

Potent Inhibition of Platelet-derived Growth Factor-induced Responses in Vascular Smooth Muscle Cells by BMS-354825*

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Running Title: Analysis of a Novel PDGF Receptor Tyrosine Kinase Inhibitor

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Number of text page: 27

Number of tables: 0

Number of figures: 7

Number of references: 39

Number of words in abstract: 246

Number of words in Introduction: 528

Number of words in Discussion: 702

Abbreviations: AoSMCs, aortic smooth muscle cells; CML, chronic myeloid leukemia;

FBS, fetal bovine serum; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth

factor receptor; IGF-1R, insulin-like growth factor-1 receptor; EGFR, epidermal growth factor

receptor; PI, propidium iodide; VSMCs, vascular smooth muscle cells;

Abstract

Abnormal migration and proliferation of vascular smooth muscle cells (VSMCs) are key events in the pathogenesis of restenosis that undermine the long-term benefit of widely performed balloon angioplasty and stenting procedures. Platelet-derived growth factor (PDGF) is a potent mitogen and motogen for VSMCs and is known to play a prominent role in the intimal accumulation of smooth muscle cells. In this study, we analyzed the effects of a novel protein tyrosine kinase inhibitor, BMS-354825 (dasatinib), on PDGF-stimulated VSMCs. BMS-354825 is an orally bioavailable dual Src/Bcr-Abl tyrosine kinase inhibitor currently undergoing clinical trials in cancer patients. We found that BMS-354825 inhibited PDGF-stimulated activation of PDGF receptor (PDGFR), STAT3, Akt, and Erk2 in rat A10 VSMCs and in primary cultures of human aortic smooth muscle cells (AoSMCs) at low nanomolar concentrations. The 50% inhibition of the PDGFR β tyrosine kinase activity in vitro by BMS-354825 was observed at 4 nM. Direct comparison of BMS-354825 and another PDGFR inhibitor, imatinib (Gleevec, STI571), in VSMCs indicated that BMS-354825 is 67-fold more potent than imatinib in inhibition of PDGFR activation. BMS-354825 also inhibited Src tyrosine kinase in A10 cells. At the cell level, PDGF stimulated migration and proliferation of A10 cells and human AoSMCs, both of which were inhibited by BMS-354825 in a concentration dependent manner in the low nanomolar range. These results suggest that BMS-354825 is a potent inhibitor of PDGF-stimulated VSMC activities and a potential agent for the development of a new therapy for vascular obstructive diseases such as restenosis.

Introduction

Balloon angioplasty and stenting are widely used procedures for coronary artery disease. Unfortunately, renarrowing (restenosis) of the dilated artery occurs in 25-40% of patients within six months after these procedures, which requires repeat angioplasty or bypass surgery (Dangas and Kuepper, 2002; Michaels and Chatterjee, 2002). Therefore, restenosis represents a major problem limiting the long-term efficacy of these revascularization therapies. Moreover, following coronary artery bypass surgery, narrowing (stenosis) of the transplanted artery can also occur that necessitates further medical treatments.

Abnormal migration and proliferation of VSMCs are critical events in the pathogenesis of artery obstructive diseases such as restenosis (Bailey, 2002; Levitzki, 2005; Sanz-Gonzalez et al., 2004). While several growth factors and cytokines are involved in the development of restenosis, many lines of evidence have indicated that PDGF plays a prominent role in the pathogenesis of restenosis. PDGF is the most potent mitogen and motogen for VSMCs (Heldin and Westermark, 1999). PDGF is present at sites of vascular injury from activated platelets, monocytes, and cells of the artery wall (Raines, 2004). Expression of exogenous PDGF in animal arteries can induce intimal thickening through stimulation of VSMC proliferation and migration and synthesis of extracellular matrix (Pompili et al., 1995). Importantly, inhibition of PDGF as well as PDGFR by immunological, molecular biological, and pharmacological methods can suppress development of restenotic lesions in animal models (Ferns et al., 1991; Levitzki, 2005; Myllarniemi et al., 1999; Sirois et al., 1997).

Imatinib (Gleevec, STI571, CGP57148B) is the first protein tyrosine kinase inhibitor that has been successfully developed into a targeted therapy drug. It is currently used to treat chronic myeloid leukemia (CML) and gastrointestinal stromal tumor based on inhibition of Bcr-Abl and

c-Kit protein tyrosine kinases, respectively (Deininger et al., 2005; Logrono et al., 2004).

Besides Bcr-Abl and c-Kit, imatinib also inhibits PDGFR tyrosine kinase (Buchdunger et al., 1996; Druker et al., 1996). Experiments in animals have shown that imatinib inhibits restenosis after balloon angioplasty and stenosis after allograft (Myllarniemi et al., 1999; Sihvola et al., 2003). Inhibition of PDGFR by imatinib, however, requires micromolar concentrations in cell-based assays (Buchdunger et al., 1996; Sanz-Gonzalez et al., 2004).

Following the findings by us and others that a Src tyrosine kinase inhibitor, PD180970, and other pyrido[2,3-d]pyrimidine derivatives could cross-inhibit Bcr-Abl (Dorsey et al., 2000; Huron et al., 2003; Wisniewski et al., 2002), several dual Src/Bcr-Abl inhibitors are now being developed as new therapeutic agents for CML. One of these is the orally bioavailable BMS-354825 (Burgess et al., 2005; Lombardo et al., 2004; O'Hare et al., 2005; Shah et al., 2004), which is currently in clinical trials in CML patients. Because the Bcr-Abl tyrosine kinase inhibitor imatinib was originally identified in the screening of PDGFR tyrosine kinase inhibitors, we asked whether BMS-354825 could also cross-inhibit PDGFR in VSMCs and suppress PDGF-stimulated migration and proliferation of VSMCs. We provide evidence here that BMS-354825 is a potent PDGFR tyrosine kinase inhibitor in VSMCs and that it inhibits cellular responses to PDGF in VSMCs at low nanomolar concentrations. These results reveal BMS-354825 as a potential agent for development of a new therapy for restenosis and stenosis, which will help to improve the long-term success of angioplasty/stenting and coronary bypass surgery.

Materials and Methods

Antibodies and Reagents: Antibodies to phosphotyrosine, phospho-Akt, and phospho-Stat3 were from Cell Signaling (Beverly, MA). Anti-PDGFR β (catalog number: 06-498, for immunoblotting) was from Upstate Biotechnology (Lake Placid, NY). Antibodies to PDGFR β (catalog number PC-17, for immunoprecipitation) and Src (catalog number OP07, for immune complex kinase assay) were from Calbiochem (San Diego, CA). Antibodies to Akt, STAT3, Erk1/2, Src (for immunoblotting), insulin-like growth factor-1 receptor (IGF-1R), and epidermal growth factor receptor (EGFR) were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-activated Erk1/2 antibody was from Promega (Madison, WI). PDGF-BB was from PeproTech (Rocky Hill, NJ). Propidium Iodide (PI) and rat tail type I collagen were from Roche (Indianapolis, IN). BMS-354825 (N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl]amino]-5-thiazolecarboxamide monohydrate) was provided by Bristol-Myers Squibb. Imatinib (Gleevec, STI571) was provided by Novartis.

Cell Culture. The A10 rat aortic smooth muscle cell line was obtained from American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μ g/ml streptomycin. Primary culture of human aortic smooth muscle cells (AoSMCs) was obtained from Cambrex (Walkersville, MD) and grown in SmGM-2 Bulletkit[®] medium (Cambrex) plus 100 units/ml penicillin and 100 μ g/ml streptomycin. Human AoSMCs were used before the 7th passage. Cells were maintained at 37 °C in a humidified 95% air and 5% CO₂ incubator.

Immunoprecipitation and Immunoblotting. Cells were lysed in cold lysis buffer [25 mM Tris-HCl (pH 7.2), 150 mM NaCl, 25 mM NaF, 1 mM benzamidine, 1% Triton X-100, 1 mM

Na₃VO₄, 20 mM *p*-nitrophenyl phosphate, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride]. Cell lysate supernatants were obtained by microcentrifugation at 4 °C for 15min and the protein concentration was determined. For immunoblotting analysis of cell lysates, cell lysate supernatants were heat-denatured in SDS loading buffer. Antibodies to activated STAT3, activated Akt, activated Erk1/2, and phosphotyrosine were used to assess the activation of these molecules and receptor tyrosine kinases by immunoblotting. Immunoblotting was performed essentially as described previously (Cunnick et al., 2001; Cunnick et al., 2002; Ren and Wu, 2003) using the SuperSignal West Pico Chemiluminescent reagent (Pierce, Rockford, IL). Each immunoprecipitation was performed using 600 μg of cell lysate supernatant, 2 μg of specific antibody indicated in the figure legends and 30 μl of Protein-A or Protein-G at 4 °C for 2 h. Immune complex was collected by microcentrifugation and washed three times with the lysis buffer. Quantification of immunoreactive band intensity was achieved using the ImageQuant program (Molecular Dynamics).

