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Farnesyltransferase inhibitor SCH-66336 downregulates secretion of matrix proteinases and inhibits carcinoma cell migration

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The *ras* oncogenes are among those most frequently found in human cancers. Blocking Ras farnesylation is a promising strategy for arresting cancer growth. Ras activates several signaling pathways with key roles in cellular proliferation, invasion, metastasis and angiogenesis. Furthermore, proteolytic activities of matrix proteinases such as urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs) are regulated by Ras isoforms. Thus, we investigated the effects of SCH-66336, a farnesyltransferase inhibitor, on secretion of components of the plasminogen activation system as well as on the gelatinases MMP-2 and MMP-9, which play pivotal roles in matrix remodeling. SCH-66336 up to 5 μM did not significantly alter the viability of prostate (PC-3) and renal (Caki-1) cancer cells incubated in serum-depleted medium. SCH-66336 partly inhibited the processing of H-Ras, while levels of mature N-Ras and K-Ras remained unaffected. Under these noncytotoxic conditions, uPA and tPA levels were lowered in culture medium but raised in cell lysates, suggesting inhibition of trafficking pathways. In contrast, SCH-66336 had no effect on uPAR expression or on secreted PAI-1 levels. As expected, the reduction of uPA and tPA activities by SCH-66336 inhibited the conversion of plasminogen to plasmin by about 25% in PC-3 cells. SCH-66336 also inhibited the levels of secreted pro-MMP-2 and pro-MMP-9 as well as the release of their inhibitors TIMP-1 and TIMP-2. SCH-66336 decreased both the adhesion and even more so the migration of PC-3 cells on gelatin. Thus, SCH-66336 inhibited farnesylation in both cancer cell types, and H-Ras functions should be reduced by the drug. In addition, the lower levels of secreted proteinases in the presence of SCH-66336 suggest that reduced matrix remodeling and cell migration should occur in treated tumors.

Key words: farnesyltransferase inhibitor; plasminogen system; metalloproteinase; extracellular matrix

A detailed understanding of the mechanisms by which mutated genes such as *ras* confer a neoplastic phenotype upon cells is anticipated to result in mechanism-based cancer therapeutics that specifically target the underlying defects in cellular growth regulation. To transduce extracellular signals, Ras must associate with the inner surface of the plasma membrane. This association is facilitated by several posttranslational modifications at the C-terminal end, farnesylation being the most essential.[1,2] Since farnesylation is crucial for the biologic activity and transforming features of oncogenic Ras, FTase has been intensively studied as a target for the development of FTIs.

SCH-66336 and other FTIs exhibit excellent cell penetration, high specificity for FTase, good inhibitory potency and little toxicity.[3,4] In cultured human tumor cells, SCH-66336 effectively inhibits the isoprenylation of H-Ras but not of K- or N-Ras as these proteins can be alternatively lipidated by GGTase I.[5] Furthermore, SCH-66336 reduces activated, GTP-bound Ras levels,[6] suggesting that pathways downstream of Ras are perturbed by the FTI. In addition, SCH-66336 selectively suppresses the anchorage-independent growth of H-, K- and N-Ras transformed cells.[5] Several reports have also explored the *in vivo* efficiency of FTIs against Ras-mutated tumors. In nude mice, SCH-66336 inhibits the growth of human tumor xenografts either with or without oncogenic Ras, including tumors derived from prostate,[7,8] colon, lung, pancreas, bladder,[7] glioblastoma multiforme[9] and transformed Bcr/Abl leukemia cells.[6] Similar results were obtained with H-Ras transgenic mice, where SCH-66336 induces mammary tumor regression, and in Bcr/Abl-positive acute lymphoblastic leukemia P190 transgenic mice, where the drug inhibits leukemogenesis and prolongs survival.[10] Preliminary results from a phase I trial of SCH-66336 in patients with advanced solid tumors show that they have a stabilized condition.[11] FTIs including SCH-66336 appear to promote tumor regression by multiple mechanisms, including apoptosis and cell cycle regulation.[6,7,12]

MMPs, TIMPs and serine proteases of the plasminogen activation system, including uPA, uPAR, PAI-1 and plasmin, play pivotal roles in ECM remodeling during cancer invasion, metastasis and angiogenesis.[13–17] Components of the plasminogen activation system are regulated by Ras isoforms. For example, transfection of NIH-3T3 fibroblast cells with constitutively active H-Ras stimulates uPAR transcription about 4-fold,[18] while disruption of mutated K-ras by homologous recombination downregulates uPAR expression.[19] These data provide evidence that the Ras oncogene is a regulator of uPAR expression. Furthermore, treatment with an antisense oligonucleotide against ERK-1/2 or with the inhibitor PD098059 against MEK in transformed PDV keratinocyte cells carrying a mutated H-Ras inhibited uPA synthesis.[20] This suggests that Ras is involved in uPA production. Similarly, Ras regulates the expression and activity of different MMPs. For instance, dominant-negative mutants of *ras* inhibit MMP-9 secretion in ovarian cancer cells[21] as well as MMP-2, MMP-9 and MT1-MMP (a membrane-bound MMP) in 3Y1 fibroblasts.[22] In addition, cell transfection with an activated H-*ras* increases MMP-9 gelatinolytic activity.[23,24] Since MMP activity is stimulated by uPA and plasmin,[13,14,16,17] plasmin, uPA, uPAR and MMPs could be regulated by FTIs through inhibition of Ras prenylation and activity.

Prostate cancer is the second leading cause of cancer death among men in industrialized countries. In its early stages, it is curable as long as it remains localized. However, it is usually fatal once cancer cells invade the area outside of the gland. An increased copy number of the uPA gene has been reported in hormone-refractory prostate carcinoma, and this was accompanied by a higher level of uPA mRNA.[25] Moreover, an invasion assay of PC-3 prostate cancer cells expressing an amplified uPA gene showed that they are very sensitive to amiloride, a uPA inhibitor.[25] Expression or activity of MMP-2, MMP-9 and their TIMPs is regulated in cancerous prostatic tissues.[26,27] RCC is a very difficult

Abbreviations: AP-1, activator protein-1; ECL, enhanced chemiluminescence; ECM, extracellular matrix; ERK, extracellular signal–regulated kinase; FTase, farnesyltransferase; FTI, farnesyltransferase inhibitor; GGTase I, geranylgeranyltransferase class I; HRP, horseradish peroxidase; MAb, monoclonal antibody; MEK, mitogen-activated protein kinase kinase; MMP, matrix metalloprotease; NF-κB, nuclear factor κB; OD_{595}, optical density at 595 nm; PAI, plasminogen activator inhibitor; PVDF, polyvinylidene difluoride; RCC, renal cell carcinoma; TBS, TRIS-buffered saline; TIMP, tissues inhibitor of matrix metalloprotease; tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

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tumor to treat, and response rates to therapies are weak. Elevated activity of MMP-2 has been associated with advanced tumors,[28] and high MMP-2 mRNA levels correlated inversely with survival in RCC lines.[29] Furthermore, both MMP-9 transcripts and zymogens were strongly associated with poor prognosis.[30] The *in vitro* invasiveness of renal cancer Caki-1 cells, which express uPA, was significantly enhanced by addition of uPA but inhibited by anti-uPA antibody and PAI-1.[31] Together, these data suggested that prostate PC-3 and renal Caki-1 cancer cells could be excellent models for investigating the ability of SCH-66336 to regulate the expression and activity of gelatinases and of components of the plasminogen activation system.

We present clear evidence that components of the plasminogen activation system, uPA and tPA secretion as well as plasmin activity are reduced by low micromolar SCH-66336 concentrations in prostate (PC-3) and renal (Caki-1) cancer cells while uPA and tPA accumulate in these cells. We also demonstrate that the secreted zymogens pro-MMP-9 and pro-MMP-2 and the MMP inhibitors TIMP-1 and TIMP-2 are decreased upon treatment with SCH-66336. In agreement with these decreased matrix proteolytic activities, we show that PC-3 cell binding and migration on gelatin are inhibited by SCH-66336.

