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A multiplicity of Anti-invasive Effects of the Farnesyl Transferase Inhibitor in Human Head and Neck Cancer

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Abstract

Metastasis is a critical event in the progression of head and neck squamous cell carcinoma (HNSCC) and closely correlates with clinical outcome. We previously showed that the farnesyl transferase inhibitor SCH66336 has antitumor activities in HNSCC by inducing insulin-like growth factor binding protein 3 (IGFBP-3) secretion, which in turn inhibits tumor growth and angiogenesis. In this study, we found that SCH66336 at a sublethal dose for HNSCC inhibited the migration and invasion of HNSCC cells. The inhibitory effect of SCH66336 was associated with the blockade of the IGF-1 receptor (IGF-1R) pathway via suppressing IGF-1R itself and Akt expression. Consistent with previous work, induction of IGFBP-3 by SCH66336 also contributed in part to the anti-invasive effect. SCH66336 treatment also reduced the expression and activity of the urokinase-type plasminogen activator (uPA) and matrix metalloproteinase 2 (MMP-2), both important regulators of tumor metastasis. The effect of SCH66336 on uPA activity was inhibited partly by knockdown of IGFBP-3 using siRNA. The inhibitory effect of SCH66336 on migration or invasion was attenuated partly or completely by knockdown of IGFBP-3, Akt, or IGF-1R expression, respectively. Our results demonstrate that the IGF-1R pathway plays a major role in the proliferation, migration, and invasion of HNSCC cells, suggesting that therapeutic obstruction of the IGF-1R pathway would be a useful approach to treating patients with HNSCC.

Keywords

Insulin-like growth factor binding protein-3; insulin-like growth factor-1 receptor; farnesyl transferase inhibitor; head and neck squamous cell carcinoma; invasion; SCH66336

Despite improvements in the locoregional control of head and neck squamous cell carcinoma (HNSCC), the survival rate of patients with this cancer has not improved substantially, indicating the need for more effective therapy [1]. In most HNSCC patients, the primary tumor has metastasized to the regional lymph nodes or distant organs by the time of diagnosis [2, 3]. Metastatic HNSCC tumors are most commonly found in the aerodigestive tract, and patients with metastatic tumors have significantly lower survival rates than patients without them [3, 4]. Consequently, inhibition of metastasis may be an important strategy for HNSCC treatment.

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Tumor metastasis is a complicated and dynamic process involving tumor cell dissociation from the primary tumor, degradation or remodeling of the extracellular matrix (ECM), infiltration of surrounding stroma, intravasation, survival of malignant cells in the circulation system, invasion of a target organ, and finally growth at a secondary site [5]. Tumor cells must interact with the ECM through a multistep process that is tightly regulated by cytokines and growth factors and requires the expression of tumor-associated proteases [5]. One of the main protease systems is the plasminogen activator system, which is composed of tissue-type plasminogen activator and urokinase-type plasminogen activator (uPA); their inhibitors, plasminogen activator inhibitors 1 and 2; and uPA receptor (uPAR). uPA is expressed as a single-chain proenzyme (pro-uPA) with little or no intrinsic enzymatic activity. Pro-uPA is converted into an enzymatically active, high-molecular-weight (HMW), two-chain form (HMW-uPA) by the action of plasmin or other enzymes that are commonly enriched in cancer cells, such as cathepsin B [6]. HMW-uPA is further degraded by plasmin into the enzymatically active, low-molecular-weight (LMW), two-chain form of uPA (LMW-uPA) and the enzymatically inactive amino-terminal fragment. These uPAs can convert inactive plasminogen to the serine protease plasmin. Plasmin can degrade most components of the ECM directly or activate other matrix metalloproteinases (MMPs), which are important in ECM modulation, composition, and maintenance and in cancer metastasis [7, 8]. uPAR binding to uPA increases plasmin activity and focuses proteolytic activity on the cell surface [7]. uPA overexpression is associated with the malignant phenotype of thyroid cancer, non-small cell lung cancer, and other solid tumors [7, 9]. It is also a potential biomarker of lymph node metastasis in oral squamous cell carcinoma [10].

While searching for agents that inhibit invasive activities in HNSCC cells, we found that SCH66336, a nonpeptide tricyclic CAAX mimetic farnesyl transferase inhibitor (FTI), which has shown inhibitory effects on tumor angiogenesis and cancer cell migration [11, 12], suppresses the ability of HNSCC cells to express matrix proteinases, leading to decreased cell migration. However, the underlying molecular mechanisms by which SCH66336 inhibits cancer cell migration and invasion have not been well studied. In the current study, we found that SCH66336 modulated expression of insulin-like growth factor (IGF) binding protein 3 (IGFBP-3), IGF-1 receptor (IGF-1R), and Akt, which in turn blocked the IGF-1R pathway and expression/activity of uPA and MMP-2.

Materials and Methods

Animals, Cells, and Materials

We purchased 5-week-old female nude mice from Harlan Sprague Dawley (Indianapolis, IN). The human HNSCC cell lines UMSCC38 and SqCC/Y1, established originally by Drs. Thomas Carey (University of Michigan, Ann Arbor, MI) and Michael Reiss (Yale University, New Haven, CT), were obtained from Dr. Reuben Lotan (The University of Texas MD Anderson Cancer Center, Houston, TX) [13]; the TR146 cell line was provided by Dr. Alfonse Balm (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cell culture inserts incorporating polyester Transwell membranes (6.4-mm diameter, 8-μm pore size) and 24-well plates for the invasion assay were from Costar (Cambridge, MA). We obtained 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma and the Amicon Ultra-4 centrifugal filter device from Millipore Co. (Bedford, MA). Antibodies against IGF-1Rβ, H-Ras, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IGFBP-3 antibody was purchased from Diagnostic Systems Laboratories, Inc. (Webster, TX), anti-uPA antibody from American Diagnostica (Stamford, CT), and anti-phosphorylated Akt (Ser^{473}) and anti-Akt antibodies from Cell Signaling Technology (Beverly, MA). SCH66336 was provided by Schering-Plough Research Institute (Kenilworth, NJ). FTI-277 was