Protein Tyrosine Kinase Assays. PDGFR was immunoprecipitated with an anti-PDGFR antibody (Calbiochem, PC17). Immunoprecipitates were washed twice with the lysis buffer and twice with kinase buffer [50mM HEPES (pH 7.4), 10mM MnCl₂, 0.1mM Na₃VO₄]. The immune complex kinase assay was performed in 50 μl reaction mixture [kinase buffer plus 20 μCi [γ-³²P]-ATP, 10 μM ATP] at 30°C for 15min. Kinase reaction was terminated by addition of 17 μl of 4 x SDS loading buffer and heat-denaturation at 95 °C for 10min. Samples were subjected to 8% SDF-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane. Phosphorylation was quantified by phosphoimage analysis or autoradiography. The immune complex kinase assay for c-Src was performed as described previously (Ren et al., 2004).

Cell Migration Assay. Cell migration was measured using the Transwell cell migration assay (Ren et al., 2004). Transwell cell culture insert polycarbonate membrane (6.5 mm, 8.0 μ m pore size, Costar, Corning, NY) was coated with rat tail type I collagen (10 μ g/ml in PBS) at 4 $^{\circ}$ C for 18 h and air-dried. VSMCs (80% confluent) were serum-starved in DMEM/0.1% BSA for 18 h, detached from plates by digestion with 1:3 diluted trypsin-EDTA (Invitrogen), washed with DMEM, and resuspended in DMEM/0.1% BSA at 5×10^4 cells/ml for A10 cells or 2.5×10^4 cells/ml for human AoSMCs. Cell suspension (0.2 ml per well) was incubated with BMS-354825 or solvent (DMSO) in 1.5-ml microfuge tube for 20 min before been placed in the upper chamber of Transwell. The lower chamber contained 0.6 ml DMEM/0.1% BSA with or without 5 ng/ml PDGF-BB (PeroTech, Rocky Hill, NJ) and BMS-354825. After incubation at 37 $^{\circ}$ C/5% CO₂ for 4 h, cells remaining on the upper membrane surface were mechanically removed with a cotton swab. Migrated cells on the lower side of membrane were fixed and stained with the HEMA3 reagents (Fisher Scientific, Swanee, GA) and enumerated under a microscope in eight randomly chosen fields with 10 x 10 lens. Each field for quantification of the migrated cell number has an area of 0.8 x 0.6 mm.

Cell Proliferation Analysis. A10 cells and human AoSMC cells were seeded in triplicate at 2×10^4 per plate in 6-cm plates in DEME/1% FBS with or without PDGF-BB (10 ng/ml). After 24 h (Day 0), cells were treated with DMSO (solvent) or 10-50 nM BMS-354825. Medium was changed on Day 3. Viable cell number was determined on Day 6 as described (Dorsey et al., 2000). For the recovery experiment, cells were treated as above for 6 days and then cultured without BMS-354825 for another 6 days.

Flow Cytometric Analysis. A10 cells (80% confluent) were serum starved in DMEM/0.1% BSA for 24 h. After which, PDGF-BB (10 ng/ml) and BMS-354825 or DMSO was added and

the incubation was continued for another 16 h. Cells were collected by trypsinization and resuspended in PBS at 5×10^6 cells/ml. Cells were fixed in cold 70% ethanol, washed with PBS, and incubated with propidium iodide (20 $\mu\text{g/ml}$) and RNase (200 $\mu\text{g/ml}$) for 1 h at room temperature at a cell concentration of 1×10^6 cells/ml. Flow cytometric analysis of cell cycle phase distribution was performed using a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and 1×10^4 events were recorded for each sample.

Results

BMS-354825 Inhibits PDGF-activated Signaling Pathways in VSMCs. To determine if BMS-354825 could inhibit PDGFR activation in VSMCs, we treated serum-starved rat A10 cells with PDGF-BB (5 ng/ml) for 0-30 min in the presence or absence of 50 nM BMS-354825. Cell lysates were analyzed for activation of PDGFR β , STAT3, Akt, and Erk2 by immunoblotting analyses (Fig. 1A). PDGFR β is the predominant PDGFR subunit in VSMCs (Raines, 2004) and the only functional PDGFR subunit in A10 cells (Rao et al., 1997). STAT3, Akt, and Erk2 signaling pathways have been reported to mediate PDGF-induced cell migration and proliferation in VSMCs (Graf et al., 1997; Kim et al., 2005; Neeli et al., 2004; Shibata et al., 2003; Vantler et al., 2005; Zhan et al., 2003). PDGF treatment markedly increased PDGFR β tyrosine phosphorylation, which was detectable at the earliest time point examined (1 min). BMS-354825 (50 nM) effectively blocked PDGF-stimulated PDGFR β tyrosine phosphorylation at all time points examined. Fig. 1A also shows that STAT3, Akt, and Erk2 were activated by PDGF in A10 cells as measured by activation-specific phosphorylation and these were inhibited by BMS-354825.

We next immunoprecipitated PDGFR β from A10 cells that had been treated with PDGF-BB (5 ng/ml, 5 min) in the presence of 0-25 nM BMS-354825 and then analyzed the PDGFR tyrosine phosphorylation by immunoblotting. As illustrated in Fig. 1B, tyrosine phosphorylation of PDGFR was induced by PDGF stimulation in A10 cells; the response was inhibited by BMS-354825 with an IC₅₀ of 3 nM in this assay.

To confirm that BMS-354825 inhibits PDGFR tyrosine kinase activity, PDGFR β was immunoprecipitated from A10 cells with or without prior PDGF stimulation (5 ng/ml, 5 min). The PDGFR tyrosine kinase activity was determined in vitro in the immune complexes by

autophosphorylation in the presence of various concentrations of BMS-354825. As shown in Fig. 1C, BMS-354825 inhibited PDGFR tyrosine kinase activity in a concentration-dependent manner. The 50% inhibition of PDGFR β phosphorylation was observed at 4 nM BMS-354825.

Comparison of PDGFR Inhibition by BMS-354825 and Imatinib. In addition to inhibiting Bcr-Abl and c-Kit, imatinib also inhibits PDGFR tyrosine kinase. To compare the inhibition of PDGFR in VSMCs by BMS-354825 and imatinib, A10 cells were pre-incubated with various concentrations of BMS-354825 or imatinib, stimulated with PDGF-BB, and activation of PDGFR, STAT3, Akt, and Erk2 were analyzed. Fig. 2A shows that PDGF-stimulated PDGFR β tyrosine phosphorylation was completely blocked by 50 nM BMS-354825. In contrast, 5 μ M imatinib was required to achieve the same level of PDGFR inhibition in parallel experiments. Similarly, complete inhibition of PDGF-stimulated Akt and Erk2 activation were observed in cells treated with 50 nM BMS-354825 but only with 5 μ M imatinib, whereas decrease in STAT3 tyrosine phosphorylation appeared at lower concentrations of imatinib (Fig. 2A). Of note, inhibition of Akt by both BMS-354825 and imatinib was observed only at the highest drug concentration tested. This may be due to the fact that PDGFR is a strong activator of the phosphoinositide-3 kinase signaling pathway because it binds phosphoinositide-3 kinase directly. Comparison of 50% inhibition of PDGFR tyrosine phosphorylation by BMS-354825 and imatinib in these experiments indicated that BMS-354825 was 67-fold more potent than imatinib in inhibiting PDGFR tyrosine phosphorylation (Fig. 2B).

Inhibition of PDGFR in Human AoSMCs by BMS-354825. To exclude the possibility that the potent inhibition of PDGFR by BMS-354825 is specific to rat VSMCs, we examined the effect of BMS-354825 in primary cultures of human AoSMCs. Human AoSMCs were pre-incubated with 0-50 nM BMS-354825 and then stimulated with PDGF-BB (5 ng/ml, 5 min).

PDGFR tyrosine phosphorylation and activation of STAT3, Akt, and Erk2 were analyzed.

PDGF markedly induced PDGFR tyrosine phosphorylation in human AoSMCs (Fig. 3). Similar to that observed in A10 cells, the PDGF-stimulated PDGFR tyrosine phosphorylation was completely blocked by 50 nM BMS-354825.

The primary culture of human AoSMCs appeared to have an elevated level of active STAT3 in the absence of PDGF stimulation (Fig. 3). Nevertheless, STAT3 was further activated by PDGF, which was blocked by BMS-354825. BMS-354825, however, was unable to reduce the active STAT3 to a level below the basal activation state in human AoSMCs. Similar to data obtained in A10 cells (Fig. 2), complete inhibition of PDGF-stimulated Akt and Erk2 activation was achieved at 50 nM BMS-354825 in human AoSMCs (Fig. 3).

Inhibition of c-Src Tyrosine Kinase by BMS-354825 in VSMCs. To determine if BMS-354825 inhibits c-Src activity in VSMCs, c-Src was immunoprecipitated from serum-starved A10 cells treated with PDGF-BB (5 ng/ml) for 0-30 min in the presence or absence of 50 nM BMS-354825, and the Src tyrosine activity was determined by an immune complex kinase assay using a GST fusion protein of Gab1 fragment as an exogenous substrate (Ren et al., 2004). c-Src isolated from A10 cells without exposure to BMS-354825 and PDGF had detectable kinase activity (Fig. 4A). This kinase activity was reduced to a residual level in c-Src isolated from BMS-354825-treated cells (Fig. 4A). PDGF treatment did not lead to a detectable increase in c-Src kinase activity in A10 cells in repeated experiments. Nevertheless, similar to that observed in unstimulated cells, the activity of c-Src tyrosine kinase in PDGF-stimulated A10 cells was suppressed by BMS-354825 to the residual level (Fig. 4A). This result indicated that c-Src was basally active in A10 cells and that BMS-354825 effectively inhibited the c-Src kinase activity in these cells.