Material and methods

Material

SCH-66336 was provided by Schering (Pointe-Claire, Canada). Mouse MAb against PAI-1 and rabbit polyclonal antibody against uPAR were purchased from American Diagnostica (Greenwich, CT). Mouse MAb to uPA was obtained from Oncogene (Boston, MA). Mouse MAb against β-actin was obtained from Sigma (St. Louis, MO). Mouse MAbs raised against H-Ras (F235), N-Ras (F155) and K-Ras (F234) as well as goat polyclonal antibody to tPA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against TIMP-1 as well as mouse MAbs to MMP-2 and TIMP-2 were from Chemicon (Temecula, CA). Mouse, rabbit and goat secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Gelatin and the plasmin substrate D-Val-Leu-Arg *p*-nitronilide were purchased from Sigma. Glu-plasminogen was from American Diagnostica.

Cell cultures

Human renal Caki-1 and prostate PC-3 cancer cell lines were purchased from the ATCC (Rockville, MD). Caki-1 cells were maintained in McCoy's 5A medium (Sigma); PC-3 cells were kept in Ham's F-12 medium (GIBCO BRL, Burlington, Canada). Calf serum (Hyclone, Logan, UT) was added to 10%, and cells were cultured under 5% CO_2 in a 95% air atmosphere.

SCH-66336 treatment, cell fractionation and concentration of medium

Cells were grown to 70% confluence in 100 mm dishes, then incubated for 24 hr with SCH-66336 dissolved in DMSO or the corresponding volume of vehicle in serum-free medium. Following medium removal, cells were washed twice in PBS and collected by scraping. Cells were centrifuged at 735*g* for 3 min at 4°C. Cells were resuspended in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS] containing a cocktail of protease inhibitors and incubated on ice for 30 min. Lysed cells were centrifuged at 1,000*g* for 5 min at 4°C to remove nuclei, cell debris and unbroken cells. Proteins of whole-cell lysates or postnuclear supernatants were used to investigate protein expression regulated by SCH-66336. To analyze secreted proteins from cancer cells, media were concentrated 20-fold by centrifugation at 4,000*g* for 20 min at 4°C using ultrafiltration units with a cut-off of 10 kDa (Ultrafree tubes; Millipore, Bedford, MA). Postnuclear supernatants and concentrated media were stored at −80°C until use.

SDS-PAGE and immunoblotting

SDS-PAGE was performed using 10% or 12.5% polyacrylamide gels in the discontinuous system described by Laemmli[32] using a Mini-Protean II apparatus (Bio-Rad, Mississauga, Canada). For immunoblotting experiments, proteins were electroblotted onto 0.45 μm pore diameter PVDF membranes (Immobilon, Millipore). Blots were blocked for 1 hr at room temperature in TBS [137 mM NaCl, 20 mM TRIS HCl (pH 7.5)] containing 0.1% (v/v) Tween-20 and 5% (w/v) nonfat dried milk (Nestlé Carnation, North York, Canada). Blots were then usually incubated with a 1:500 dilution of primary antibody in TBS containing 0.1% (v/v) Tween, 3% BSA and 0.01% NaN_3 overnight, followed by 1 hr incubation with a 1:2,500 dilution of secondary antibody conjugated to HRP in TBS containing 0.1% (v/v) Tween. Immunoreactive proteins were detected using an ECL Western blotting kit as described in the manufacturer's instructions (Amersham, Oakville, Canada) with preflashed Fuji (Tokyo, Japan) film. To quantify the immunodetected proteins, autoradiograms were scanned with a personal densitometer (Molecular Dynamics, Sunnyvale, CA).

Gel zymography

Gelatinolytic activity of MMP-2 and MMP-9 was monitored by gelatin zymography as previously described.[33] Soluble proteins from concentrated media and cell lysates were separated on 7.5% SDS-polyacrylamide gels containing 0.1% (w/v) gelatin under nonreducing conditions. Activities of tPA and uPA were determined by casein zymography. Proteins were loaded without reduction or heating onto 12.5% SDS-polyacrylamide gels containing 50 μg/ml plasminogen and 0.2% (w/v) β-casein as substrate. Following electrophoresis, proteins were refolded in 2.5% (v/v) Triton X-100 to remove the SDS, washed with distilled water and incubated in 50 mM TRIS (pH 7.6), 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% (v/v) Brij-35 overnight at 37°C for gelatin zymography or in 500 mM TRIS (pH 8.0) and 75 mM EDTA for 2.5 hr for casein zymography. Coomassie blue–stained zymograms were scanned with a personal densitometer.

Cell adhesion and migration assays

Cells were dislodged after brief trypsinization, dispersed into homogeneous single-cell suspensions, washed extensively and resuspended in medium at a concentration of 10^6 cells/ml. For adhesion assays, 10^5 cells were put into wells coated with 0.15% (w/v) gelatin and filled with serum-free medium containing the appropriate amounts of SCH-66336. Cells were allowed to adhere for 24 hr at 37°C in a 5% CO_2/95% air atmosphere. Medium was removed, cells were stained with 0.1% (w/v) crystal violet/20% (v/v) methanol and lysed in 10% (v/v) acetic acid. To assess migration from established monolayers, cells (10^5) were dispersed onto 0.15% (w/v) gelatin/PBS-coated chemotaxis filters (Costar, Cambridge, MA; 8 μm pore size) within Boyden chamber inserts that contained serum-free medium and various concentrations of SCH-66336. Migration was allowed to proceed for 24 hr at 37°C. Cells which remained attached to the upper surface of the filters were carefully removed with a cotton swab. Cells that had migrated to the lower surface of the filters were fixed with formaldehyde and stained with crystal violet as described above. OD_{595} was used for quantification of cells that had attached to or migrated through the gelatin-coated filters.

Plasmin generation assays

Enzymatic activities of tPA and uPA were measured with a colorimetric assay using the peptide substrate D-Val-Leu-Arg *p*-nitronilide, which was cleaved by plasmin to generate the fragment *p*-nitronilide that absorbs at 405 nm. Reactions were performed in a 200 μl volume consisting of 50 mM TRIS HCl (pH 7.5), 150 mM NaCl, 50 mM $CaCl_2$, peptide substrate (0.25 mg) and culture medium containing tPA/uPA activities with or without exogenous plasminogen (27.5 nM). Reaction mixtures were incubated at 37°C, and product formation was monitored by measuring

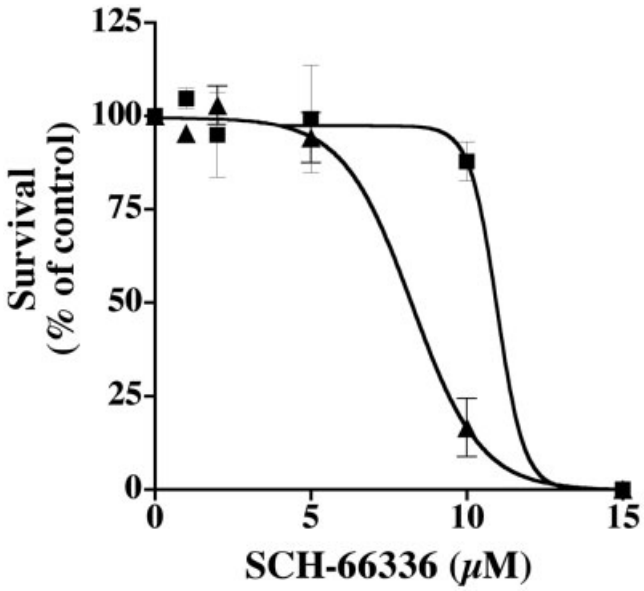

FIGURE 1 – Dose–response analysis of SCH-66336 effects on cell viability. Prostate PC-3 (triangles) and kidney Caki-1 (squares) cells were treated in serum-free medium with various concentrations of SCH-66336 for 24 hr. Following FTI treatment, cells were washed and resuspended in medium containing 10% serum. Cells were colored with trypan blue and evaluated as being dead or alive. Data are expressed as percentages of control cells incubated with vehicle, DMSO, and are means of 2 experiments.