purchased from Calbiochem (San Diego, CA). SCH66336 and FTI-277 were dissolved in dimethyl sulfoxide at various concentrations to establish dose responses.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from cells using Trizol reagent (Invitrogen). cDNA was synthesized, and the reverse transcription-polymerase chain reaction was performed as described elsewhere [14]. The primer sequences were as follows: 5′-CAAGAGTGCGCTGACCATCC-3′ (sense) and 5′-CCGGA CTCACGCACCAAC-3′ (antisense) for H-Ras; 5′-GCGTGCGGCAAGACGTCTG-3′ (sense) and 5′-TCATAGCACCTTGCAGCAGTT-3′ (antisense) for RhoB; and 5′-GGTGAA GGTCGGTGTGAACGGATTT-3′ (sense) and 5′-AATGCCAAAGTTGTCATGGATGACC-3′-3′ (antisense) for GAPDH. The thermocycler conditions used for amplification were 94°C for 6 min (hot start), 94°C for 45 sec, 56–60°C for 45 sec, and 72°C for 1 min. The control polymerase chain reaction was performed in 26 or 27 cycles with 0.5 μL of cDNA solution to allow quantitative comparisons among the cDNAs developed from identical reactions with primers for GAPDH. Amplification products (8 μL) were resolved in 2% agarose gel, stained with ethidium bromide, visualized in a transilluminator, and photographed using a Kodak Gel Logic 100 imaging system (Eastman Kodak, Rochester, NY).

Small Interfering RNA Transfection

H-Ras and *RhoB* small interfering RNAs (siRNAs) were purchased from Ambion (Austin, TX). *IGFBP-3*, *Akt1*, *Akt2*, *Akt3* and nonspecific control siRNAs were synthesized at Dharmacon (Chicago, IL), and *IGF-1R* siRNA was synthesized at Bioneer (Seoul, Korea). UMSCC38 cells were transfected with siRNA using oligofectamine (Invitrogen) and incubated in a medium with 10% FBS containing 0.1% DMSO or SCH66336 (5 μmol/L) for 2 days. Cells were then harvested for Western blot or RT-PCR analysis. Conditioned medium (CM) for the zymography assay was also collected from cells that had been incubated in 10% FBS medium with or without SCH66336 (5 μmol/L) for 2 days and transferred to medium without FBS for 1 day. CM was concentrated using the Amicon Ultra-4 centrifugal filter device. Protein concentrations were measured using the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). UMSCC38 cells were pretreated with SCH66336 and subjected to an *in vitro* invasion assay.

Adenoviral Studies

Construction and amplification of adenoviruses expressing IGFBP-3 (Ad-BP3) or uPA (Ad-uPA) have been previously described [15]. UMSCC38 cells that had been infected with several doses of empty vector (Ad-EV), Ad-BP3, or Ad-uPA for 2 days were used for the invasion assay. CM was also collected from cells that had been infected with adenoviruses, incubated in 10% FBS medium with or without SCH66336 (5 μmol/L) for 2 days, and transferred to medium without FBS for 1 day. To assess the involvement of Hsp90 in the SCH66336-mediated suppression of invasion, UMSCC38 cells that had been infected with Ad-EV or adenovirus expressing Hsp90 (Ad-Hsp90) were incubated in 0.1% FBS medium with or without SCH66336 (5 μmol/L) for 1 day.

Western Blot Analysis

Total cell extracts were harvested from HNSCC lines after treatment. Whole-cell lysate preparation, protein quantification, gel electrophoresis, and Western blotting were performed as described elsewhere [13]. UMSCC38 and SqCC/Y1 cells were incubated for 24 h in the medium containing 0.1% FBS with or without 5 μmol/L SCH66336. Equivalent amounts of proteins from the cell lysate or CM from each treatment group were resolved

using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with primary antibodies. Loading of equal amounts of proteins in CM samples was confirmed by Coomassie blue staining of duplicate gels and Ponceau staining of the membrane.

Migration and Invasion Assays

In vitro migration and invasion assays were performed as described elsewhere [16]. In brief, CM obtained by culturing for 18 h in DMEM with 10% FBS was placed into the lower chamber of each well as a chemoattractant, and 5×10^4 cancer cells were placed in the upper chamber in DMEM without FBS. For the migration assay, filters were coated with a 0.1 mg/mL solution of collagen type IV (Trevigen, Gaithersburg, MD) in PBS. The invasion assay was performed in the same manner except the Transwell units were coated with Matrigel (Becton Dickinson Labware, Bedford, MA) at a concentration of 50 μg/mL in PBS. UMSCC38 cells were infected with Ad-BP3 or EV (10 and 50 plaque-forming units [pfu]/cell) for 24 h (control cells were not infected), harvested by trypsinization, washed, and placed into the Transwell. Within 24 h of incubation of SqCC/Y1 and TR146 cells in 0.1% FBS culture medium, with or without 5 μmol/L SCH66336, cells on the membranes were fixed and stained with hematoxylin and eosin. The numbers of cells in four independent fields were counted under a light microscope (Leica, Wetzlar, Germany). The results are expressed as the percentage of cells that migrated or invaded compared with control cells.

MTT Assay

The inhibitory effect of SCH66336 on growth of HNSCC cells was determined using a MTT assay. Cells were plated in 96-well plates (3×10^3/well) and cultured in medium with or without FBS. The cells then were grown for an additional total incubation of 24 or 72 h.