We next treated A10 cells with various concentrations of BMS-354825, immunoprecipitated c-Src and assayed its tyrosine kinase activity to determine the concentration-dependent effect of BMS-354825 on c-Src in A10 cells. Fig. 2B shows that BMS-354825 potently inhibited c-Src tyrosine kinase in A10 cells. The IC_{50} for inhibition of c-Src autophosphorylation was 2.25 nM while the IC_{50} for inhibition the exogenous substrate (GST-Gab1CT) was 2.0 nM.

IGF-1R and EGFR in A10 cells are not sensitive to BMS-354825 inhibition. Besides PDGFR, A10 cells also express IGF-1R and EGFR. To assess if BMS-354825 inhibits other receptor tyrosine kinases in VSMCs, we examined the effects of BMS-354825 on these two receptor tyrosine kinases in A10 cells. As shown in Fig. 5, IGF-1 induced IGF-1R tyrosine phosphorylation, whereas EGF induced EGFR tyrosine phosphorylation in A10 cells. No detectable inhibition of IGF-1R and EGFR tyrosine phosphorylation was observed when A10 cells were treated with up to 50 nM BMS-354825. These data are consistent with a previous report that the IC_{50s} for inhibition of IGF-1R and EGFR by BMS-354825 in the *in vitro* kinase assays were $>5 \mu\text{M}$ and 180 nM, respectively (Lombardo et al., 2004).

BMS-354825 Inhibits PDGF-stimulated VSMC Migration. Migration of VSMCs plays a critical role in the development of restenosis. PDGF is a potent migratory stimulus for VSMCs. Transwell cell migration assay was performed to determine the effect of BMS-354825 on PDGF-induced VSMCs migration. A10 cells had a low basal migration activity. PDGF (5 ng/ml) stimulated A10 cell migration 7-fold in our assay. This response was inhibited by BMS-354825 in a concentration-dependent manner (Fig. 6A-B). Consistent with the biochemical data, complete inhibition of PDGF-stimulated A10 cells migration was observed at 50 nM BMS-354825.

The primary human AoSMCs had a higher basal migration activity, which is 4 times that of A10 cells. In the presence of PDGF (5 ng/ml), migration of human AoSMCs was increased 3-fold. Again, PDGF-stimulated human AoSMC cell migration was inhibited by BMS-354825 (Fig. 6C).

Inhibition of VSMC proliferation by BMS-354825. To determine the effect of BMS-354825 on PDGF-stimulated VSMC proliferation, rat A10 cells were incubated in medium containing low serum (1% FBS), PDGF (10 ng/ml), and/or 0-50 nM BMS-354825 for 6 days and viable cell numbers were determined. As shown in Fig. 7A, A10 cell proliferation was stimulated 5-fold by PDGF. BMS-354825 effectively inhibited PDGF-stimulated A10 cell proliferation. Complete inhibition was achieved at 50 nM of BMS-354825. Similar results were obtained in human AoSMCs (Fig. 7B).

We next analyzed the effect of BMS-354825 on PDGF-induced change in cell cycle phase distribution. Fig. 6C shows that PDGF decreased the percentage of cells in G1 phase from 88.65% to 65.44%, while it increased the percentages of cells in S and G2 phases from 10.4% and 0.99% to 23.83% and 10.74%, respectively. The effect of PDGF was suppressed by BMS-354825, such that in the percentages of cells in G1, S, and G2 phases were 88.29%, 7.99% and 3.75% in cells treated with PDGF in the presence of 50 nM BMS-354825 (Fig. 7C).

A previous study showed that the PDGFR specific tyrosine kinase inhibitor AG-1295 could inhibit porcine SMC proliferation and the effect was reversible and not toxic (Banai et al., 1998). To evaluate if the inhibitory effect of BMS-354825 on A10 cell proliferation is reversible, two sets of A10 cells were treated with 0-50 nM BMS-354825 for 6 days. After this time, BMS-354825 was removed from one set of cells and cells were cultured for another 6 days. Viable cell numbers were determined on Day 12. As shown in Fig. 7D, A10 cells proliferation resumed

after BMS-354825 withdrawal. Thus, the inhibitory effect of BMS-354825 on A10 cell proliferation was reversible, suggesting that BMS-354825 does not exert a cytotoxic effect on these cells.

Discussion

BMS-354825 is an orally bioavailable protein tyrosine kinase inhibitor currently in human clinical trials as a dual Src/Bcr-Abl inhibitor. We illustrate here that BMS-354825 is a potent inhibitor of PDGF-stimulated cellular responses in VSMCs. It is possible that inhibition of Src tyrosine kinase could contribute to the inhibitory effects of BMS-354825 in VSMCs, such as the STAT3 pathway (Bowman et al., 2001; Wang et al., 2000). However, biochemical evidence suggests that inhibition of PDGFR plays a major role in suppression of PDGF-stimulated responses in VSMCs by BMS-354825.

First, our data show that BMS-354825 is a potent inhibitor of PDGFR. The IC_{50} for inhibition of PDGFR β tyrosine kinase in PDGF-stimulated A10 cells is 3 nM. A similar IC_{50} (4 nM) was obtained in the in vitro kinase assay. While this work was in progress, Lombardo et al reported the kinase selectivity profile of BMS-354825 (Lombardo et al., 2004), which showed that BMS-354825 inhibited PDGFR in vitro with an IC_{50} of 28 nM. Although no cell-based assay was performed in the study by Lombardo and co-workers, data from these two studies are consistent in demonstrating that BMS-354825 is a potent PDGFR inhibitor in vitro. The slight difference in IC_{50} for inhibition of PDGFR may reflect different assay conditions used to determine the PDGFR kinase activity between these two studies.

We found that Src is basally active in A10 cells and that BMS-354825 inhibits the basal Src activity ($IC_{50} = 2$ nM). Src kinase activity may be essential for cellular activities such as migration (Heldin and Westermark, 1999). Thus, blocking Src activity could contribute to the overall effect of BMS-354825 in inhibition of PDGF-stimulated VSMC migration. However, we did not detect measurable PDGF-induced Src activation in A10 cells, whereas marked activation of PDGFR was readily detectable. Thus, Src kinase activity alone is insufficient to

account for the higher migratory activity of VSMCs observed in the presence of PDGF. Furthermore, while imatinib has no Src tyrosine kinase inhibitor activity (Druker et al., 1996), it suppressed the PDGF-stimulated activation of PDGFR, STAT3, Akt, and Erk2 in A10 cells. Therefore, inhibition of the PDGFR tyrosine kinase activity alone could have major effects on blocking PDGF-stimulated responses in VSMCs.

Several studies have shown that PDGFR inhibitors such as imatinib and AG-1295 are effective in suppressing restenosis and stenosis in experimental animals (Fishbein et al., 2000; Levitzki, 2005; Myllarniemi et al., 1999; Sihvola et al., 2003). Inhibition of PDGFR in VSMCs by imatinib and AG-1295 requires micromolar concentrations (Fishbein et al., 2000; Sanz-Gonzalez et al., 2004). In contrast, near-complete inhibition of PDGFR activation and PDGF-stimulated activities in VSMCs was observed with 50 nM BMS-354825 in this study. Direct comparison of BMS-354825 and imatinib in A10 cells indicates that BMS-354825 is 67-fold more potent than imatinib (Fig. 2). Thus, BMS-354825 is a much more potent PDGFR inhibitor than imatinib.

Inhibition of PDGF-stimulated VSMC proliferation by BMS-354825 is reversible. It has been reported that the vascular endothelial growth factor receptor is not sensitive to BMS-354825 inhibition (Lombardo et al., 2004). These observations suggest that BMS-354825 is unlikely to cause unmanageable damage to the vascular system. In support of this notion, imatinib, which also inhibits PDGFR, does not cause an adverse effect in the cardiovascular system in CML patients who have been treated with the drug for over 4 years (Lahaye et al., 2005). The reversibility of the inhibitory effect of PDGF-stimulated VSMC proliferation by BMS-354825 also raises the question of whether BMS-354825 will be effective in suppressing restenosis. Conceivably, restenosis-promoting activities peak shortly after vascular injury and

lessen afterward. Therefore, one would predict that VSMC proliferation is not likely to resume when BMS-354825 is terminated after sufficient period of treatment unless there is new vascular injury.

BMS-354825 is currently being developed as an anti-cancer drug. We demonstrated here that it is very effective in inhibiting PDGF-stimulated VSMC migration and proliferation. VSMC migration and proliferation are critical events in the development of restenosis, which is a major problem for the long-term efficacy of the widely performed angioplasty and stenting procedures for coronary artery disease. Thus, in addition to being a promising anti-cancer drug, BMS-354825 is a potential novel therapeutic agent for cardiovascular diseases involving abnormal VSMC activities such as restenosis and stenosis.

