

# Selective Platelet-derived Growth Factor Receptor Kinase Blockers Reverse *sis*-Transformation<sup>1</sup>

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## ABSTRACT

A novel class of tyrosine kinase blockers represented by the tyrphostins AG1295 and AG1296 is described. These compounds inhibit selectively the platelet-derived growth factor (PDGF) receptor kinase and the PDGF-dependent DNA synthesis in Swiss 3T3 cells and in porcine aorta endothelial cells with 50% inhibitory concentrations below 5 and 1  $\mu$ M, respectively. The PDGF receptor blockers have no effect on epidermal growth factor receptor autophosphorylation; weak effects on DNA synthesis stimulated by insulin, by epidermal growth factor, or by a combination of both; and over an order of magnitude weaker blocking effect on fibroblast growth factor-dependent DNA synthesis. AG1296 potently inhibits signaling of human PDGF  $\alpha$ - and  $\beta$ -receptors as well as of the related stem cell factor receptor (c-Kit) but has no effect on autophosphorylation of the vascular endothelial growth factor receptor KDR or on DNA synthesis induced by vascular endothelial growth factor in porcine aortic endothelial cells. Treatment by AG1296 reverses the transformed phenotype of *sis*-transfected NIH 3T3 cells but has no effect on *src*-transformed NIH 3T3 cells or on the activity of the kinase p60<sup>c-src(F527)</sup> immunoprecipitated from these cells. These potent and selective compounds represent leads for the development of novel agents to combat tumors driven by PDGF or to inhibit PDGF action in other diseases in which PDGF plays a key role, such as restenosis.

## INTRODUCTION

PDGF<sup>5</sup> is a potent mitogen for mesenchymal, glial, and capillary endothelial cells (see Refs. 1 and 2 for reviews). The three isoforms of PDGF, PDGF-AA, PDGF-AB, and PDGF-BB, interact differentially with structurally related receptors designated  $\alpha$ - and  $\beta$ -receptors; each of the receptors has an extracellular part with 5 immunoglobulin-like domains and an intracellular part with a tyrosine kinase domain containing a characteristic insert sequence (3–5). The tyrosine kinase activity of the receptors is essential for transmission of the mitogenic signal into the cell (6). PDGF and its receptors participate in various physiological processes such as embryonal development and wound healing. An abnormally high activity of PDGF is believed to play a central role in the etiology of certain adverse pathophysiological situations, such as atherosclerosis and restenosis (7, 8), as well as in other nonmalignant diseases such as pulmonary fibrosis (9), glomerular nephritis (10), and rheumatoid arthritis (11). Moreover, the PDGF

B-chain was acquired as the *sis* oncogene by the acutely transforming simian sarcoma virus (12, 13). The expression of a PDGF-like growth factor in cells infected with simian sarcoma virus or transfected with the *sis* oncogene leads to their transformation due to the persistent autocrine stimulation of the resident PDGF receptors. Furthermore, certain human tumors possess PDGF receptors and express the genes for PDGF which suggests that autocrine growth stimulation via PDGF receptors contributes to the malignant phenotype of these tumors (2, 14). The fact that PDGF is likely to be involved in the development of certain disorders has prompted the search for agents to block the action of PDGF. The approaches for interference with PDGF-induced signaling include peptides competing with PDGF for receptor binding (15), dominant negative mutants of PDGF (16, 17) or of PDGF receptor (18), and low molecular weight blockers of the receptor tyrosine kinase activity known as tyrphostins (19). Certain tyrphostins which block PDGF-dependent proliferation of rabbit vascular smooth muscle cells (20) and of human bone marrow fibroblasts (21) have already been reported. In this study we describe a novel class of potent tyrphostins which possess the quinoxaline moiety, which show high selectivity towards the PDGF receptor kinase, and which reverse the transformation of fibroblasts transformed with the oncogene *sis*. These compounds are new leads for drugs which could potentially combat restenosis, atherosclerosis, and tumors in which PDGF plays a prominent role.