Gelatin and Fibrinogen/Plasminogen Zymography

The proteolytic activity of MMP-2/MMP-9 and uPA in CM was analyzed by substrate gel electrophoresis [16] using sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels containing 0.2% (m/v) gelatin or 0.12% (m/v) fibrinogen and 0.01 NIH unit/mL plasminogen. CM from each treatment group was concentrated with the Amicon Ultra-4 and loaded onto gels. After analysis, gels were washed with 2.5% Triton X-100 and incubated in a zymogram incubation buffer (50 mmol/L Tris-HCl, 0.15 M NaCl, 10 mmol/L $CaCl_2$, 0.02% NaN_3) overnight at 37°C. Clear bands, which are indicative of gelatinolytic activity, were visualized by staining with Coomassie blue.

Animal experiment

All animal procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Usage Committee. Orthotopic tongue tumor model was established as described elsewhere [11]. Female nude mice under anesthesia were injected with UMSCC38 cells (2×10^6) into the lateral tongue (n=5). One week later, when tumors started to develop, 40 mg/kg of SCH66336 or 20% hydroxyl-propyl-betacyclodexatrin control vehicle was given orally twice a day for 3 weeks. The tongues were removed, fixed, embedded in paraffin, and sectioned for uPA staining.

Statistical Analysis

Data are shown as means + standard deviations or means + standard error. Cell proliferation, migration, and invasion data in HNSCC cells were analyzed by Student's t-test using Excel 2007 software (Microsoft Corp., Redmond, WA) to determine the statistically significant difference between groups. All statistical tests were two-sided. A p value of less than 0.05 was considered statistically significant.

RESULTS

Effects of SCH66336 on HNSCC Cell Migration and Invasion

Because an important process in cancer metastasis is local invasion into the host stroma [5], we first assessed the effect of SCH66336 on the invasiveness of UMSCC38 cells by *in vitro* migration and invasion assays. Pretreatment with 1–5 μmol/L SCH66336 for 24 h, which showed a mild effect on cell proliferation, significantly reduced the number of migrating and invading cells (Fig. 1A). We also observed consistent inhibitory effects of SCH66336 on the migration and invasion of SqCC/Y1 and TR146 cells (Fig. 1B). Although the development of FTIs was based on the finding that oncogenic Ras is posttranslationally prenylated to transform cells [17], FTIs are known to inhibit anchorage-independent growth in many human cancer cell lines irrespective of whether they express wild-type or mutated Ras [18, 19]. Hence, we first determined whether silencing H-Ras or RhoB expression by siRNA abolished the activity of SCH66336 in UMSCC38 cells. Transfection with siRNAs targeting H-Ras or RhoB specifically inhibited H-Ras or RhoB RNA expression in UMSCC38 cells, whereas control scrambled siRNA did not affect expression (Fig. 1C, left). Cell viability was not specifically altered in any cells (data not shown). An *in vitro* invasion assay performed on cells transfected with siRNAs that targeted H-Ras or RhoB revealed no attenuation of SCH66336's inhibitory effects (Fig. 1C, right). This finding indicates that SCH66336 suppressed the invasive activity of HNSCC cells by an H-Ras-or RhoB-independent mechanism in this cell line.

Effects of SCH66336 on uPA and MMP-2 Activity in HNSCC Cells

Because uPA is considered to be the critical trigger for converting plasmin, which degrades various components of the ECM (e.g., fibrin, fibronectin, laminin) under physiologic and pathologic conditions (such as invasion) through plasmin or MMP activation, we analyzed the effects of SCH66336 on the activity of uPA. To this end, we performed Western blot and zymography analyses and analyzed expression and activity of uPA in CM from UMSCC38 cells. As shown in Fig. 2A (top), SCH66336 markedly decreased the levels of uPA protein, especially LMW-uPA (Fig. 2A, top). Results of the plasminogen/fibrinogen zymography assay on CM also showed that SCH66336 treatment reduced the activity of uPA, with dramatic effects on LMW-uPA (Fig. 2A, middle). Because MMP activity is stimulated by uPA or plasmin, we also tested the effect of SCH66336 on MMP activity. A gelatin-zymography assay on CM revealed that SCH66336 decreased MMP-2 activity (Fig. 2A, bottom). MMP-9 activity was suppressed by SCH66336 to a much lesser extent.

We further tested the effects of SCH66336 on uPA expression in HNSCC *in vivo* in a mouse model. An immunohistochemical analysis of uPA in tongue tumor tissue revealed that treatment of mice bearing orthotopic tongue tumors with oral SCH66336 (40 mg/kg) markedly inhibited uPA expression in the tumors (Fig. 2B).These findings suggest that oral administration of SCH66336 was sufficient to decrease uPA expression *in vivo*.

Because uPA expression in stromal cells has been shown to contribute to tumor metastasis [5], we evaluated the effect of SCH66336 on UMSCC38 cell invasiveness stimulated by Wi38 fibroblasts. The plasminogen/fibrinogen zymography assay also showed reduced activity of uPA in CM from SCH66336-treated Wi38 cells (data not shown). Furthermore, UMSCC38 cell invasiveness was significantly decreased when attracted with medium collected from SCH66336-treated Wi38 cells compared with medium collected from untreated Wi38 cells (Fig. 2C). These findings suggest that downregulation of uPA expression in HNSCC and stromal cells may decrease the invasive potential of HNSCC cells.

To determine the specific role of uPA in the invasion of HNSCC cells, we performed an invasion assay on UMSCC38 cells infected with Ad-uPA followed by SCH66336 treatment.