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Footnotes

*This work was supported by American Heart Association Grant 0455429B. We also acknowledge the H. Lee Moffitt Cancer Center & Research Institute core facilities and the Tsai-fan Yu Research Endowment Fund.

Figure Legends

Fig. 1. Effect of BMS-354825 on PDGF-stimulated activation of PDGFR, STAT3, Akt, and Erk in rat A10 VSMCs. (A) Time-dependent activation of PDGFR, STAT3, Akt, and Erk2 in A10 cells. Subconfluent A10 cells were serum starved for 18 h, pre-incubated with BMS-354825 (50 nM, 20 min) or DMSO (-) for 20 min, and then treated with PDGF-BB (5 ng/ml) for the indicated time. Cell lysate supernatants (20 µg/each) were analyzed by immunoblotting with antibodies to phosphotyrosine, phospho-STAT3, phospho-Akt, phospho-Erk1/2, PDGFRβ, total STAT3, total Akt, and total Erk1/2. (B) Serum-starved A10 cells were pre-treated with indicated concentrations of BMS-354825 and then stimulated with PDGF (5 ng/ml, 5 min). PDGFβ was immunoprecipitated from cell lysates. The immunoprecipitates were analyzed by antibodies to phosphotyrosine (pTyr) and PDGFβ (inset). The relative pTyr signal intensities were compared. The graph represents data from three experiments, one of which was performed in duplicate (n = 4). Each data point in the graph represents mean ± SEM (this applies to all subsequent graphs). (C) BMS-354825 inhibited PDGFR tyrosine kinase activity in vitro. PDGFR was immunoprecipitated from A10 cells stimulated with PDGF-BB (5 ng/ml, 5 min). A portion of each immunoprecipitate (5/7) was used for in vitro kinase assay in the presence of BMS-354825 at the indicated concentrations. The remaining immunoprecipitates were used for immunoblotting analysis with anti-PDGFRβ antibody (inset). The graph is derived from three experiments.

Fig. 2. Comparison of the inhibitory effects of BMS-354825 and imatinib in A10 cells. (A) A10 cells were serum-starved, pre-incubated with indicated concentrations of BMS-354825 or imatinib for 20 min, and then stimulated with PDGF-BB (5 ng/ml, 5 min). Cell lysate

supernatants were analyzed by immunoblotting similar to that described in Fig. 1A legend. (B) Quantification of PDGFR pTyr signal intensities. Data were from two experiments.

Fig. 3. Inhibitory effects of BMS-354825 in primary culture of human AoSMCs. Human AoSMCs were deprived from serum for 18 h, pre-incubated with indicated concentrations of BMS-354825 for 20 min and then stimulated with PDGF-BB (5 ng/ml, 5 min). Cell lysate supernatants were analyzed for activation of PDGFR, STAT3, Akt, and Erk2 by immunoblotting.

Fig. 4. Inhibition of Src tyrosine kinase by BMS-354825. (A) c-Src was immunoprecipitated from serum-starved A10 cells (0) or A10 cells stimulated with PDGF (5 ng/ml) for the indicated time in the presence or absence of 50 nM BMS-354825. Src kinase activity was determined by autophosphorylation and phosphorylation of GST-Gab1CT in the immune complex kinase assay (Ren et al., 2004). After autoradiography, the filter was used for immunoblotting analysis with an anti-Src antibody (lower panel). (B) c-Src was immunoprecipitated from serum-starved A10 cells treated with the indicated concentrations of BMS-354825 for 20 min and Src kinase activity was determined. (C) Quantification of radioactive signal from c-Src autophosphorylation and phosphorylation of GST-Gab1CT. The data were from two experiments performed in duplicate (n = 4).

Fig. 5. IGF-1R and EGFR are insensitive to BMS-354825. A10 cells were serum-starved, pretreated with indicated concentrations of BMS-354825 (20 min) and then stimulated with IGF-1 (10 ng/ml, 10 min) or EGF (10 ng/ml, 10 min). IGF-1R or EGFR was immunoprecipitated from cell lysate supernatants and analyzed by antibodies to pTyr and IGF-1R (A) or pTyr and EGFR (B).

Fig. 6. Effect of BMS-354825 on PDGF-stimulated cell migration. Transwell cell migration assay was performed using PDGF-BB (5 ng/ml) as the chemoattractant in the presence or

absence of BMS-354825 as indicated. (A) For A10 cells, 1×10^4 cells were loaded into the upper chamber of each well. Each field was 0.8×0.6 mm. The data were from 2 (lanes 2, 3, 4) to 4 experiments performed in duplicate. (B) Representative views of migrated cells from each sample. Different microscopes were used to acquire data for (A) and (B). Each area in these photos is 1.0×0.8 mm. (C) For human AoSMCs, 0.5×10^4 cells per well were used. The results were from two experiments performed in duplicate.

Fig. 7. Inhibition of PDGF-stimulated cell proliferation and cell cycle progression by BMS-354825. (A) and (B) A10 cells (2×10^4 cells/well) or human AoSMCs (3.5×10^4 cells/well) were cultured in DMEM/1% FBS plus indicated PDGF-BB and BMS-354825 for 6 days and viable cell number were determined. Data represent two triplicate experiments. (C) A10 cells were incubated with or without PDGF-BB (10 ng/ml) and indicated concentrations of BMS-354825 for 16 h and processed for propidium iodide staining and flow cytometric analysis. The data represent two experiments performed in duplicate. (D) A10 cells (2×10^4 cells/well) were cultured in DMEM/1% FBS plus PDGF-BB in the presence of indicated concentrations of BMS-354825 for 6 days. After which, cells were washed and cultured in DMEM/1% FBS plus PDGF-BB with or without BMS-354825 for another 6 days. Viable cell number was determined on Day 12. The data were from three experiments performed in duplicate.