## MATERIALS AND METHODS

**Synthesis.** The synthetic methods and analytical data of the used compounds will be described elsewhere. The formulas of the tyrphostins used in this study are shown in Fig. 1.

**Cells and Reagents.** Swiss 3T3 cells (obtained from E. Rozengurt, London, United Kingdom) and NIH 3T3 cells, stably transfected with a constitutively active mutant (F527) of chicken *c-src* gene (generously provided by S. Courtneidge, Heidelberg, Germany), were grown in DMEM supplemented with 4 g/liter glucose, glutamic acid, antibiotics, and 10% FCS. *sis*-transfected NIH 3T3 fibroblasts (kindly provided by Dr. S. Aaronson, Bethesda, MD) were cultivated in the same medium in the presence of 0.4 mg/ml geneticin. Porcine aortic endothelial cells stably expressing human PDGF  $\beta$ -receptors (22),  $\alpha$ -receptors (23), Kit/SCF receptor (24), or KDR (25) have been described previously and were cultured in Ham's F-12 medium supplemented with geneticin (0.4 mg/ml) and 10% FCS. All cell culture reagents were from Gibco. PDGF was the recombinant human BB homodimer. Murine EGF, human VEGF, and recombinant human basic FGF were kindly provided by E. Spitzer (Berlin, Germany), D. Gospodarowicz (San Francisco, CA), and B. Stoyanov (Sofia, Bulgaria), respectively. The anti-PDGF receptor antiserum DIG1 was raised against a peptide corresponding to amino acid residues 1075–1089 in the human PDGF  $\alpha$ -receptor but recognized PDGF  $\alpha$ - and  $\beta$ -receptors equally well.<sup>6</sup> The antiserum PDGF-R3 against PDGF receptor (5) and antisera against FGF receptor 1 (26) and KDR (25) have been described. [methyl-<sup>3</sup>H]Thymidine and [ $\gamma$ -<sup>32</sup>P]ATP were purchased from DuPont/NEN (Dreieich, Germany).

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<sup>5</sup> The abbreviations used are: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PAE, porcine aortic endothelial cells; SCF, stem cell factor; TCA, trichloroacetic acid; IC<sub>50</sub>, 50% inhibitory concentration.

<sup>6</sup> L. J. Gonez, unpublished data.

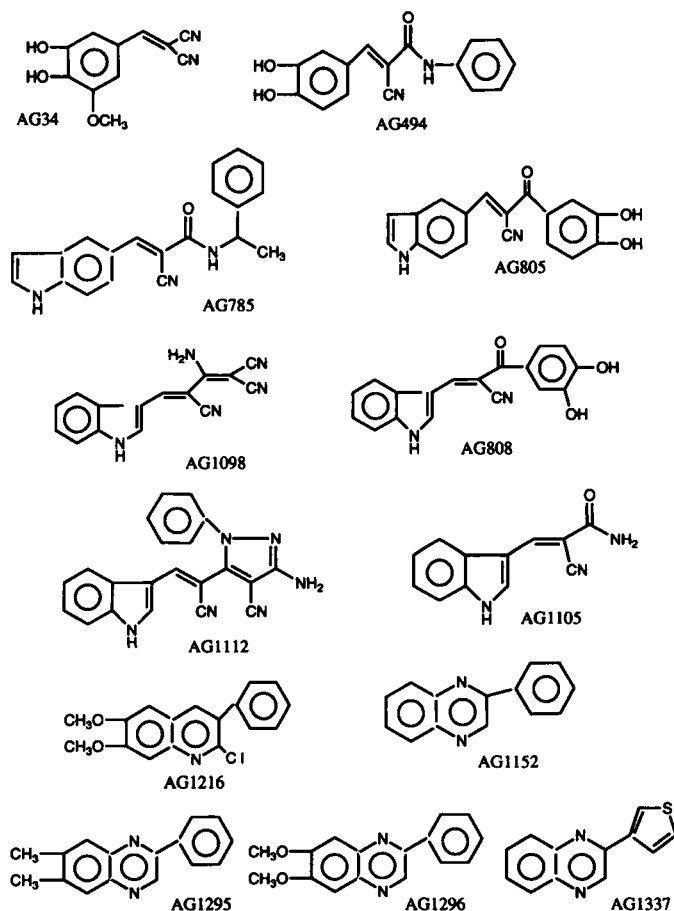


Fig. 1. Structure of tyrphostins used in the study.