OD_{405} every 5 min for 5 hr with a Microplate Thermomax Autoreader (Molecular Devices, Palo Alto, CA).

Cell survival assays

PC-3 and Caki-1 cells were treated with various concentrations of SCH-66336 for 24 hr in serum-free medium. Media containing detached cells were then collected. Attached cells were washed and suspended by trypsinization into 10% serum-culture media. Each suspension was pooled with the complementary preparation of detached cells and centrifuged for 5 min at 1,000*g*. Pelleted cells were resuspended in media containing 10% serum, then stained with trypan blue and counted. Round and pale cells were considered alive, while blue cells were considered dead. The number of dead cells in untreated controls was subtracted from values obtained in PC-3 cells treated with SCH-66336. Survival rates were established by dividing the living cells by the total number of cells.

Statistical analysis

Results are expressed as means ± SEM and analyzed with Student's *t*-test. Only significant differences ($p < 0.05$) are indicated in the figures.

Results

Elevated SCH-66336 doses reduce cell viability

In preliminary experiments, the cytotoxicity of SCH-66336 on cancer cell lines used as models was determined. The viability of prostate PC-3 cells treated with SCH-66336 concentrations was assessed by the trypan blue exclusion test. PC-3 cells treated with the FTI remained viable when exposed to up to 5 μM SCH-66336, but the drug rapidly became cytotoxic at higher concentrations (Fig. 1). Similarly, the FTI did not alter the viability of renal Caki-1 cells at up to 10 μM (Fig. 1). However, SCH-66336

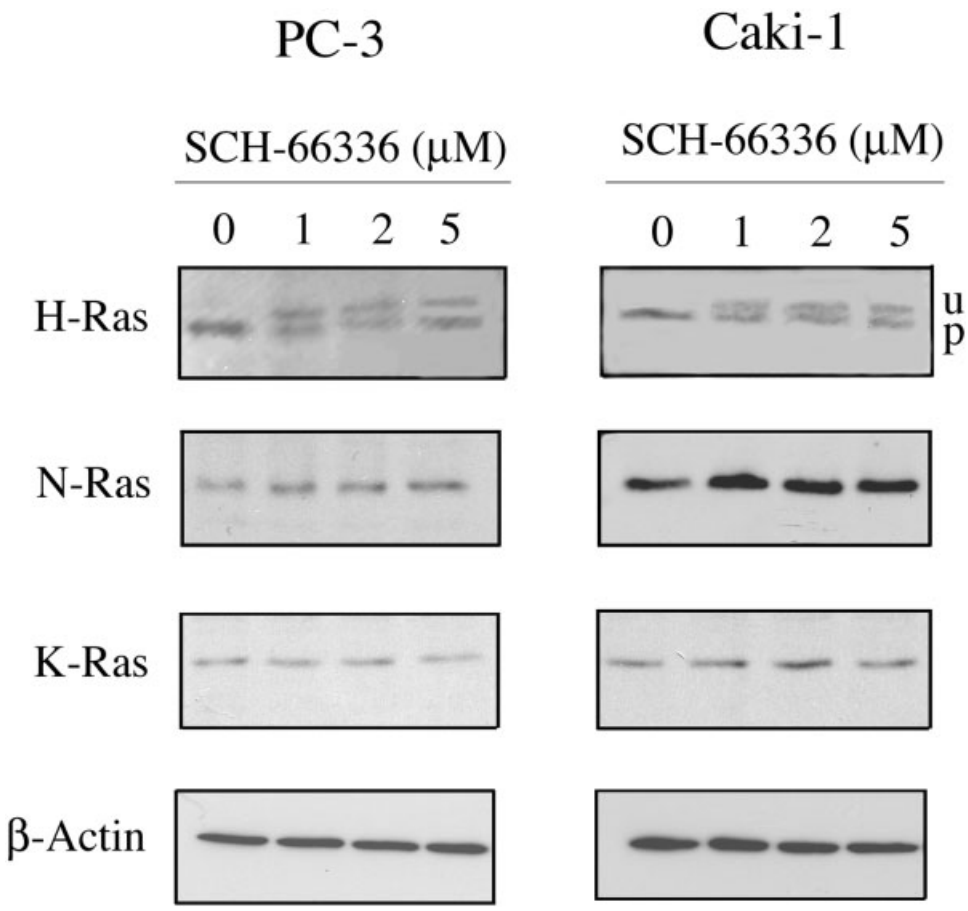

FIGURE 2 – Effects of SCH-66336 on Ras processing. PC-3 and Caki-1 cells were treated in serum-free medium with increasing concentrations of SCH-66336 for 24 hr. Proteins in cell lysates were analyzed by SDS-PAGE for assessment of processed (p) and unprocessed (u) forms of H-Ras, N-Ras and K-Ras following SCH-66336 treatment. Ras isoforms and β-actin were detected by immunoblotting with appropriate antibodies. Results are representative of 3 experiments.

treatment at 15 μM for 24 hr killed all PC-3 and Caki-1 cells (Fig. 1). Interestingly, 1–5 μM SCH-66336 corresponds to serum concentrations measured in patients with advanced solid tumors in a phase I trial.[34] Thus, the potential regulatory effects of SCH-66336 on Ras prenylation as well as on expression and secretion of serine proteinases and gelatinases were next investigated, only in the range 1–5 μM to avoid cytotoxicity in incubated PC-3 and Caki-1 cells and because these concentrations are representative of those found in treated humans.

SCH-66336 regulates H-ras prenylation in PC-3 and Caki-1 cells

Both Caki-1 and PC-3 cells expressed the tested Ras isoforms: H-Ras, N-Ras and K-Ras (Fig. 2). Then, SCH-66336 effects on