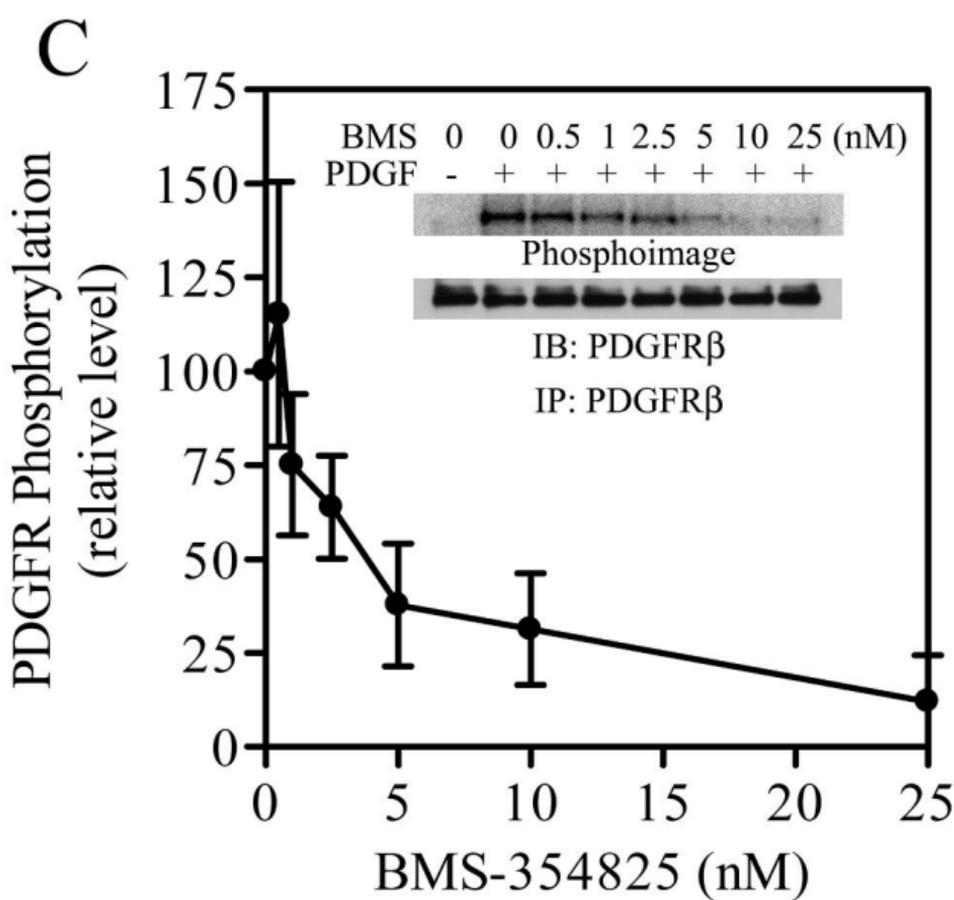
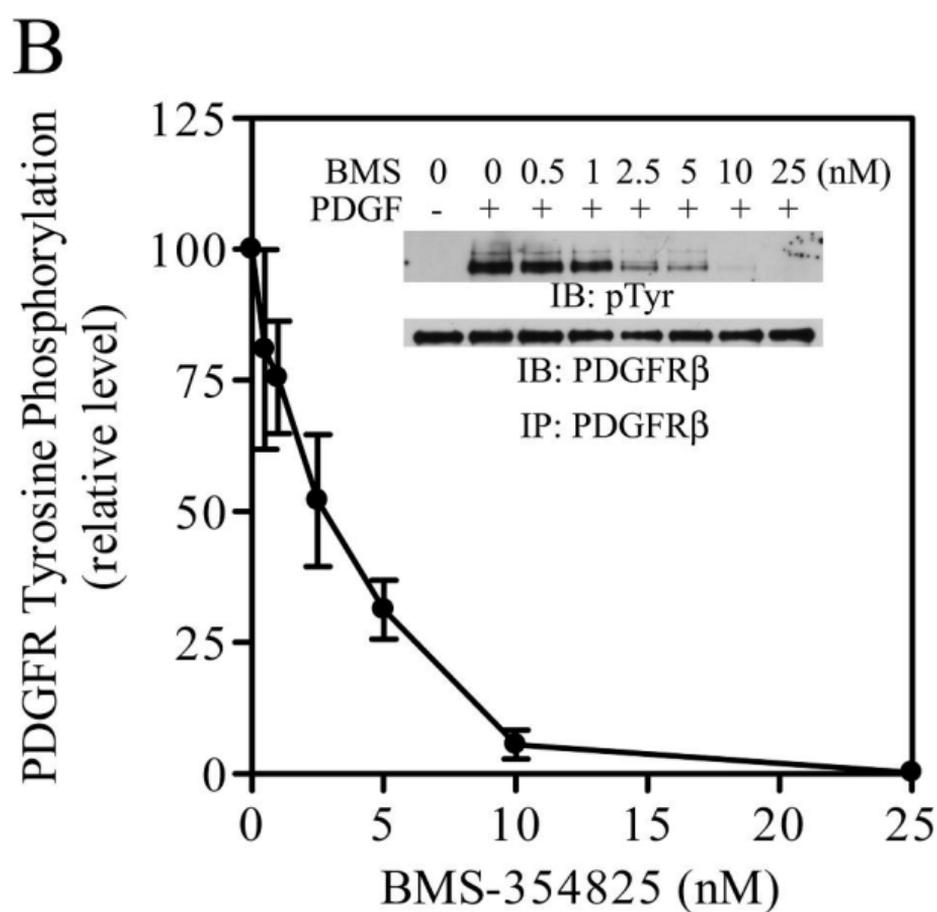
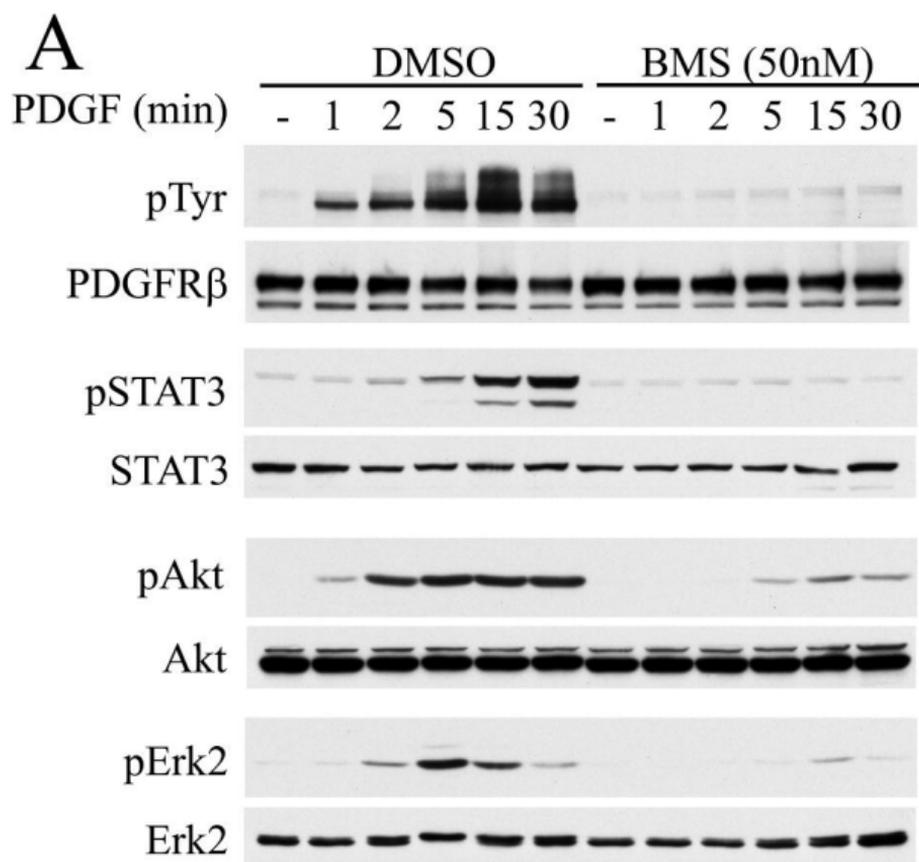


Fig. 1

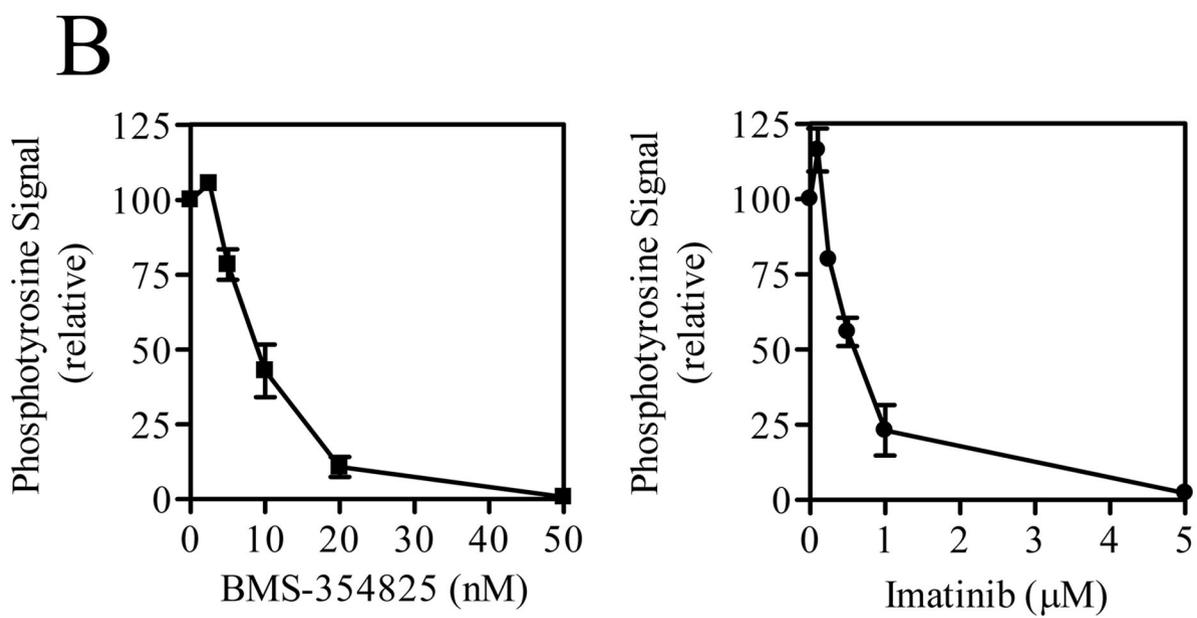
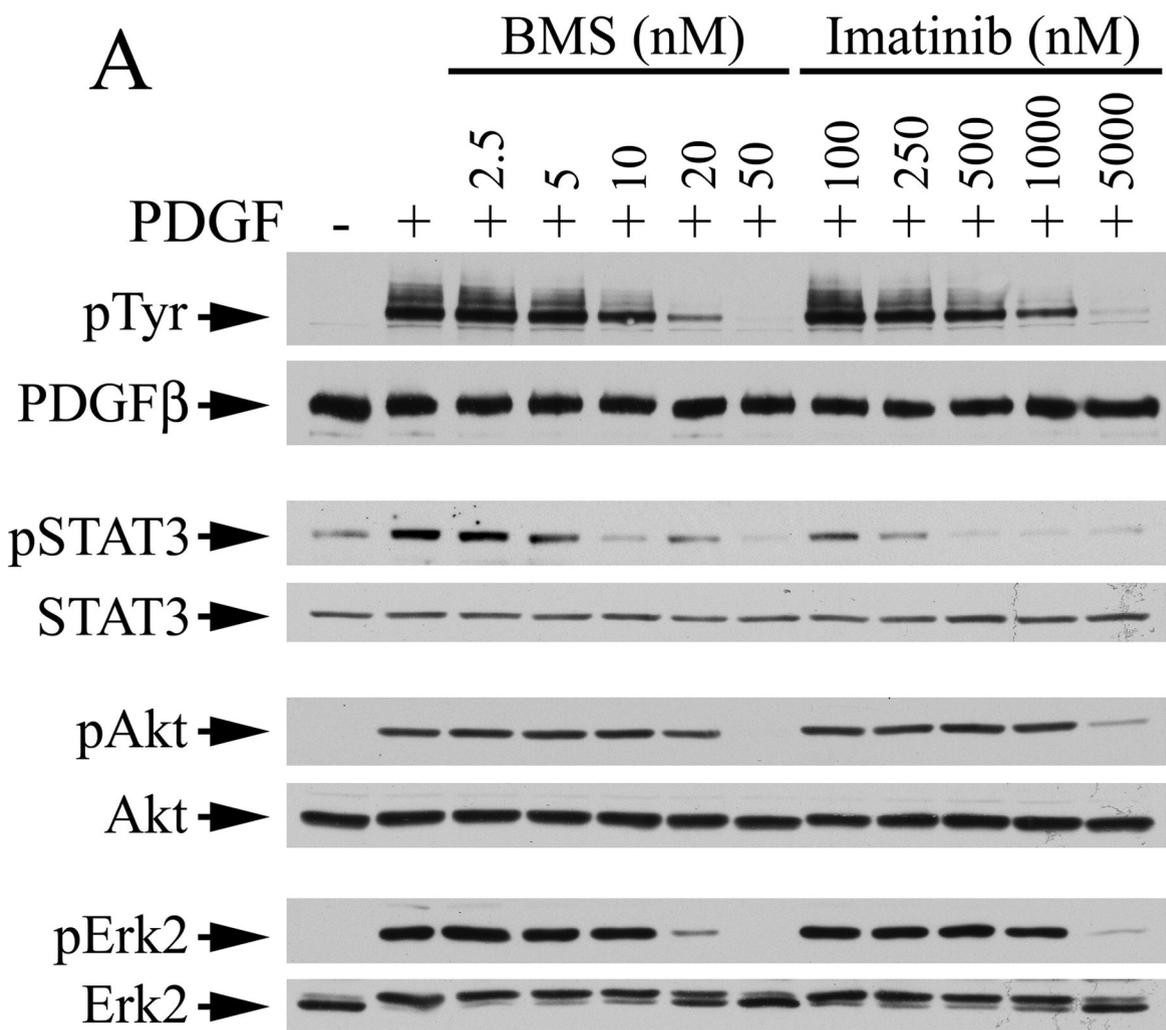