As shown in the representative picture of UMSCC38 cells (Fig. 2D) and by statistical analysis (Fig. 2E), Ad-uPA increased the invasiveness of UMSCC38 cells and attenuated the anti-invasive effects of SCH66336 in a dose-dependent manner. These findings suggest that uPA is a major target for SCH66336's inhibitory effects on HNSCC cell invasion.

uPA Downregulation by SCH66336 Through IGFBP-3 Induction

We studied the mechanism by which SCH66336 regulates uPA expression. We previously showed that the antiangiogenic effect of SCH66336 was mediated by IGFBP-3 induction[11]. Hence, we sought to determine whether IGFBP-3 was implicated in the anti-invasive effect of SCH66336 on HNSCC cells. We first tested whether expression of IGFBP-3 induced by Ad-BP3 could inhibit the invasive activities of UMSCC38 cells. As shown by Western blotting and plasminogen/fibrinogen and gelatin zymography assays on CM from IGFBP-3 adenovirus-infected cells, IGFBP-3 induced a marked decrease in the activity of MMP-2, HMW-uPA, and LMW-uPA (Fig. 3A). Moreover, UMSCC38 cell invasiveness was significantly inhibited by Ad-BP3 (Fig. 3B) at doses of 10–50 pfu/cell, which showed no effects on UMSCC38 cell proliferation (data not shown). To determine the importance of IGFBP-3 in mediating SCH66336-induced anti-invasive effects in HNSCC cells, we tested the effects of SCH66336 on UMSCC38 cells transfected with IGFBP-3-targeting siRNA, which effectively suppressed IGFBP-3 mRNA and protein expression (Fig. 3C, top). UMSCC38 cells showed a slight increase in basal uPA activity after IGFBP-3 siRNA transfection. The effects of SCH66336 on HMW-uPA and LMW-uPA activities were partially attenuated in UMSCC38 cells transfected with IGFBP siRNA (Fig. 3C, bottom). The inhibitory effect of SCH66336 on the invasion of IGFBP-3 siRNA-transfected cells was also partially attenuated compared with control siRNA-transfected cells (Fig. 3D). These findings indicate that IGFBP-3 is an SCH66336-induced inhibitory molecule against these ECM degraders.

SCH66336 Exerts Anti-invasive Activities in HNSCC Cells by Blocking IGF-1R and Akt Expression

Because the inhibition of IGFBP-3 expression did not completely attenuate the anti-invasive activity of SCH66336 in UMSCC38 cells, we sought other molecules that acted as SCH66336-modulated regulators of invasion. Because the IGF-1R pathway is known to upregulate uPA expression [20, 21], we sought to determine whether the IGF-1R signaling axis is a target of anti-invasive actions of SCH66336 in HNSCC cells. We found that SCH66336 treatment for 24 h suppressed the expression of IGF-1R and Akt in UMSCC38 and SqCC/Y1 cells cultured in 0.1% FBS medium, in which migration and invasive activities of the cells were analyzed (Fig. 4A). We further assessed the effects of FTI-277, another FTI, on the expression of IGF-1R and Akt in SqCC/Y1. An obvious decrease in IGF-1R and Akt protein levels was observed (Fig. 4B). No change was detected in the expression of MAPK1/2 after FTI277 treatment. To determine the specific role of the IGF-1R/Akt pathway in mediating anti-invasive actions of SCH66336 in HNSCC cells, we tested the inhibitory effect of SCH66336 on the migration and invasion of UMSCC38 cells that were transfected with siRNA targeting IGF-1R. We found that IGF-1R expression was efficiently inhibited by siRNA (Fig. 4C, left). Inhibition of IGF-1R expression suppressed migration (Fig. 4C, middle) and invasion (Fig. 4C, right) of UMSCC38 cells. The effects of SCH66336 on migration and invasion were dramatically attenuated when IGF-1R expression was blocked compared with control siRNA-transfected cells. We further assessed the role of Akt in anti-invasive activities of SCH66336. To completely block the expression of Akt, we transfected UMSCC38 cells with siRNAs targeting specific Akt1, Akt2, and Akt3 isoforms. Transfection with these siRNAs markedly inhibited Akt protein expression in UMSCC38 cells, whereas control scrambled siRNA did not affect Akt protein expression (Fig. 4D, left). The inhibitory effect of SCH66336 on migration was completely blocked in

UMSCC38 cells transfected with Akt siRNAs compared with those transfected with control siRNA (Fig. 4D, right). These results indicate that SCH66336 suppressed the invasive activity of HNSCC cells by modulating IGF-1R signaling components, including IGF-1R and Akt.

We investigated the mechanism that mediates down-regulation of IGF-1R pathway by SCH66336. SCH66336 had been originally developed to inhibit posttranslational activation of Ras by blocking farnesylation [22]. However, none of the cells used in this study have a *ras* mutation (data not shown), suggesting that proteins other than Ras are responsible for the effects of SCH66336 on IGF-1R pathway. SCH66336 has been shown to inhibit Hsp90 function, resulting in decreased levels of Hsp90 client proteins [14], such as IGF-1R and Akt [23]. Hence, we studied the role of Hsp90 in SCH66336-mediated effects on IGF-1R pathway in UMSCC38 cells. To this end, we tested whether overexpression of Hsp90 would protect UMSCC38 cells from SCH66336-induced decrease in Akt protein levels. To this end, we infected UMSCC38 cells with an adenoviral vector containingHA-tagged Hsp90 (Ad-Hsp90) [24]. Induction of Hsp90 protein expression in the cells infected with Ad-Hsp90 was determined by the increase in the HA expression in Western blot analysis (Fig. 5). Indeed, cells infected with Ad-Hsp90 were completely rescued from the effect of SCH66336 on Akt and pAkt levels, unlike cells infected with Ad-EV, suggesting an impact of Hsp90 in the action of SCH66336 against invasive activities of HNSCC.

DISCUSSION

In this study, we found that HNSCC cell invasion is significantly inhibited by the FTI SCH66336 at a concentration reported to be achievable in serum [25]. We were led to follow this line of research by early observations of tumor regression in clinical trials involving SCH66336 [26]. Increasing evidence has demonstrated that the Ras oncogenes are among those most frequently found in human cancers and that signaling pathways mediated by these oncogenes play critical roles in cancer cell proliferation, angiogenesis, and metastasis. Hence, the antineoplastic FTIs were developed to inhibit the catalytic farnesylation step in Ras [17]. However, it has become clear that Ras may not be the only critical target of FTIs [17–19], and other molecules (e.g., RhoB, Akt, and prostacyclin receptor) have been implicated in the suppression of transformed morphologic characteristics and the induction of apoptosis by FTIs [13, 27–29]. We found that the blockade of H-Ras and RhoB by specific siRNAs did not influence SCH66336-induced inhibitory effects on the invasion of UMSCC38 cells. Therefore, the anti-invasive activity of SCH66336 in HNSCC cells does not seem to depend on Ras and RhoB but to operate by other mechanisms of action.

