, the most widely used TGFβR1 inhibitor, has been shown to inhibit tumor metastasis in breast cancer (15), glioma (16), and renal cell carcinoma (20) in the preclinical stage. However, SB-431542 is not as selective as previously believed. The compound has multiple targets. The half maximal inhibitory concentration for its targets are 3.3 nM for ALK4, 94 nM for TGFβR1, 2.8 nM for ALK7, 19 nM for Tyk2, and 323 nM for JAK3 (21). A possible reason for SB-431542 not having progressed to the clinical setting for cancer is the potential to promote tumor cell growth because the most significant clinical response to SB-431542 inhibitor is induction of cellular growth (24). Therefore, it has been proposed that SB-431542 may have potential clinical use in wound healing and Chagas disease, for which TGFβ inhibition appears to decrease muscle injury, thereby preventing heart damage (22–24). In comparison, YR-290 functions at relatively lower effective concentration in vivo and has no notable cellular and mouse toxicity and no effect on growth of both normal and tumor cells. YR-290 could thus be a novel agent effective for inhibiting tumor growth, invasion, and metastasis. In fact, it has been shown that lifetime exposure...
to a soluble TGFβ antagonist could protect mice against metastasis without adverse side effects (47), suggesting that inhibition of TGFβ could be an effective and safe option for cancer therapy (48).

Besides tumor metastasis, the TGFβ/Smad signaling pathway is also involved in numerous human diseases. It's been reported that the TGFβ/Smad signaling pathway is constitutively activated in myelodysplastic syndrome. Knockdown or pharmacologic inhibition of TGFβR1 reverses TGFβ-modulated cell-cycle arrest in bone marrow and stimulates hematopoiesis in vivo (49). Camurati–Engelmann disease, which is the typical progressive diaphyseal dysplasia in human disease, also has been characterized as having high levels of active TGFβ in bone marrow cells, and a TGFβR1 inhibitor partially rescued the uncoupled bone remodeling and prevented fractures (50). Inhibition of TGFβ/Smad signaling also has therapeutic effects on skin (51), pulmonary (52), and hepatic fibrosis (53). Therefore, it is reasonable to speculate that YR-290 has effects on hyper-TGFβ/Smad signaling pathway–related human diseases. However, further careful evaluation will be required in other TGFβ-related diseases.

This study had a few limitations. In the three different tumor metastasis mouse models, we treated the mice only by intraperitoneal injection, not oral or intravenous application of YR-290. The drug metabolism and pharmacokinetics and pharmacodynamics of this compound need to be further investigated to determine whether the amount of absorbed YR-290 is enough to function in breast cancer patients. Another limitation is that we were not blind to the dimethyl sulfoxide–treated control mice and YR-290-treated mice, which could lead to biased interpretation of the results.

In conclusion, our current preclinical study demonstrated that YR-290, a small molecule inhibitor, impairs the kinase activity of TGFβR1 and thus inhibits tumor migration in human breast cancer. Furthermore, mouse models indicated that systemic administration of YR-290 prevents both lung and bone metastasis of breast cancer in vivo, and it improved the survival of YR-290-treated mice. We therefore conclude that the small molecule YR-290 may be a good potential candidate for clinical treatment in prevention of breast cancer metastasis.

References

10. Xu X, Tate Y, Jin L, et al. TGFβ-receptor kinase inhibitor LY2109761 reverses the anti-apoptotic effects of TGFβ1 in myeloid-mono


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