Fig. 2

BMS (nM)	-	-	10	20	50
PDGF	-	+	+	+	+

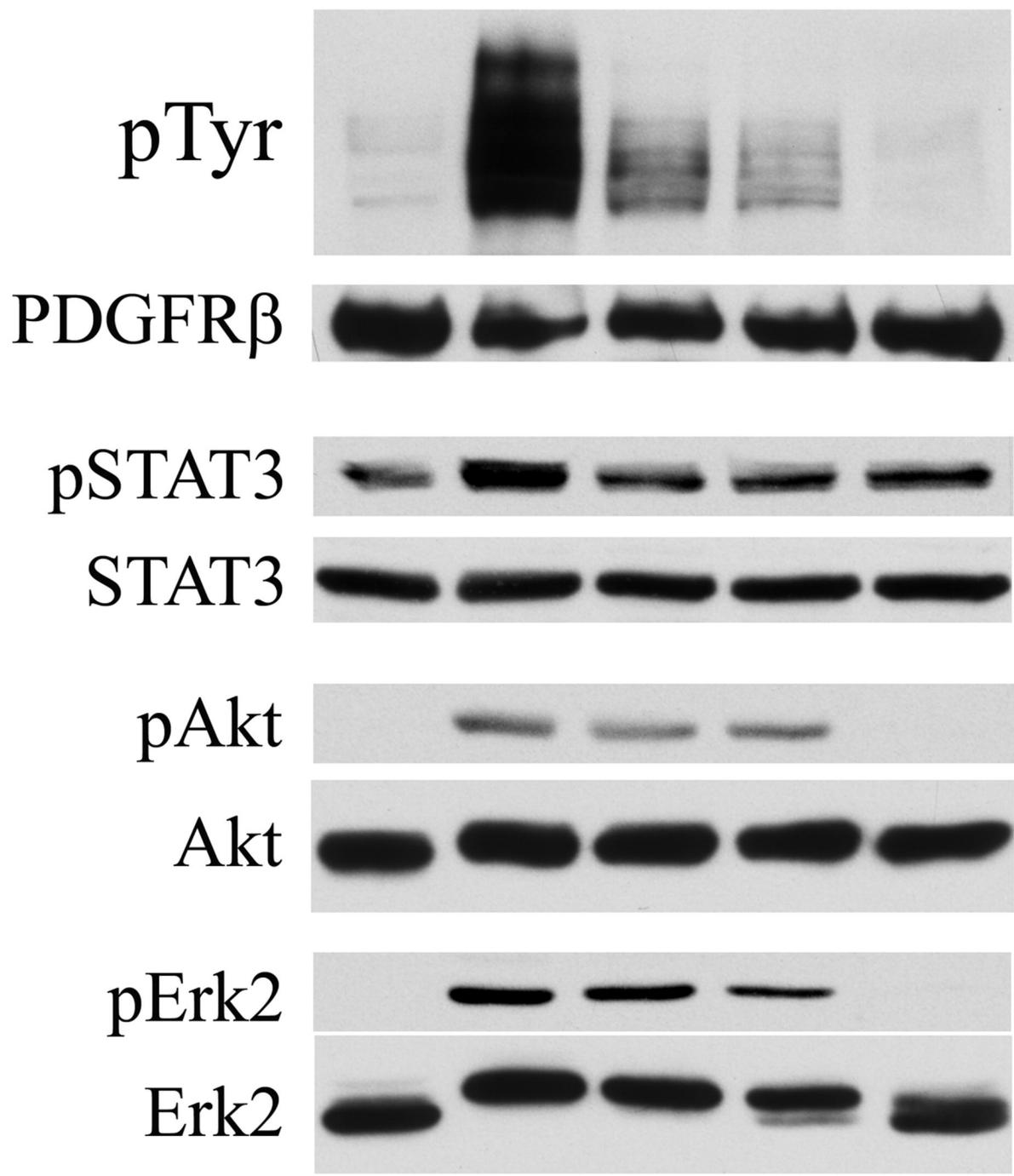


Fig. 3

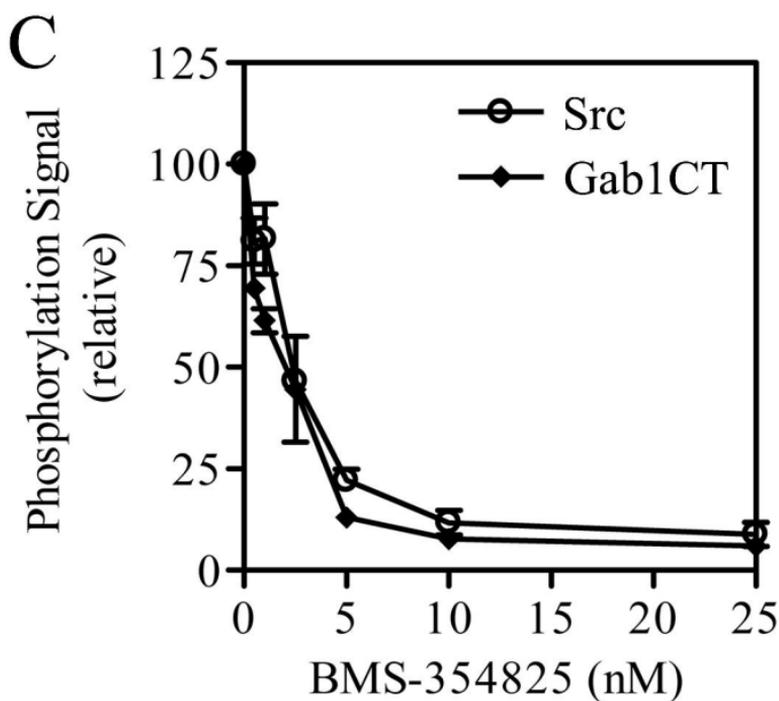
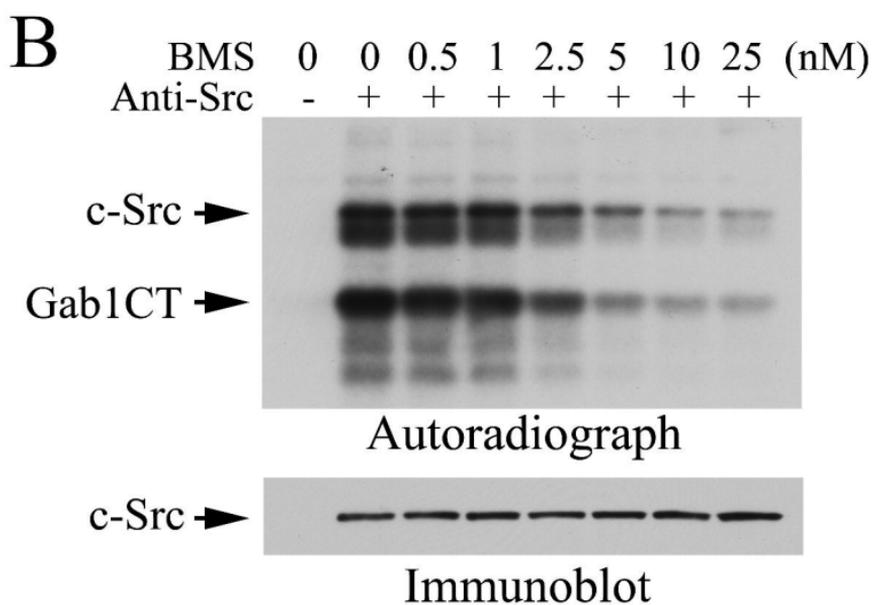
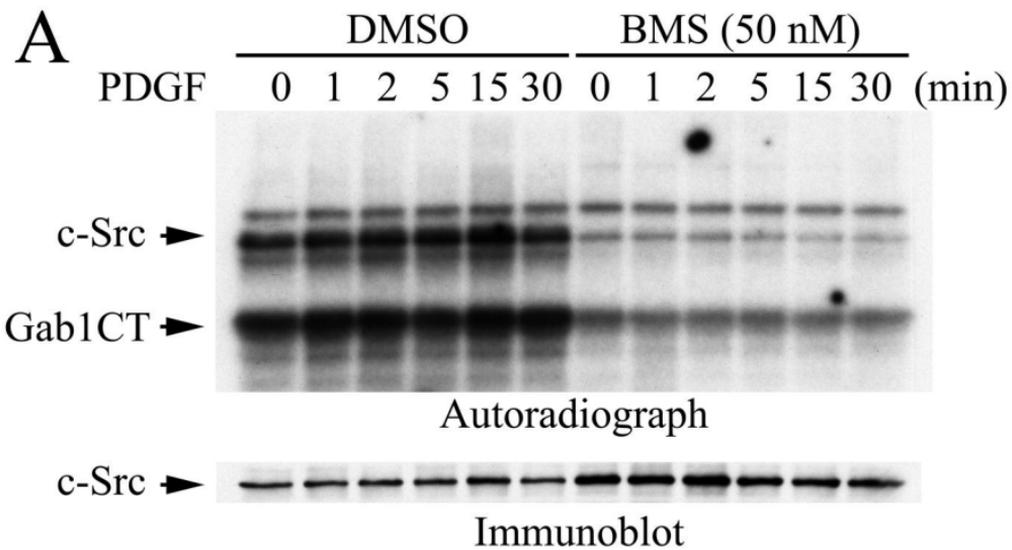