We found that SCH66336 treatment reduced the amount of secreted HMW- and LMW-uPA and MMP-2 in supernatant from UMSCC38 cells, consistent with previous findings [12]. uPA and MMP-2 are important regulators of metastasis and are highly correlated with poor prognosis in patients with HNSCC [10, 30]. The effects of SCH66336 on uPA expression seem to have a role in its anti-invasive activities in the cells, because overexpression of uPA induced by Ad-uPA attenuated SCH66336's actions at least in part. The pro-uPA secreted from cells is converted to active uPA when bound to uPAR. Inactive uPA–plasminogen activator inhibitor-1 complexes bound to uPAR are promptly internalized and degraded in lysosomes, whereas unoccupied uPAR recycles to the cell surface [7]. The decreased levels of secreted uPAs from SCH66336-treated cells could be the result of downregulation of uPAR expression, direct inhibition of uPA transcription or translation, or interference with secreted uPA conversion in UMSCC38 cells. In our study, SCH66336 did not appear to suppress uPAR expression or to inhibit secreted uPA. This conclusion is based on our results showing that uPAR levels remained unchanged (data not shown) after the SCH66336 treatment, and levels of secreted HMW-uPA and LMW-uPA (as measured by plasminogen/fibrinogen

zymography) were reduced in agreement with levels assessed by Western blot analysis. SCH66336 has been shown to increase IGFBP-3 expression, which mediates IGF-independent inhibition of uPA transcription [11, 16]. In our study, overexpression of IGFBP-3 dramatically decreased HMW- and LMW-uPA and MMP-2 activities. Hence, the effect of SCH66336 in decreasing levels of secreted uPA could result from suppression of the expression of genes encoding uPA. However, SCH66336's actions on secreted uPA levels and invasive activities in UMSCC38 cells were only marginally abolished by the siRNA inhibition of IGFBP-3 expression, indicating that uPA gene expression is not the only operative factor.

The results described above suggest that SCH66336 may have other targets that are responsible for invasive activities in UMSCC38 cells. In our study, SCH66336 also inhibited the activation of MMP-2 in UMSCC38 cells, which could have been mediated by the decreased plasminogen-to-plasmin conversion activity of uPA [8] or by the direct inhibition of the expression of MMP-2 transcription and translation. SCH66336 is known to affect several proteins, such as tissue-type plasminogen activator, MMP-9, tissue inhibitor of MMP 1 (TIMP-1), and TIMP-2, all of which play important roles in ECM remodeling in prostate cancer cells [12]. Akt has also been implicated in the antitumor activity of SCH66336 [13, 31]. SCH66336 has the ability to inhibit Hsp90 function [14], which stabilizes client proteins, such as IGF-1R and Akt. Given that the IGF-1R/Akt pathway has a major role in cell invasion and metastasis [32], we attempted to test whether IGF-1R and Akt regulation contributes to the action of SCH66336 against invasion of HNSCC cells and whether Hsp90 function was involved in the action. We were surprised to find that SCH66336-mediated anti-invasive effects on UMSCC38 cells were almost completely blocked by the siRNA-mediated knockdown of IGF-1R or Akt expression. These findings suggest that IGF-1R signaling is a major operative factor for anti-invasive activity of SCH66336 in HNSCC. In support of this suggestion, IMC-A12 (the therapeutic antibody to IGF-1R) effectively inhibited the invasion of UMSCC38 cells cells *in vitro* (data not shown). We also noted that overexpression of Hsp90 rescued the cells from anti-invasive activities of SCH66336. These findings indicate that IGF-1R pathway is regulated by SCH66336, at least in part, through an Hsp90-dependent pathway.

In conclusion, SCH66336 inhibits uPA and MMP-2 expression/activity and has anti-invasive activity by inducing IGFBP-3 expression and by decreasing IGF-1R and Akt protein levels, leading to effective inhibition of the IGF-1R signaling pathway. SCH66336 (Lonafarnib) was well tolerated and demonstrated disease control in patients with HNSCC [33]. Unfortunately, the development of the FTIs has been discontinued. However, our current study on the mechanism of action of these compounds provides rationale for the use of the previously discredited anticancer drug in patients with HNSCC, in which activity of IGF-1R is frequently deregulated through high levels of systemically circulating or tissue-derived IGFs, overexpression of IGF-1R and/or decreased expression of IGFBPs, loss of heterozygosity for *IGF-2R*, and biallelic expression of IGF-2 [34–38]. IGF-1R has been shown to stimulate cell proliferation and survival, protecting cells from apoptosis. There are numerous preclinical and clinical studies underway to test two major anti-IGF-1R strategies, which involve either monoclonal antibodies (mAbs) or small-molecule tyrosine kinase inhibitors (TKIs) against IGF-1R [39, 40]. Anti-IGF-1R monoclonal antibodies and TKIs have shown favorable profiles in phase I clinical trials [41, 4243]. However, recent clinical studies in patients with recurrent and/or metastatic head and neck squamous cell carcinoma (HNSCC) have revealed only very modest therapeutic benefit from anti-IGF-1R mAbs [44–46]. Previously, we and others have demonstrated that the interplay between the EGFR and IGF-1R pathways and overlapping downstream signaling mediators, including Akt, induce resistance to tyrosine kinase inhibitors of EGFR and IGF-1R [47–49]. A previous study clearly shows that inhibition of IGF-IR stimulates activation of EGFR [50]. Our recent study

identified activation of Akt in HNSCC cells as a result of blockade of IGF-1R by IMC-A12 (data not shown). Given the multiplicity of effects of SCH66336 on the IGF system, FTIs have unique features that make it an effective IGF-1R-targeting antineoplastic drug in clinical trials. Further studies are warranted to identify predictive biomarkers for selecting likely responders to FTIs-based therapy and then evaluate the efficacy of the drugs in therapy for patients with HNSCC.