**Membrane Autophosphorylation Assays.** Membranes were prepared from confluent cultures of Swiss 3T3 cells as described (27). For measuring receptor autophosphorylation, 10  $\mu$ g membrane protein per assay were incubated for 20 min on ice in the presence of 1.2  $\mu$ g/ml EGF or 2  $\mu$ g/ml PDGF, or both; 50 mM Hepes (pH 7.5); and 3 mM  $MnCl_2$  (final concentrations) in a volume of 45  $\mu$ l. In order to test the effects of tyrphostins, these were added in a volume of 0.5  $\mu$ l (in DMSO; final concentration, 0.5%) 15 min before addition of the growth factors. Phosphorylation was initiated by addition of [ $\gamma$ - $^{32}P$ ]ATP (5  $\mu$ l, 3–5  $\mu$ Ci; final concentration, 2  $\mu$ M) and terminated after 2 min by addition of 10  $\mu$ l of a solution containing 6% SDS, 30%  $\beta$ -mercap-

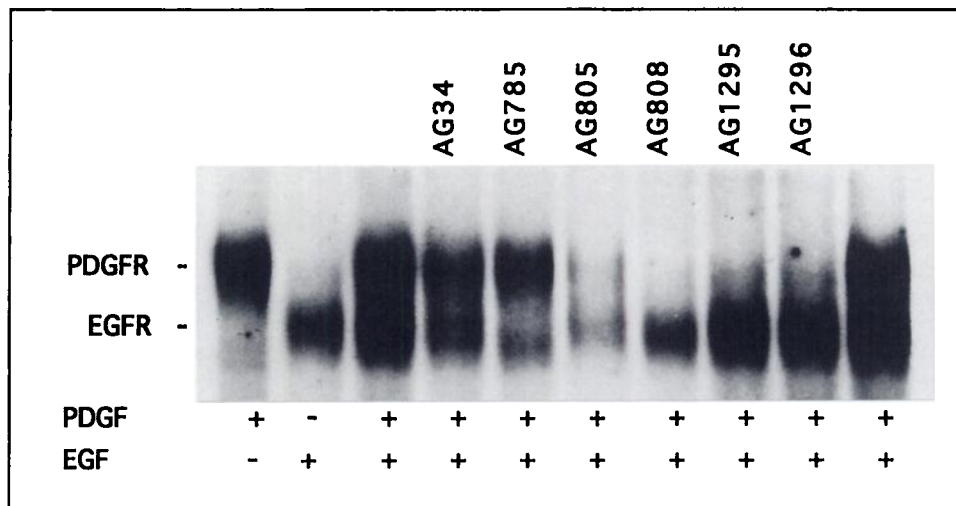
toethanol, 40% glycerol, and 0.5 mg/ml bromophenol blue. The samples were heated for 5 min at 95°C and subjected to SDS-PAGE according to the method of Laemmli (28) using 10% acrylamide gels. The gels were stained and dried and subjected to autoradiographic analysis.

For quantification of radioactivity in electrophoresis gels, a PhosphorImager (Molecular Dynamics, Fuji, or Bio-Rad) was used according to the instructions of the manufacturers. To obtain autoradiograms, objects were exposed to X-ray film (Fuji RX or Kodak X-OMAT) with intensifying screens at -70°C.