Fig. 4

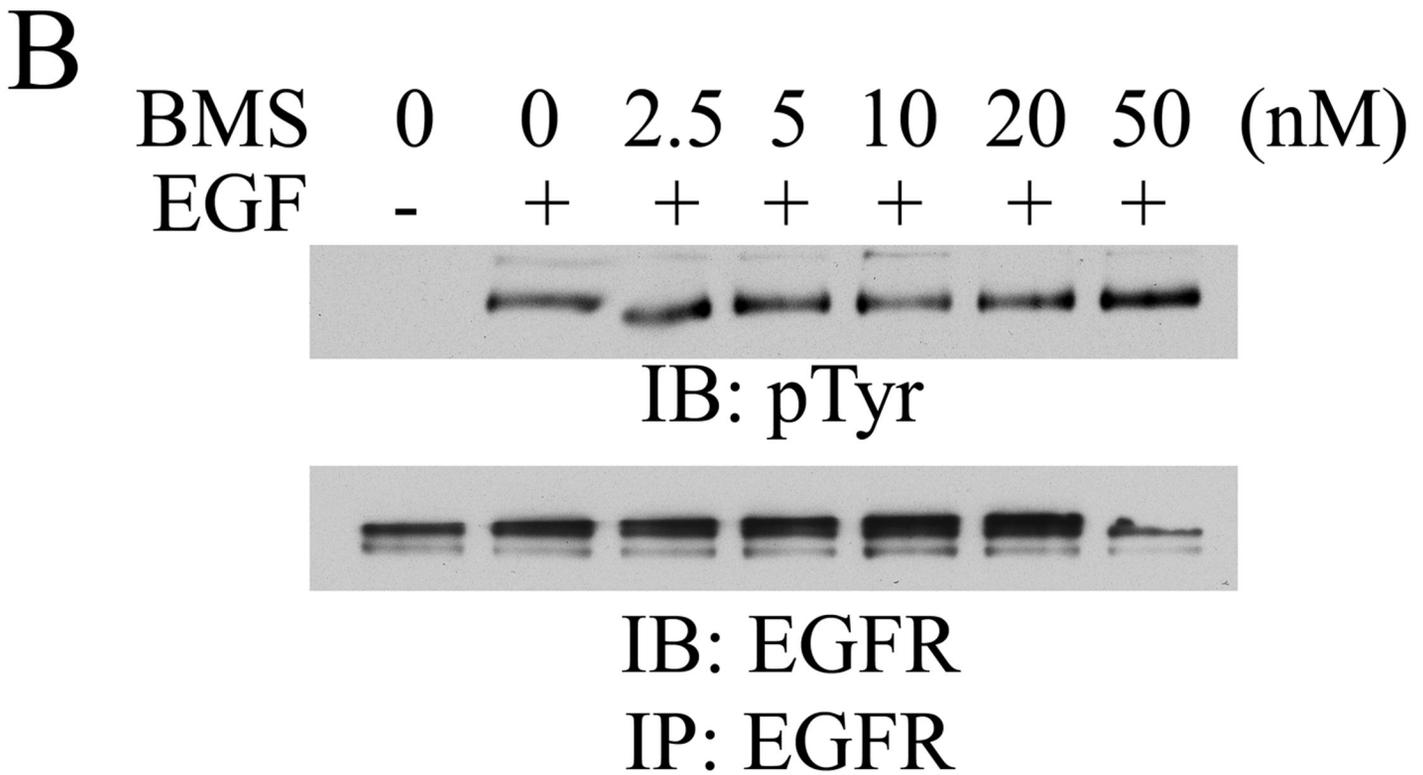
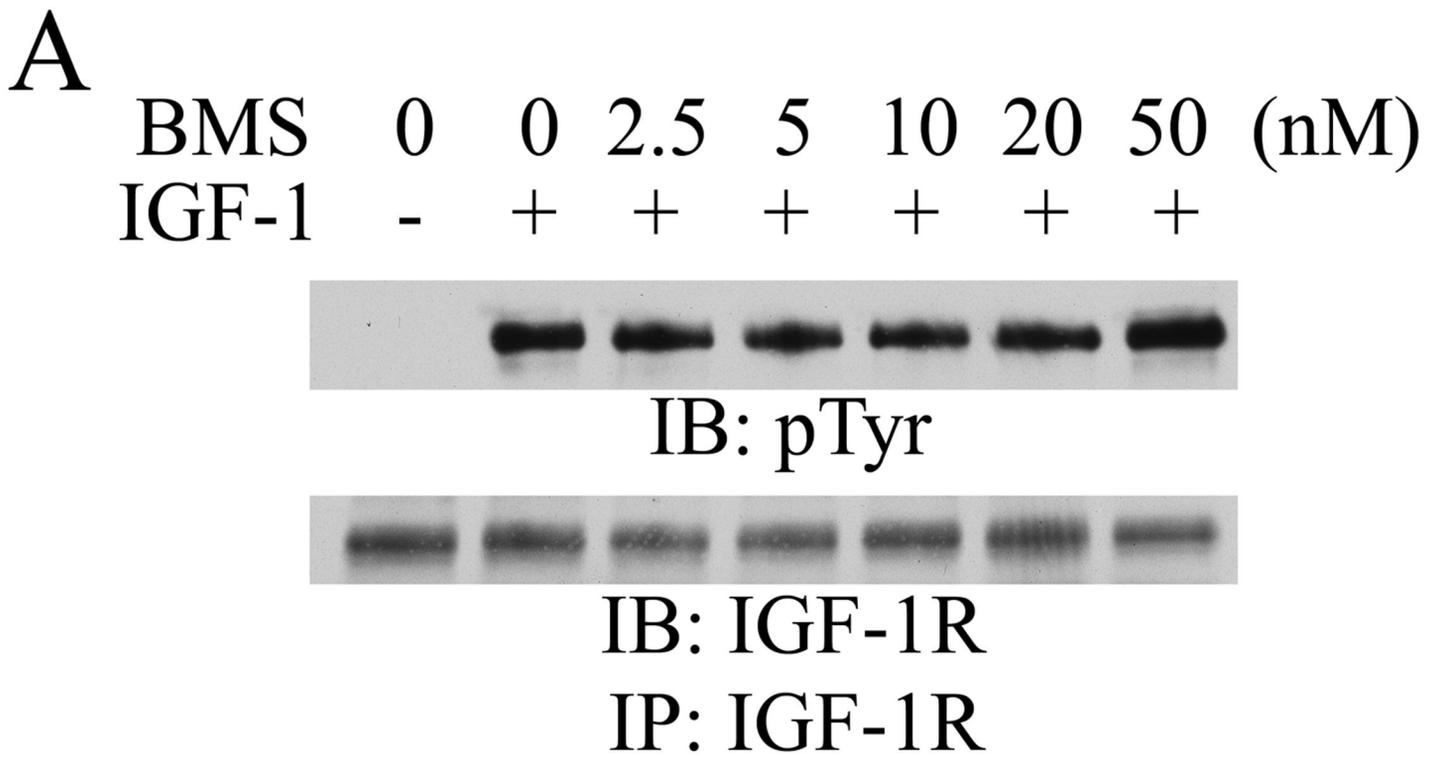
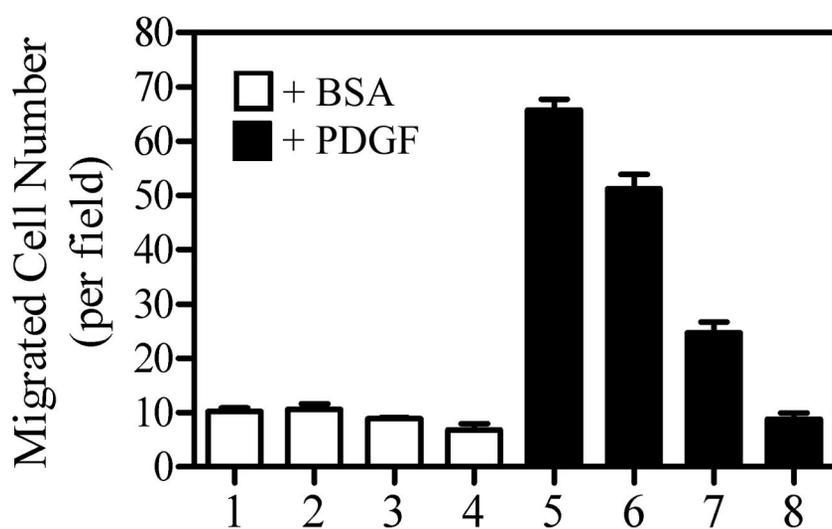