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ABBREVIATIONS

CM	conditioned medium
ECM	extracellular matrix
EV	parental vector
FTI	farnesyl transferase inhibitor
FBS	fetal bovine serum
HNSCC	head and neck squamous cell carcinoma
HMW	high molecular weight
IGF	insulin-like growth factor
IGF-1R	IGF-1 receptor
IGFBP-3	IGF binding protein 3
LMW	low molecular weight
MMP	matrix metalloproteinase
siRNA	small interfering RNA
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor

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The novelty and impact of this article

We found SCH66336 inhibits uPA and MMP-2 expression/activity and has anti-invasive activity in head and neck cancer cells. The anti-invasive effect was mediated by inducing IGFBP-3 expression and by decreasing IGF-1R and Akt protein levels, leading to effective inhibition of the IGF-1R signaling pathway.

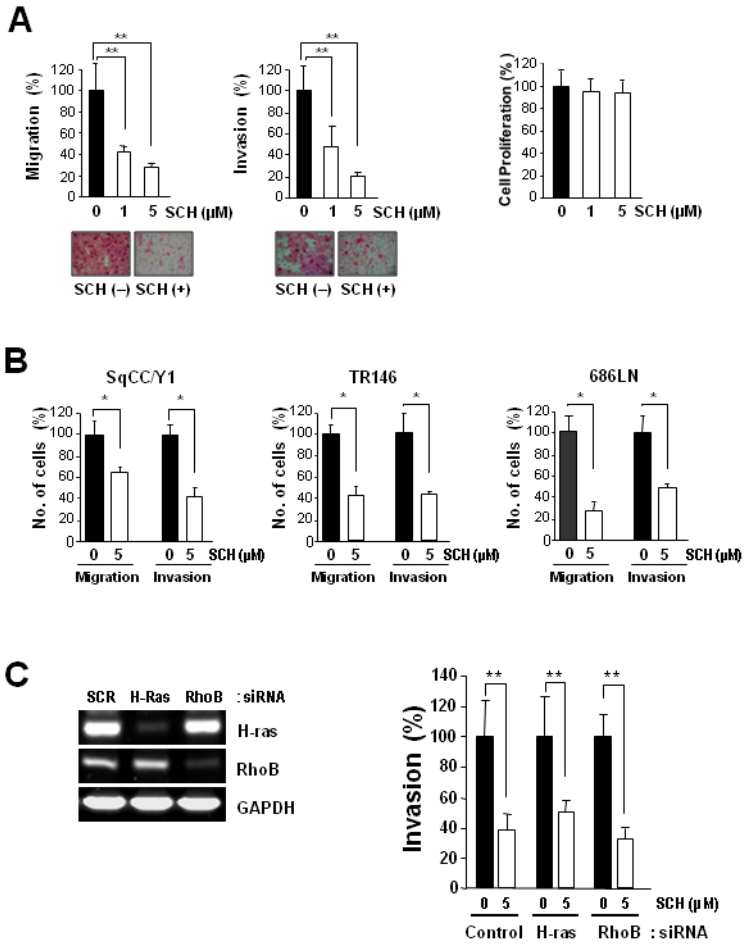

Fig. 1.

Anti-invasive activity of SCH66336 in HNSCC. A, Migration and invasion of UMSCC38 cells were inhibited by treatment with SCH66336 (1 or 5 μmol/L) for 24 h. UMSCC38 cell proliferation was marginally inhibited by treatment with SCH33663 for 24 h. B, Migration and invasion of SqCC/Y1 and TR146 cells were inhibited by treatment with SCH66336 (5 μmol/L) for 24 h. C, Invasion of UMSCC38 cells after H-Ras or RhoB siRNA transfection was inhibited by SCH66336. Values are means ± SEM. * $p < 0.05$; ** $p < 0.01$ compared with the control group. SCH, SCH66336.