**Assay of Receptor Autophosphorylation in Intact Cells.** Confluent Swiss 3T3 cells in 24-well plates (Nunc) were incubated for 20–24 h in serum-free DMEM. Subsequently, tyrphostins were added at concentrations ranging from 0 to 100  $\mu$ M (final DMSO concentration, 0.5%) and the incubation was continued for 6–8 h. The cells were then stimulated with 100 ng/ml PDGF-BB for 5 min at room temperature or 600 ng/ml EGF for 2.5 min on ice. The growth factor treatment was terminated by washing twice with ice-cold PBS and the cells were scraped off the wells in 60  $\mu$ l lysis buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate, 50 mM NaF, 2 mM sodium *o*-vanadate, 20  $\mu$ M zinc acetate, 10 mM EDTA, 2 mM [ethylenebis(oxyethylenitrilo)]tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 5  $\mu$ g/ml leupeptin. *sis*-transfected cells were grown to near confluency and treated with the tyrphostins in the presence of DMEM/10% FCS for 48 h. Cell extracts were obtained as above.

The cell lysates were clarified by centrifugation (cooled microfuge, 17,000 rpm, 15 min) and analyzed by SDS-PAGE (6.5% gels) and immunoblotting with anti-phosphotyrosine antibodies (either PY 20, ICN and subsequently a peroxidase-coupled secondary antibody, or RC20-peroxidase conjugate; Affinit, Nottingham, United Kingdom). The blots were developed with a chemiluminescence detection system (Western Light, Tropix, or ECL; Amersham). In some experiments PDGF receptors were immunoprecipitated with PDGF-R3 or DIG1 antibodies as described (23) prior to the analysis by immunoblotting with anti-phosphotyrosine antibodies. To measure autophosphorylation of PDGF  $\alpha$  or PDGF  $\beta$ -receptors expressed in PAE cells, subconfluent cultures were treated with serum-free medium in the presence of various concentrations of the inhibitors or DMSO for 1 hour at 37°C. Then, PDGF-AA or PDGF-BB were added at 50 ng/ml and the incubation was continued for 5 min. The cultures were placed on ice and the cells were lysed with ice-cold lysis buffer containing 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.15 M NaCl, 20 mM Tris/HCl (pH 7.4), 5 mM EDTA, 30 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, and 200  $\mu$ M sodium *o*-vanadate. The lysates were then incubated for 1 h at 4°C with wheat germ agglutinin-Sepharose beads (29). The beads were washed 3 times with the lysis buffer and once with H<sub>2</sub>O and were then eluted with SDS-PAGE sample buffer. Then, receptor phosphorylation was analyzed by SDS-PAGE (7.5% gels) and immunoblotting with anti-phosphotyrosine antibodies.

Fig. 2. Selective effects of tyrphostins on the autophosphorylation of PDGF receptor (PDGFR) versus EGF receptor (EGFR) in Swiss 3T3 cell membranes. Membrane samples were preincubated with tyrphostins for 15 min on ice. Then, PDGF and/or EGF at saturating concentrations were added for 20 min and receptor autophosphorylation was allowed to proceed for 2 min in the presence of 2  $\mu$ M [ $\gamma$ - $^{32}P$ ]ATP as described in "Materials and Methods." The reaction was quenched with SDS-PAGE sample buffer and the samples were subjected to electrophoresis in 10% acrylamide gels and autoradiography. Only the portion of the gel containing the receptor bands is shown; the localization of the PDGF receptors and EGF receptors is indicated.



To measure autophosphorylation of KDR expressed in PAE cells, subconfluent cultures were treated with Ham's F-12 medium containing 0.5% FCS for 16 h in the presence of the inhibitors or DMSO. Then, the cells were treated with 50 ng/ml VEGF for 8 min at 37°C and subsequently extracted as described above. The receptors were immunoprecipitated with a KDR-specific antiserum (25) and the immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies.