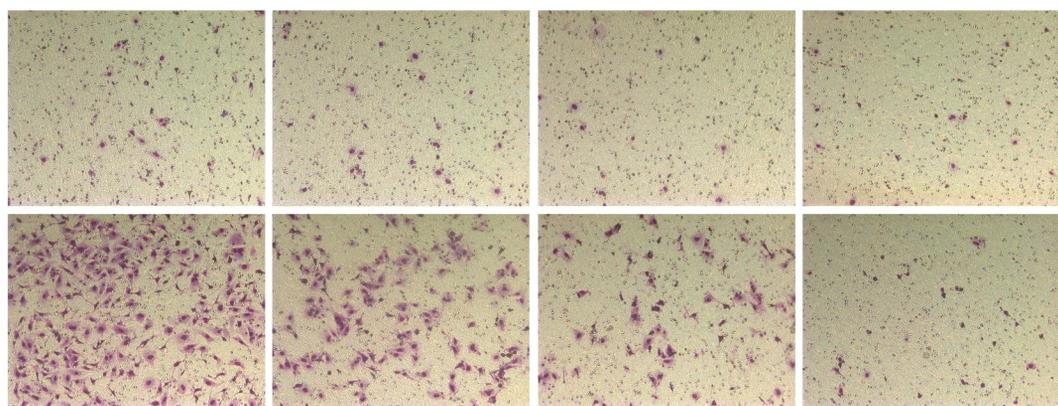
Fig. 5

A**B**

BMS-354825 0 10 20 50 0 10 20 50 (nM)

BSA

PDGF



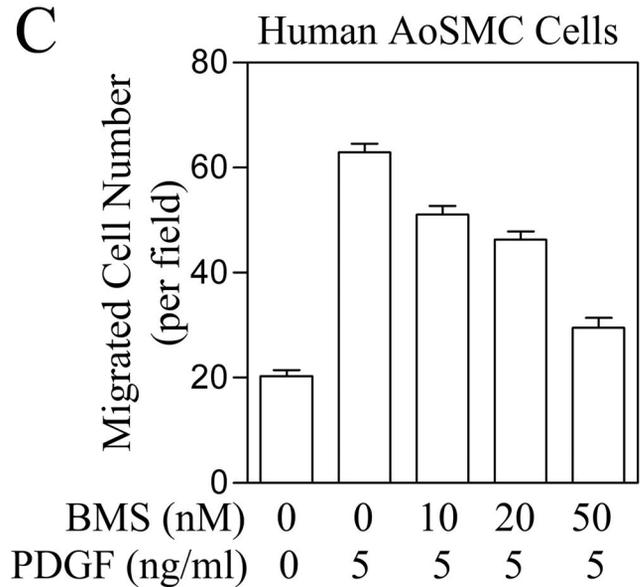
BMS-354825

0

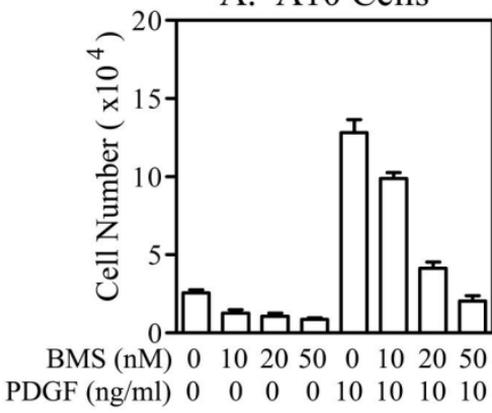
10

20

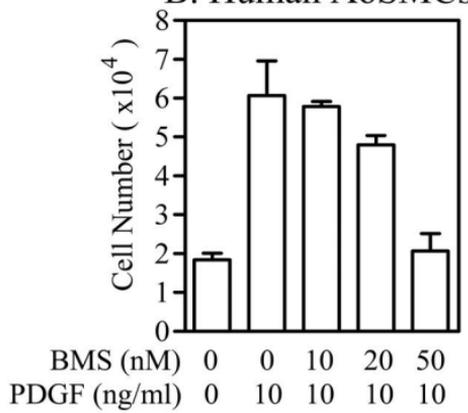
50 (nM)

C**Fig. 6**

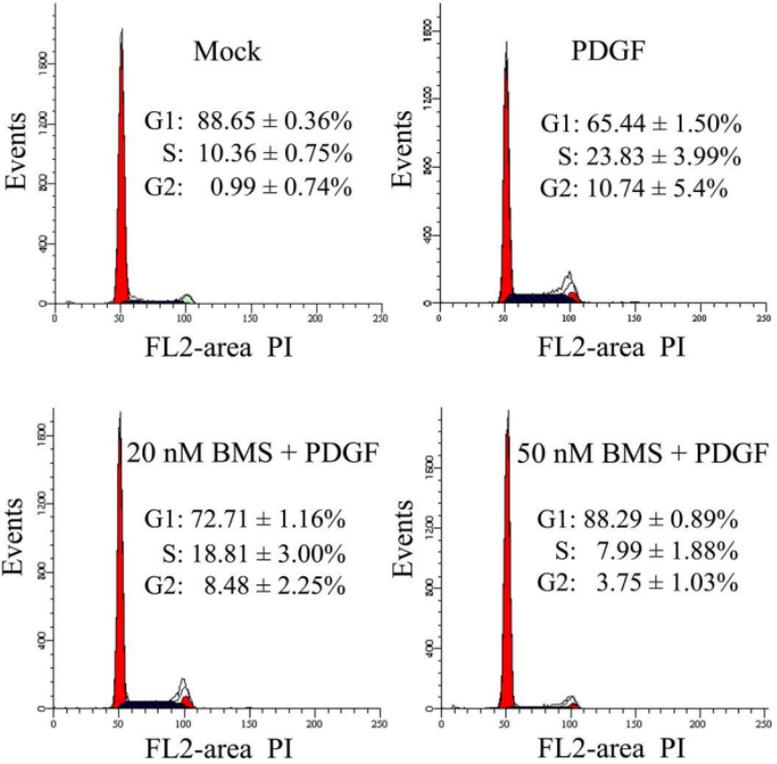
A. A10 Cells



B. Human AoSMCs



C. A10 Cells



D. A10 Cells

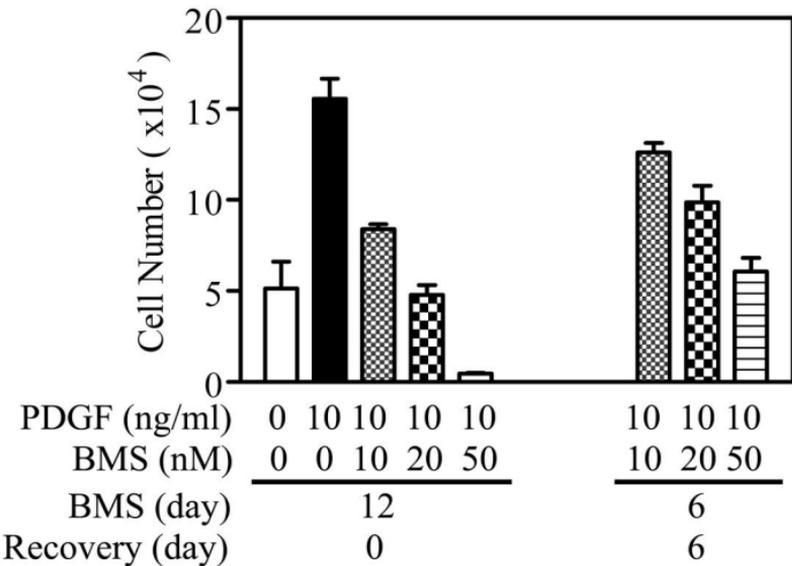


Fig. 7