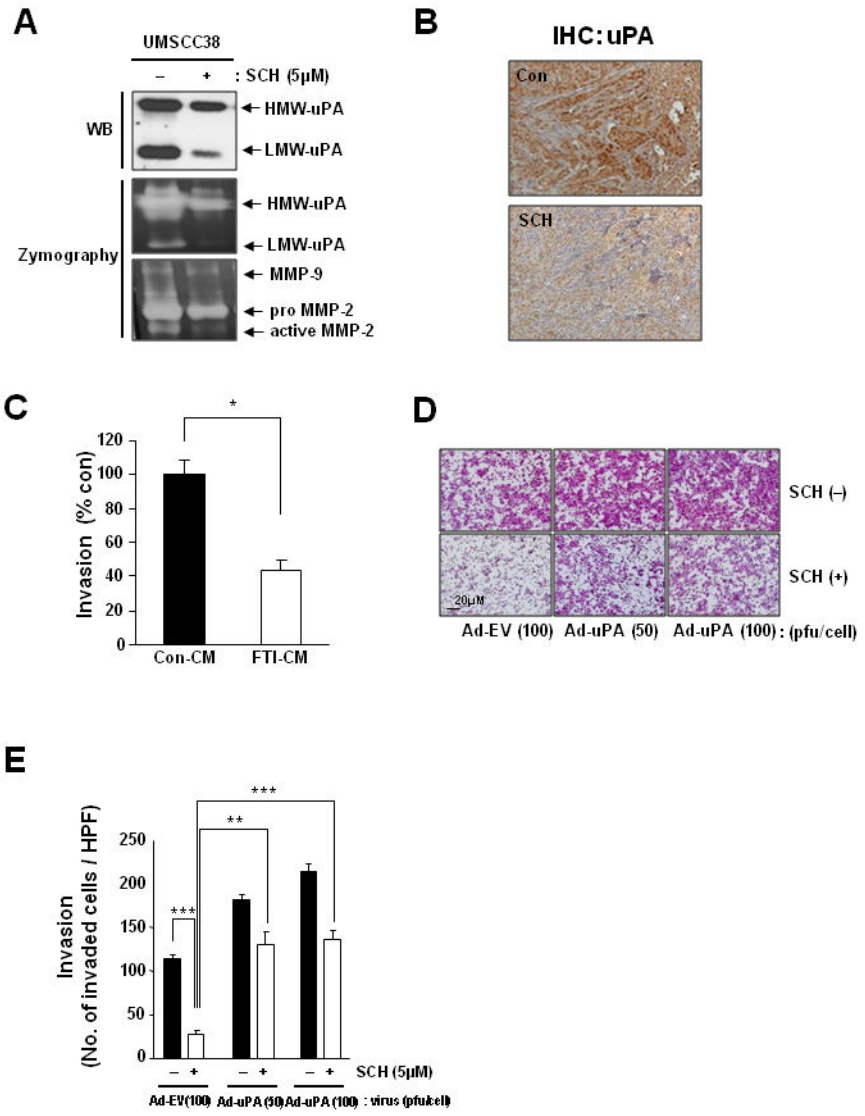

Fig. 2.

Effects of SCH66336 on the expression and activities of uPA, MMP-2, and MMP-9. A, Effects of SCH66336 on uPA expression in CM secreted from SCH66336-treated UMSCC38 cells (top). Effects of SCH66336 on uPA, MMP-2, and MMP-9 activities were determined by gelatin or plasminogen/fibrinogen zymography (middle, bottom). One representative result from two independent experiments is shown. B, Orthotopic tongue tumor tissues from mice were stained with an anti-uPA antibody. C, UMSCC38 cell invasion was analyzed in the CM obtained from overnight incubation of Wi-38 lung fibroblasts with (FTI-CM) or without (Con-CM) SCH66336 (5 μmol/L) in the lower compartment of the Transwell unit. D, UMSCC38 cells were infected with control adenoviruses (Ad-EV) or adenoviruses expressing uPA (Ad-uPA) at a concentration of 50 or 100 pfu/cell for 48 h and used for the invasion assay. Shown are representative pictures of UMSCC38 cells that invaded through the Matrigel. E, SCH66336 inhibited invasion of UMSCC38 cells in Ad-EV- or Ad-uPA-infected cells by 76.0% (EV), 27.4% (uPA 50), and 35.2% (uPA 100). Data shown are means ± SEM of three experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. SCH, SCH66336. pfu, plaque forming unit.

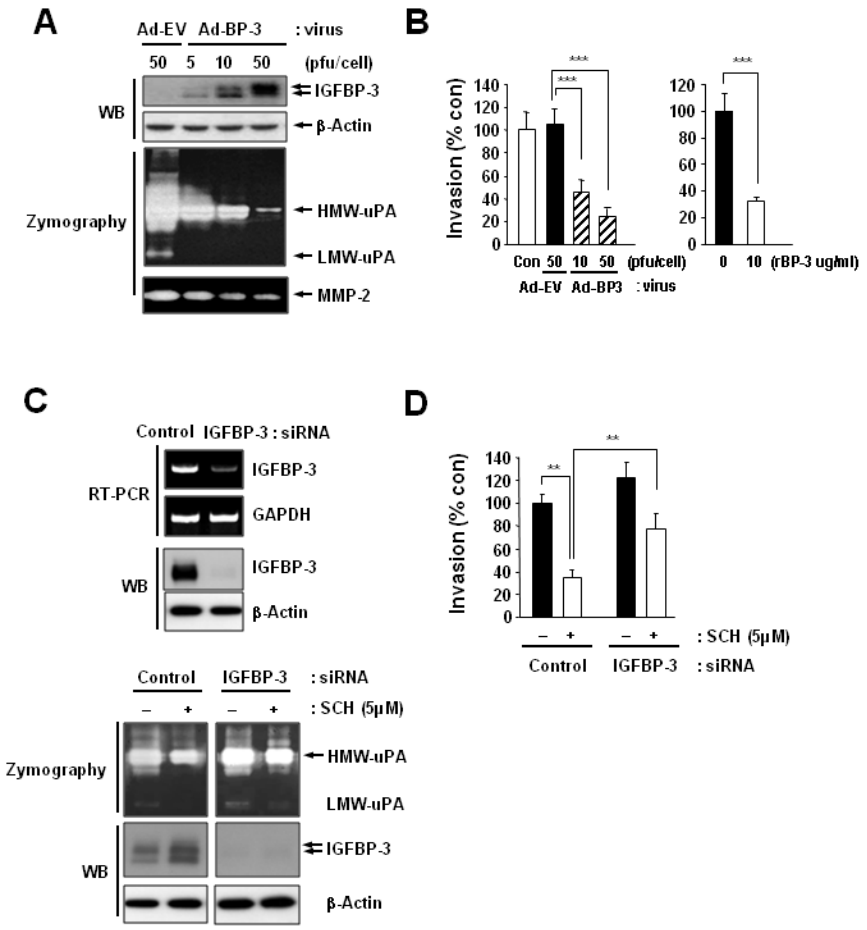

Fig. 3.