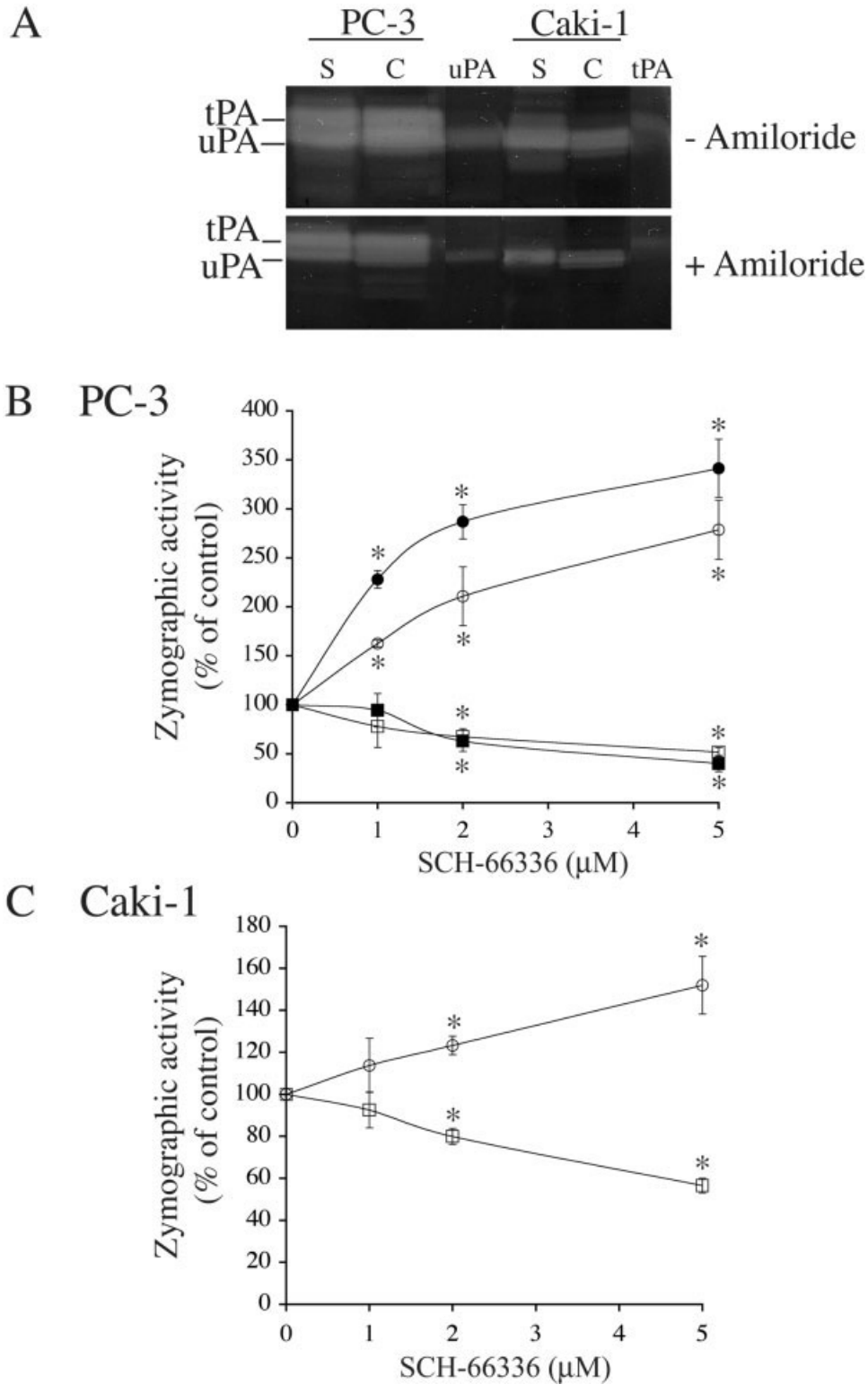

FIGURE 3 – Casein/plasminogen zymography of SCH-66336 effects on secreted and cellular uPA and tPA levels. *(a)* The capacity of PC-3 and Caki-1 cells to produce tPA and uPA was analyzed by casein/plasminogen zymography. To clearly identify where secreted (S) and cellular (C) tPA and uPA migrate, purified uPA and tPA were used as standards. Furthermore, gels were treated with or without 300 μM amiloride during protein refolding and proteolysis to block uPA activity. Then, SCH-66336 effects on levels of secreted and cellular tPA and uPA were investigated in PC-3 *(b)* and Caki-1 *(c)* cells. SCH-66336 concentrations up to 5 μM were used to treat cells in serum-free medium for 24 hr. Conditioned media were collected and concentrated while cells were lysed. Proteins from conditioned media and cell lysates were dissolved in Laemmli sample buffer and analyzed by casein zymography in the presence of plasminogen under nonreducing conditions. Zymographic activities of secreted (open squares) and cellular (open circles) uPA as well as of secreted (solid squares) and cellular (solid circles) tPA were evaluated by densitometry. Data are means ± SEM of 3 experiments. Significant differences (*$p < 0.05$) compared to untreated cells are indicated.

Ras processing and expression were determined by examining protein mobility shifts following SDS-PAGE and immunoblotting (Fig. 2). SCH-66336 treatment resulted in accumulation of one slower-mobility form of H-Ras in Caki-1 and PC-3 cells, likely reflecting the formation of unprenylated and/or unprocessed proteins. Indeed, this was accompanied by lower amounts of mature H-Ras in the presence of SCH-66336. These data confirmed that SCH-66336 doses employed to treat renal and prostate cancer cells for 24 hr were able to partially inhibit H-Ras processing. For N-Ras and K-Ras, no unprocessed forms appeared in the presence of the drug. In addition, levels of mature N-Ras and K-Ras remained unaffected by SCH-66336 treatment. These latter results are supported by a previous report showing that while SCH-66336 blocks H-Ras farnesylation, K-Ras and N-Ras are alternatively lipidated by GGTase I in cells treated with the FTI.[5] As a negative control, β-actin levels were examined and found to be unaffected by FTI treatment in both cell types. Thus, these data show that low micromolar concentrations of SCH-66336 inhibit prenylation in Caki-1 and PC-3 cancer cells and that H-Ras functions should be reduced in these treated cells.

Low SCH-66336 concentrations promote the cellular accumulation and reduce the secretion of uPA and tPA

Both directly and through activation of plasmin and MMPs, components of the uPA/uPAR system play critical roles in ECM remodeling during tumor invasion and metastasis as well as in tumor angiogenesis. Oncogenic Ras regulates the expression of uPA, uPAR and certain MMPs. Consequently, the ability of SCH-66336 to affect the production of the serine protease uPA as well as tPA was studied. Initial experiments were performed to evaluate the capacity of PC-3 and Caki-1 cells to produce and secrete uPA and tPA. Casein/plasminogen zymography exhibited 2 proteolytic activities that comigrated with purified uPA or tPA (Fig. 3*a*). When zymograms were allowed to develop in the presence of amiloride, a uPA inhibitor, the proteolytic activity comigrating with purified uPA was reduced (Fig. 3*a*). Comigration with purified uPA and tPA, and the inhibition by amiloride supported the assumption that the fastest migrating proteolytic activity was uPA while the slowest corresponded to tPA. In contrast to PC-3 cells, which strongly expressed both uPA and tPA (Fig. 3*a,* lanes 1, 2), Caki-1 cells produced uPA mainly (Fig. 3*a,* lanes 4, 5).

Next, the effect of SCH-66336 on the production and secretion of uPA and tPA was examined. Densitometric analysis of casein/plasminogen zymographic activities showed that amounts of secreted uPA and tPA were progressively reduced following treatment with increasing concentrations of FTI for 24 hr in PC-3 and Caki-1 cells (Fig. 3*b,c*). Results from 3 experiments demonstrated that 5 μM SCH-66336 significantly decreased levels of secreted uPA and tPA by about 50% in both cancer cell types (Fig. 3*b,c*). The reduced secretion of uPA and tPA into culture medium in the presence of SCH-66336 could be caused by reduced synthesis of the zymogen, impairment of its secretion or enhanced proteolysis following secretion. To investigate the involvement of these mechanisms, the effect of SCH-66336 on uPA and tPA production was determined by casein/plasminogen zymography of cellular proteins separated under nonreducing conditions following cell treatment with various FTI concentrations. SCH-66336 treatment induced accumulation in cellular levels of uPA and tPA in PC-3 cells and, to a lesser extent, of uPA in Caki-1 cells, which was inversely related to the reduction seen in culture medium (Fig. 3*b,c*). These results, showing accumulation of uPA and tPA in both cancer cell types, support the hypothesis that low micromolar SCH-66336 doses block secretory pathways rather than inhibit pro-uPA synthesis or stimulate extracellular proteolysis of the plasminogen activators.

Low doses of SCH-66336 do not alter urokinase receptor expression

uPA is secreted as a proenzyme that binds to the cellular receptor uPAR, where it is cleaved and activated by plasmin and eventually inhibited by PAI-1. The uPA–PAI-1 complex is internalized together with uPAR but becomes degraded, while the unoccupied uPAR returns to the plasma membrane. Since uPAR plays a crucial role in regulating the level of available secreted uPA, the effect of SCH-66336 on uPAR expression was studied. Immunoblot analysis of PC-3 cells showed that cellular uPAR expression, unlike uPA and tPA (Fig. 3*b*), was similar between controls and cells treated with SCH-66336 up to 5 μM (Fig. 4*a*). These observations suggest that uPAR is not responsible for the reduced levels of secreted pro-uPA/uPA at SCH-66336 concentrations lower than 5 μM because the level of receptor remained constant.