**Src Activity Assay.** *src*-transformed NIH 3T3 cells were grown in 24-well plates to confluency; rinsed twice with a solution containing 20 mM Tris, 0.1 mM sodium *o*-vanadate, and 150 mM NaCl (pH 7.5); and lysed in 100 µl/well of lysis buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 2.5 mM EDTA, 10 mM NaF, 1% Trasylol, and 20 µM leupeptin. The lysate was clarified by centrifugation and subjected to immunoprecipitation with the anti-Src monoclonal antibody 327 (Oncogene Science; 0.5 µg antibody/80 µg protein) for 1 h at 4°C. Then, goat anti-mouse IgG (Sigma; 0.2 µg/0.5 µg monoclonal antibody 327) was added and incubation was continued for 30 min followed by another 40 min of incubation with 10 µl of protein A-Sepharose Cl-4B (Pharmacia). The immunoprecipitates were washed 5 times with the lysis buffer and twice with kinase buffer containing 40 mM Hepes (pH 7.4), 0.5 mM dithiothreitol, 5 mM MnCl<sub>2</sub>, and 0.1 mM sodium *o*-vanadate. The immunoprecipitates were suspended in kinase buffer and aliquots corresponding to 40 µg of cell lysate protein were preincubated with or without AG1296 for 15 min at 30°C. Then, the kinase reaction was performed in the presence of 2 µg of acid-treated enolase (30) and 10 µCi of [ $\gamma$ -<sup>32</sup>P]ATP (2 µM) in a final volume of 30 µl. The reaction was terminated by addition of SDS-PAGE sample buffer and the incorporation of radioactivity in the enolase was analyzed by SDS-PAGE and autoradiography as well as PhosphorImager quantification.

**Assay of DNA Synthesis.** Swiss 3T3 cells were seeded into 24-well plates (Nunc, 1–2 × 10<sup>4</sup> cells/well) and cultured for 3 days in DMEM supplemented with 10% FCS. Thereafter, the medium was changed to serum-free DMEM for 16–18 h. Then, tyrosinostins were added at the indicated concentrations for 6–8 h (final DMSO concentration, 0.5%). Growth factors (EGF, PDGF, or basic FGF at 10 ng/ml; insulin at 1 µg/ml) and [*methyl*-<sup>3</sup>H]thymidine (final concentration, 1 µCi/ml, 2 µM) were added together and cultivation was continued for 20–24 h. Then, the cells were washed twice with PBS, treated for 10 min with 5% TCA, washed once with TCA, twice with PBS, and twice with methanol (all at 4°C) and dried. 0.5 ml NaOH (0.1 N) containing 1% SDS was added per well, the plates were incubated for 30 min at 37°C, and 0.2-ml aliquots were taken for measuring radioactivity.

To test the reversibility of the inhibitory effect of tyrosinostins, the cells were washed once for 30 min with serum-free DMEM after 6–8 h of preincubation with the inhibitors. Then, growth factors and [<sup>3</sup>H]thymidine were added and the procedure was continued as described above.

DNA synthesis in PAE cells expressing PDGF receptors or Kit/SCF receptor was measured as described (23, 24). Briefly, the cells were serum deprived for 48 h. Then, the medium was changed to serum-free medium containing

Table 1 Structure of tyrosinostin derivatives and effects on PDGF receptor function in Swiss 3T3 cells

Compound AG no.	IC <sub>50</sub> (µM) for inhibition of PDGF-stimulated			Selectivity for inhibition of PDGF-stimulated DNA synthesis
	PDGF receptor autophosphorylation		DNA synthesis	
	<i>In vitro</i>	In cells		
AG34	>30	ND <sup>a</sup>	ND	None
AG494	ca.10	ND <sup>b</sup>	ND	ND
AG805	ca.10	ND <sup>b</sup>	ND	ND
AG808	ca.10	>100	10–30	None
AG1098	ca.10	>100	10–30	None
AG1105	3–10	>100	30–100	None
AG1112	ca.10	ND <sup>b</sup>	ND	ND
AG1152	3–10	ca.25	10–30	Partial
AG1216	1–3	10–50	10–30	None
AG1295	0.3–0.5	0.5–1	2.5	Yes
AG1296	0.3–0.5	0.3–0.5	1.5	Yes
AG1337	ca.1	ND	≥10	ND

<sup>a</sup> ND, not determined.