Role of IGFBP-3 in SCH66336-induced inhibition of HNSCC cell invasion. A, UMSCC38 cells were infected with control adnoviruses (Ad-EV) or adenoviruses expressing IGFBP-3 (Ad-BP-3) at a concentration of 1, 10, or 50 pfu/cell for 48 h. Ad-EV- or Ad-BP-3-infected UMSCC38 cells and CM from them were used for Western blotting and gelatin and fibrinogen/plasminogen zymography. B, Invasion of UMSCC38 cells treated as in A through the Matrigel-coated Transwell filter was inhibited by IGFBP-3. C, UMSCC38 cells were transfected with control or IGFBP-3 siRNA and treated with SCH66336. CM and cells were used for fibrinogen/plasminogen zymography and Western blotting, respectively. D, Cells transfected as in C were used for the invasion assay in the presence or absence of SCH66336 for 24 h. Data shown are means ± SEM of three experiments. $^{**}p < 0.01$, $^{***}p < 0.001$. SCH, SCH66336. pfu, plaque forming unit.

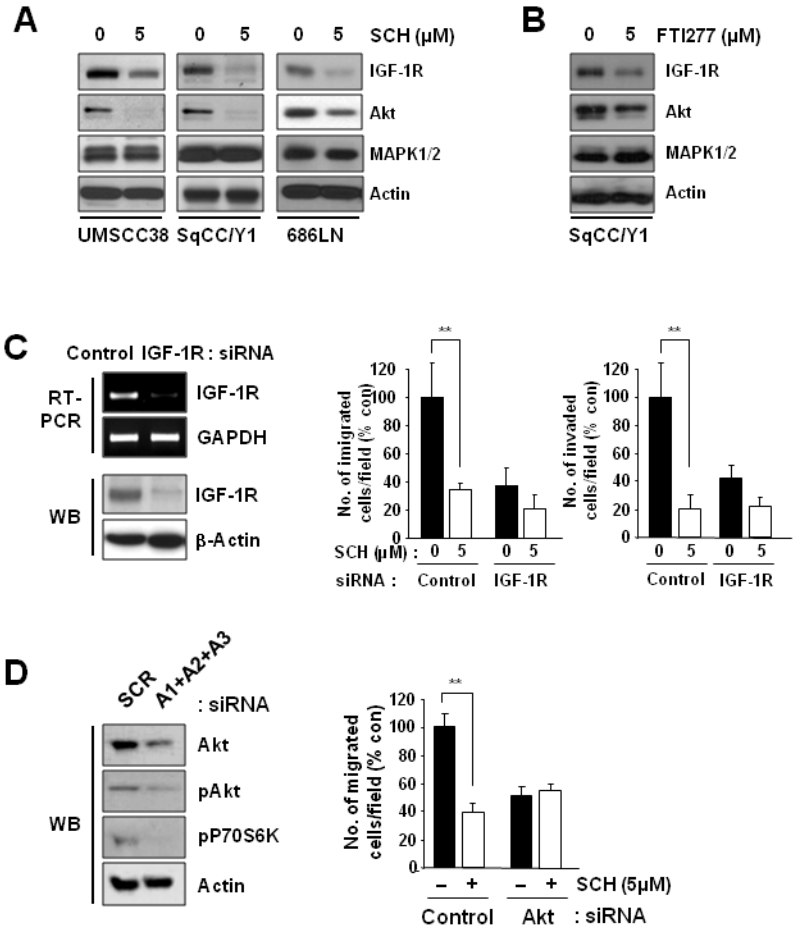

Fig. 4.

Role of the IGF-1R/Akt pathway in SCH66336-induced inhibition of HNSCC cell invasion. A, Western blot analysis of UMSCC38 and SqCC/Y1 cells incubated for 24 h in the medium containing 0.1% FBS with or without 5 μmol/L SCH66336. B, Western blot analysis of UMSCC38 cells incubated for 24 h in the medium containing 0.1% FBS with or without 5 μmol/L FTI277. C, RT-PCR and Western blot analysis using UMSCC38 cells after transfection of siRNAs targeting IGF-1R (left). Migration and invasion assay in UMSCC38 cells treated with 5 μmol/L SCH66336 after transfection with IGF-1R siRNA (right). D, Western blot analysis using UMSCC38 cells after transfection of siRNAs targeting Akt1, Akt2, and Akt3 (left). Migration assay using UMSCC38 cells treated with SCH66336 after transfection with control or Akt siRNAs (right). Data shown are means ± SEM of three experiments. $^{**}p < 0.01$ compared with untreated control cells. SCH, SCH66336.

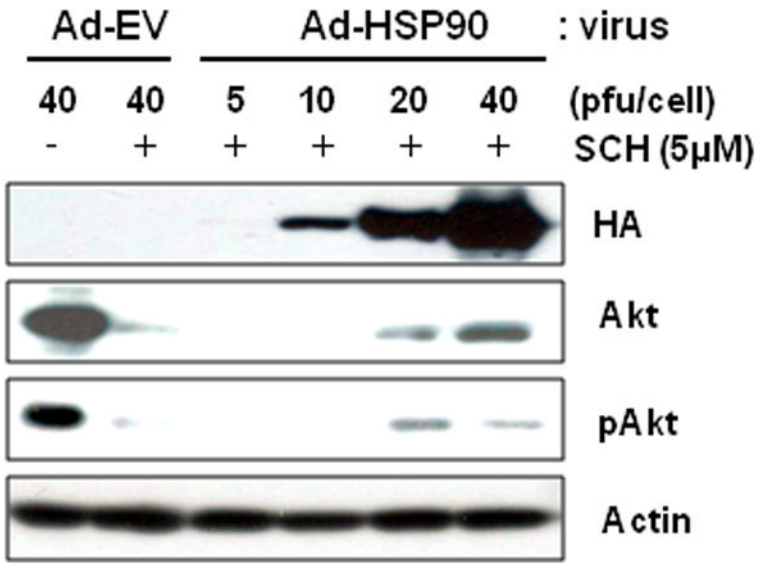

Fig. 5.

Hsp90 mediated stabilization of Akt. Western blot analysis in the UMSCC38 cells which were infected with an adenovirus carrying Hsp90 (Ad-Hsp90) or empty control (Ad-EV) and incubated for 24 h in the medium containing 0.1% FBS with or without 5 μmol/L SCH66336. SCH, SCH66336.. pfu, plaque forming unit.