PAI-1 production is unaffected by SCH-66336 at low concentrations

While SCH-66336 did not appear to modulate uPAR functions, it could inhibit the formation of uPAR–uPA–PAI-1 complexes, leading to PAI-1 accumulation in the medium of FTI-treated cells. Alternatively, PAI-1 secretion could be reduced by SCH-66336, as happens to uPA and tPA. The antibody was unable to immunodetect PAI-1 in PC-3 medium (data not shown), suggesting that these cells secrete PAI-1 at a very low level. Interestingly, levels of secreted PAI-1 were stable in Caki-1 cells treated with SCH-66336 up to 5 μM (Fig. 4*b*). These results show that SCH-66336 up to 5 μM is not likely to alter the formation of the uPA–PAI-1 complexes required for internalization with uPAR. These data agree with the conclusion above that the decrease in secreted uPA level caused by FTI is not due to enhanced internalization by uPAR.

SCH-66336 inhibits plasmin generation

The uPA function in matrix remodeling is dependent on its capacity to convert the proenzyme plasminogen into plasmin. Consequently, the inhibition of uPA secretion in PC-3 cells treated

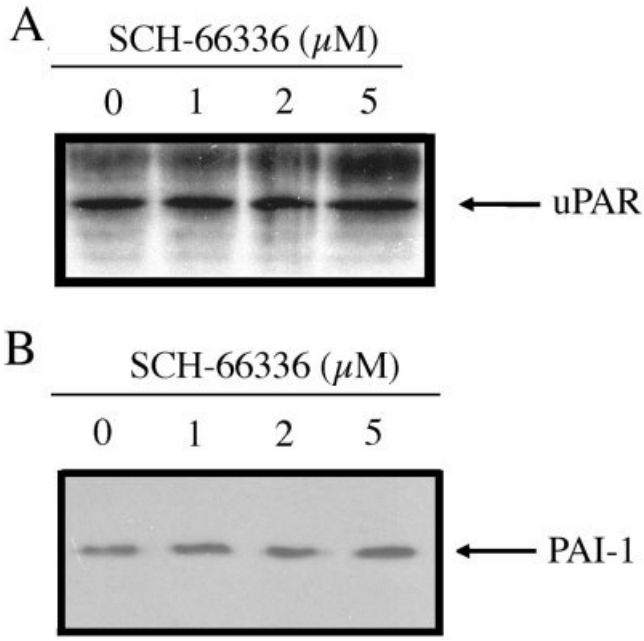

FIGURE 4 – Lack of effect of SCH-66336 on uPAR expression and on secreted PAI-1 level. PC-3 and Caki-1 cells were treated with concentrations of SCH-66336 up to 5 μM in serum-free medium for 24 hr. Cells were harvested and postnuclear supernatants prepared while conditioned medium was concentrated. *(a)* Proteins were dissolved in Laemmli sample buffer and analyzed by SDS-PAGE. uPAR was immunodetected in PC-3 cells. *(b)* Secreted PAI-1 was immunodetected in media from Caki-1 cells. Results are representative of 2 experiments.

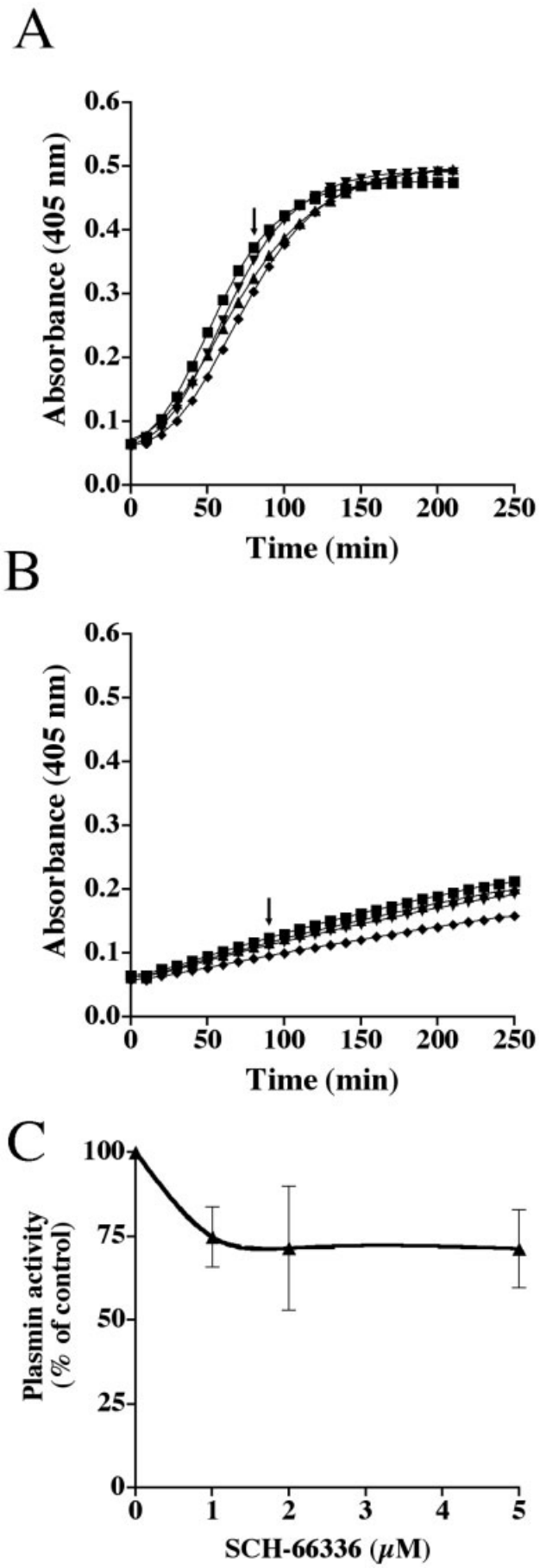

FIGURE 5

with SCH-66336 should decrease the conversion of plasminogen into plasmin. To test this hypothesis, PC-3 cells were treated with various concentrations of SCH-66336, and plasmin generation in the culture medium was measured. Addition of exogenous plasminogen to culture medium stimulated plasmin generation about 3-fold compared to basal levels (Fig. 5*a,b*). When the cleavage of plasminogen into plasmin was evaluated during the linear portion of the reaction at 80 min, increasing concentrations of SCH-66336 partly inhibited plasmin formation (Fig. 5*c*). Indeed, plasmin generation was inhibited by 25% at 1 μM SCH-66336 and then maintained a plateau at this level (Fig. 5*c*). This clearly shows that SCH-66336 inhibits plasminogen activation in PC-3 cancer cells.

SCH-66336 reduces secretion of gelatinases and their TIMPs

Aside from degrading a variety of ECM components, plasmin also activates several MMPs.[13] In addition, Ras regulates the expression and activity of different MMPs. These metalloproteinases play key roles in matrix remodeling processes that are essential for tumor growth and invasion.[13] Consequently, the effects of SCH-66336 on the secretion of 2 gelatinases, MMP-2 and MMP-9, were investigated. Gelatin zymography showed that increasing doses of SCH-66336 gradually reduced levels of secreted pro-MMP-2 from PC-3 cells (Fig. 6*a*) as well as of pro-MMP-2 and pro-MMP-9 from Caki-1 cells (Fig. 6*b*). Densitometric analysis indicated that the amounts of secreted pro-MMP-2 and pro-MMP-9 in culture media were significantly reduced by 5 μM SCH-66336 to 40–50% compared to control PC-3 and Caki-1 cells (Fig. 6*b,c*). MMP-9 proteolytic activity was difficult to reveal by gelatin zymography in PC-3 media because it was present in trace amounts (Fig. 6*a*). In contrast to uPA and tPA, whose reduced secretion by SCH-66336 was accompanied by cellular accumulation (Fig. 3*a,b*), the gelatinolytic activities of pro-MMP-2 and pro-MMP-9 could not be detected in lysates of PC-3 and Caki-1 cells (Fig. 6*a,b*). These data demonstrate that SCH-66336 reduces the gelatinolytic activities of secreted pro-MMP-2 and pro-MMP-9 in treated cancer cells, as well as of serine proteinases uPA and tPA.

An imbalance between MMPs and TIMPs is linked to ECM degradation in cancer invasion and angiogenesis. Thus, the effects of SCH-66336 on secreted TIMP-1 and TIMP-2 were also analyzed by immunoblotting. The FTI clearly inhibited TIMP-1 accumulation in PC-3 culture media, and only 63% of the normal TIMP-1 level was found in the presence of 5 μM SCH-66336 (Fig. 7*a*). Since TIMP-2 was not immunodetectable in PC-3 culture media, the regulation of TIMP-2 levels by FTI was analyzed in Caki-1 media. SCH-66336 inhibited the production of secreted TIMP-2, reaching 50% inhibition at 2 μM (Fig. 7*b*). Levels of secreted PAI-1 were unaffected by the same FTI treatments (Fig. 4*b*). Together, these data show SCH-66336 specificity for MMPs and TIMPs and that, at the same time, the FTI reduces levels of pro-MMP-2, pro-MMP-9 and their TIMPs in 2 cancer cell lines.

FIGURE 5 – Kinetic analysis of plasmin generation in medium of PC-3 cells treated with SCH-66336. PC-3 cells were incubated in serum-free medium with DMSO (squares) or 1 (upward triangles), 2 (downward triangles) or 5 (diamonds) μM SCH-66336 for 24 hr. Proteins (300 μg) from conditioned media and the plasmin substrate D-Val-Leu-Arg *p*-nitronilide (250 μg) were mixed together. Reactions took place with *(a)* or without *(b)* 275 nM of exogenous plasminogen. Reaction mixtures were kept at 37°C, and *p*-nitroaniline formation was monitored by measuring absorbance at 405 nm every 5 min for several hours. Conditions under initial reaction velocities at 80 min, as indicated by arrows, were chosen to evaluate SCH-66336 effects on plasmin formation. *(c)* Cleaved *p*-nitronilide appearance was calculated as the difference between absorbance in the presence and in the absence of plasminogen. Data are expressed as percentages compared to cells incubated with vehicle only. Data are means of 2 experiments.

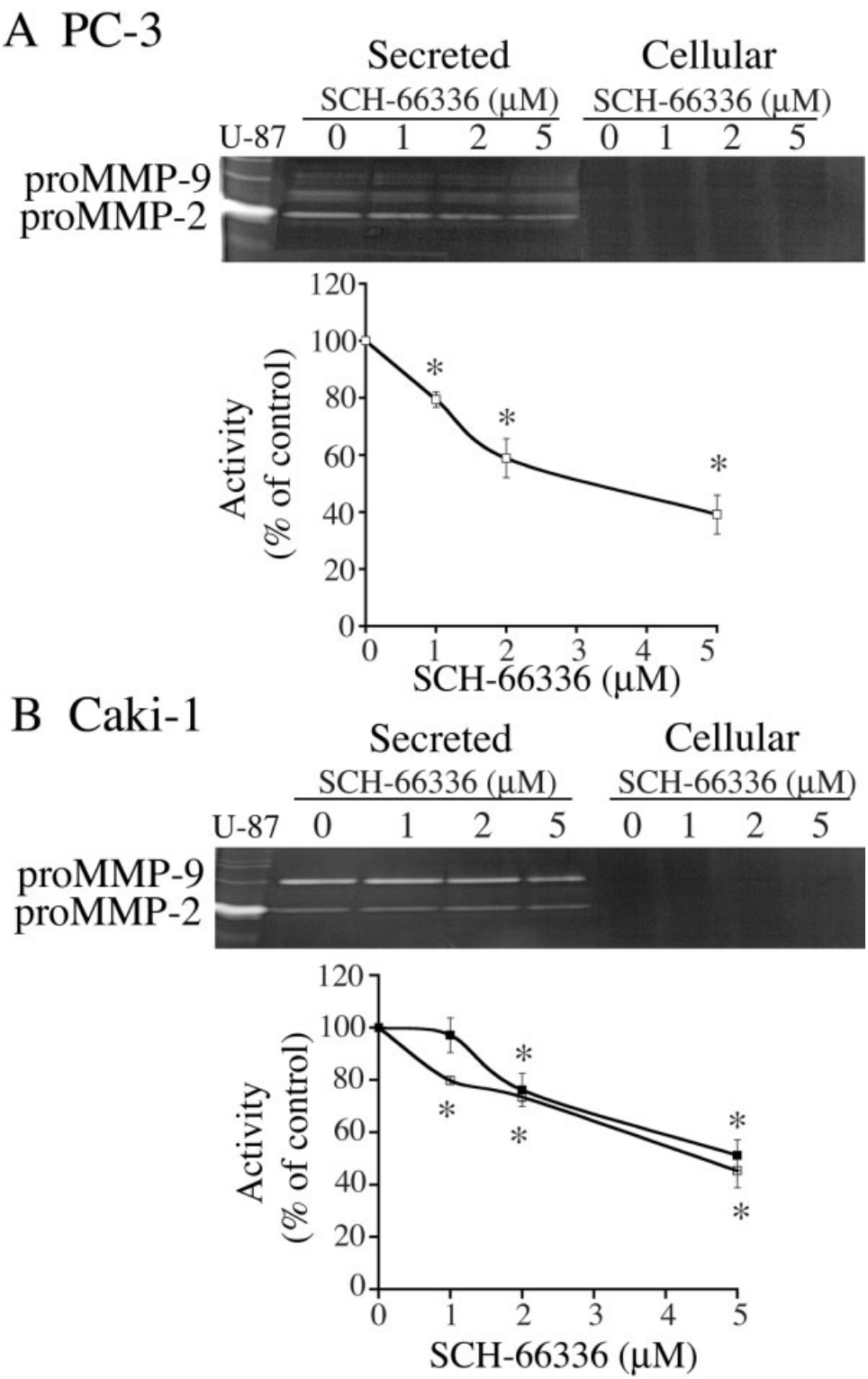

FIGURE 6 – Gelatin zymography of SCH-66336 effects on secreted and cellular MMP-2 and MMP-9 levels. To determine whether SCH-66336 affected MMP-2 and MMP-9 production, PC-3 *(a)* and Caki-1 *(b)* cells were incubated in serum-free medium with various concentrations of SCH-66336 for 24 hr. Proteins from concentrated conditioned media and cell lysates were analyzed by gelatin zymography. Concentrated conditioned media from glioblastoma U-87 cells were used as positive controls to localize MMP-2 and MMP-9 migration under these conditions. Gelatinolytic activities of pro-MMP-2 (open squares) and pro-MMP-9 (solid squares) were evaluated by densitometry. Data are means ± SEM of 3 experiments. Significant differences ($*p < 0.05$) compared to untreated cells are indicated.

SCH-66336 reduces cancer cell attachment and migration

The reduction of secreted uPA and tPA, plasmin generation and gelatinases by SCH-66336 suggested that the drug could regulate cancer cell adhesion and migration. Since PC-3 cells are highly invasive, they are an excellent model for investigating SCH-66336 effects on cancer cell migratory properties. Adhesion of PC-3 cells on gelatin was reduced 26% at 5 μM FTI compared to control cells (Fig. 8*a*). Since Caki-1 cells did not bind to gelatin, the ability of the FTI to prevent binding could not be tested (data not shown). For motility assays, migration of PC-3 cells was analyzed in a Boyden chamber coated with gelatin. PC-3 motility was significantly blocked by 77% at a concentration of 5 μM SCH-66336 (Fig. 8*b*). These data indicate that inhibition of cell migration by SCH-66336 is due not only to reduced capacity of cells to bind to gelatin but mainly to inhibition of migration *per se.* The marked reduction of prostate cancer cell motility by SCH-66336 supports

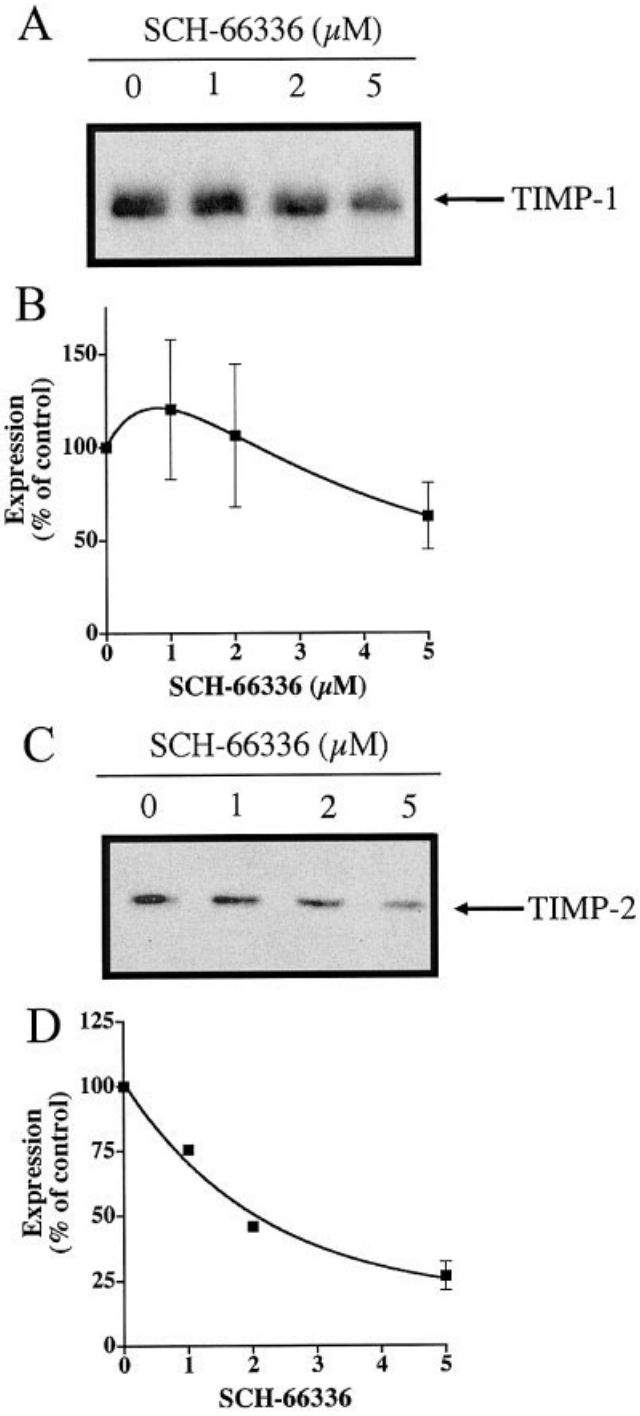

FIGURE 7 – SCH-66336 inhibits TIMP-1 and TIMP-2 expression. PC-3 and Caki-1 cells were treated with various concentrations of SCH-66336 in serum-free medium for 24 hr. Conditioned media were concentrated, and proteins were dissolved in Laemmli sample buffer and analyzed by SDS-PAGE. Secreted TIMP-1 from PC-3 cells *(a)* and TIMP-2 from Caki-1 cells *(b)* in conditioned media were immunodetected with the respective antibodies. Levels of secreted TIMP-1 and TIMP-2 were measured by densitometry. Results are means of 2 experiments.

the assumption that this FTI could decrease cell invasiveness in tumors.

Discussion

In this report, we demonstrate that the FTI SCH-66336, at a concentration found in the serum of treated patients (1–5 μM),[34] acts on several proteins that play pivotal roles in ECM remodeling during cancer cell growth. The affected proteins include tPA, uPA, plasmin, pro-MMP-2, pro-MMP-9, TIMP-1 and TIMP-2. In addition, we show that adhesion and migration of prostate cancer PC-3 cells are strongly reduced by SCH-66336. Overall, our data provide new insight into the molecular mechanisms of action of SCH-66336. Several distinct mechanisms may be responsible for the inhibition of tumor growth by the FTI since serine proteases, metalloproteases and their inhibitors are key enzymes involved in matrix remodeling during cancer invasion, metastasis and angiogenesis. This multiplicity of effects may provide therapeutic advantages in cancer treatment.

Under serum-free conditions, SCH-66336 does not produce demonstrable cytotoxic effects on PC-3 cells at concentrations up to 5 μM or on renal Caki-1 cells at concentrations up to 10 μM. At higher concentrations, SCH-66336 rapidly becomes toxic, with most cells being killed by 15 μM, likely by induction of apoptosis as previously reported.[35,36] Interestingly, SCH-66336 doses of 1–5 μM are similar to serum concentrations measured in treated patients with advanced solid tumors during a phase I trial.[34] Thus, we investigated the regulatory effects of SCH-66336 only in the range of 1–5 μM because of the lack of FTI cytotoxicity in PC-3 and Caki-1 cells and the fact that these concentrations are representative of those in serum of SCH-66336-treated humans.

In culture media of PC-3 and Caki-1 cells, secreted uPA and tPA levels are gradually reduced by exposure to increasing concentrations of SCH-66336 for 24 hr. Indeed, the FTI concentrations required to block half of the secretion of specific proteinases or their inhibitors are quite variable: these values include 2 μM SCH-66336 for pro-MMP-2 and TIMP-2 but 5 μM SCH-66336 for pro-MMP-9 and TIMP-1. In contrast, secreted PAI-1 as well as cellular levels of uPAR, K-Ras, N-Ras and β-actin are unaffected by SCH-66336 concentrations up to 5 μM. Since secreted PAI-I levels remain stable while those of released proteinases are variously affected by the FTI, the inhibition of protein secretion may not be due to a common process, *e.g.,* associated with cell cycle arrest where the reorganization of the Golgi complex could limit the capacity to secrete. Furthermore, it has been reported that cells do not undergo any appreciable cell cycle arrest in the presence of 3 μM SCH-66336.[37] These differences in effective drug concentrations are likely due to FTI inhibition at different steps in vesicle trafficking of exocytic pathways. This assumption is supported by the simultaneous accumulation of uPA and tPA in SCH-66336-treated PC-3 and Caki-1 cells. Interestingly, FTIs also block insulin secretion.[38] Increasing evidence suggests that several Rho family members are localized to vesicular compartments and play major roles in vesicle trafficking of exocytic pathways.[39] A possible explanation for the inhibition of secreted proteins by SCH-66336 is that the FTI impairs their secretion by blocking the farnesylation of certain Rho GTPases involved in vesicle trafficking. These differences could also reflect the turnover rates of the secreted proteins since those with short half-lives should be more rapidly depleted. Together, these results suggest that regulation of protein secretion by SCH-66336 is a complex process requiring further study.

As with most proteinases, uPA is secreted from cells in its precursor form. The zymogen pro-uPA is converted to uPA when bound to uPAR. Inactive uPA–PAI-1 complexes bound to uPAR are rapidly internalized and degraded in lysosomes, whereas unoccupied uPAR returns to the cell surface.[40] SCH-66336 does not appear to directly interfere with these processes. For instance, while the total amount of secreted uPA from PC-3 cells decreases

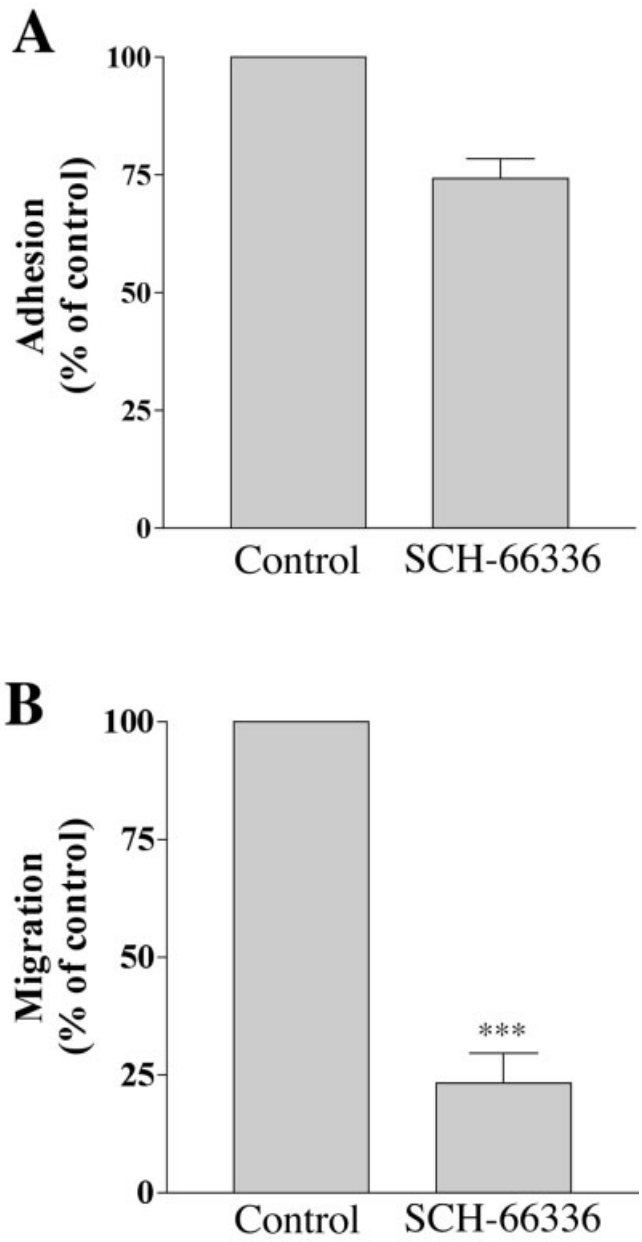

FIGURE 8 – SCH-66336 inhibits cell adhesion and migration. *(a)* For adhesion assays, PC-3 cells were incubated for 24 hr at 37°C on gelatin-coated microtiter wells in serum-free medium containing vehicle (Control) or 5 μM SCH-66336. *(b)* For migration assays, PC-3 cells were loaded on filters coated with gelatin in Boyden chambers filled with serum-free medium and the appropriate SCH-66336 concentrations as in adhesion assays. PC-3 cells were allowed to migrate for 24 hr. Cells that had adhered or migrated were fixed with formaldehyde, washed with PBS and stained with crystal violet. Absorbance of stained cells was determined with a microplate reader at 595 nm. Data represent means of 2 experiments for adhesion assays and means ± SEM of 4 experiments for migration assays. Significant differences (***$p < 0.001$) compared to untreated cells are indicated.

in the presence of SCH-66336, uPAR levels remain stable, likely allowing pro-uPA processing into active uPA. Since uPAR levels are stable in PC-3 cells treated with SCH-66336, this also suggests that the drug does not disrupt receptor recycling.

Gelatin zymography indicates that SCH-66336 reduces the amount of secreted gelatinases from PC-3 and Caki-1 cells. Similar to its effects on the components of plasminogen activator systems, SCH-66336 does not appear to directly inhibit secreted pro-MMP-2 or pro-MMP-9. This is based on our results showing that levels of secreted pro-MMP-2 and pro-MMP-9 as measured by gelatin zymography are reduced in agreement with levels assessed by Western blotting (data not shown). In contrast to uPA, which increased in SCH-66336-treated cells, the decrease of secreted pro-MMP-2 and pro-MMP-9 is not accompanied by cellular accumulation. Thus, these lower levels of secreted gelatinases in the presence of SCH-66336 could result from downregulation of the expression of genes encoding for pro-MMP-2 and pro-MMP-9, decreased stability of their mRNA or reduced protein synthesis. It appears that different molecular mechanisms are regulated by SCH-66336 for the inhibition of secreted uPA and gelatinases in both cancer cell lines.

A critical step that is affected at low concentrations (1–5 μM) of SCH-66336 is the generation of plasmin from plasminogen, which is reduced by about 25%. This likely arises from the decrease of secreted uPA in the presence of SCH-66336. Decreased plasmin formation when cancer cells are treated with SCH-66336 should reduce the cleavage of ECM proteins by this serine protease. In addition, the activation of pro-MMPs, including pro-MMP-2 and pro-MMP-9, by plasmin could be decreased. A proteolytic imbalance has been reported in malignant prostate tissues where ratios of MMP-2 and MMP-9 to TIMP-1 are increased.[27] Thus, the beneficial effects of SCH-66336 due to lower plasmin activity should reduce ECM proteolysis during tumor growth and angiogenesis.

Invasiveness requires multiple cellular functions, such as adhesion, detachment and pericellular proteolysis. Both adhesion and migration are markedly decreased when PC-3 cells are treated with increasing SCH-66336 concentrations. The higher sensitivity of prostate cancer cell migration than cell adhesion to the FTI is illustrated at 5 μM SCH-66336, where these activities are inhibited by about 26% and 77%, respectively. Although the reduction in cell adhesion by the drug could contribute to the lower migration, it is clear that many mechanisms are involved. It has been demonstrated that uPAR-positive cells express higher invasive capacity than uPAR-negative cells on gelatin-coated filters.[41] However, the stable expression of uPAR in the presence of SCH-66336 suggests that other factors are involved in the reduced migration of PC-3 cells on filters coated with gelatin. The lower activities of uPA, plasmin and gelatinases in cancer cells treated with SCH-66336 could contribute to reduced migratory capacity. This is supported by results showing that Caki-1 cells exhibit lower invasive capacity when uPA activity is reduced by added anti-uPA antibody and PAI-1.[31] Interestingly, an *in vivo* assay with another FTI, L-744,832, also showed inhibition of growth of 3 human prostate tumor xenographs in nude mice.[42]

High levels of plasminogen activators as well as MMP-2 and MMP-9 have been correlated with aggressive phenotypes in different cancers, including prostate[27] and renal[43] cancers. Since Ras plays critical roles in multiple signaling pathways, inhibition of its farnesylation by SCH-66336, as observed for H-Ras in PC-3 and Caki-1 cells and as reported for the FTI L-744,842 in another prostate cancer cell line,[44] should reduce transcription and/or secretion of gelatinases and plasminogen activators. This conclusion is based on multiple observations. Functional analysis of the gene promoters of MMP-2 and MMP-9 has allowed identification of several binding domains for transcription factors such as AP-1 and NF-κB.[45–47] Promoters of genes encoding TIMP-1 and TIMP-2 also contain consensus sequences for AP-1.[48,49] uPA expression is regulated through AP-1 binding sites in human prostate cancer.[50,51] NF-κB signal blockade downregulates MMP-9 and uPA but upregulates TIMP-1, TIMP-2 and PAI-2.[52] Since AP-1 and NF-κB are Ras-responsive promoter elements, transcriptional regulation of uPA, MMP-2, MMP-9 and TIMP promoters by SCH-66336 likely occurs through the ERK pathway. This conclusion has been supported by a report showing that

SCH-66336 reduced phosphorylated ERK1/2 levels in H-Ras transformed fibroblasts.[35] However, further work is required to determine how SCH-66336, through the reduction of H-Ras prenylation, regulates the expression and secretion of tPA, uPA, pro-MMP-2 and pro-MMP-9 as well as reduces the migration of Caki-1 and PC-3 cancer cells.

Overall, we have demonstrated that SCH-66336 at noncytotoxic concentrations similar to those found in patients under treatment downregulates levels of secreted uPA and tPA as well as plasmin formation. This is accompanied by increased cellular uPA and tPA levels. Furthermore, release of pro-MMP-2, pro-MMP-9, TIMP-1 and TIMP-2 from cancer cells is decreased by the FTI. This reduction of secreted proteinases involved in ECM proteolysis is accompanied by a marked inhibition of *in vitro* migration capacity of prostate cancer PC-3 cells. Prevention of cancer cell migration should decrease the invasive potential of cancer cells in tumors treated with SCH-66336.

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