<sup>b</sup> These compounds were not selective in primary screening and were hence not further investigated.

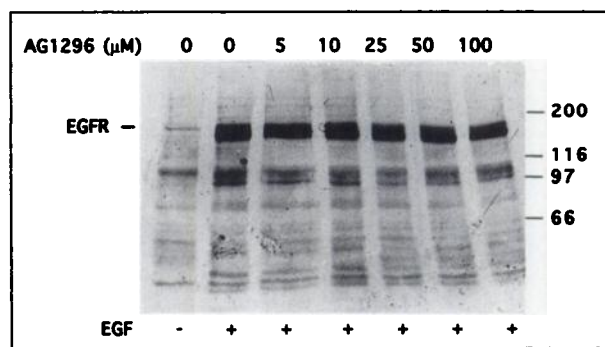
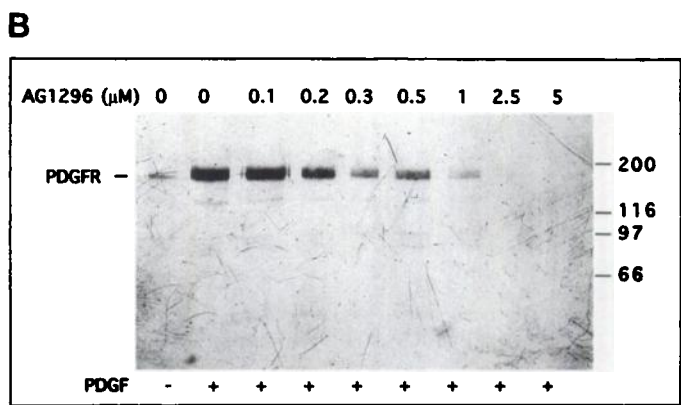
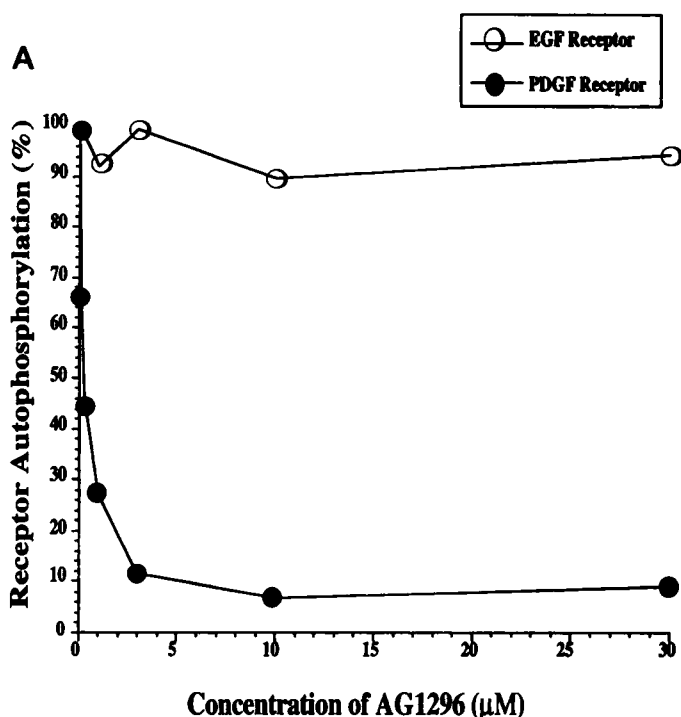


Fig. 3. Concentration dependence of the inhibition of PDGF receptor and EGF receptor autophosphorylation in Swiss 3T3 cells by AG1296. A, membranes. The assay was performed as described in the legend to Fig. 1, except that phosphorylation was measured in the presence of only one ligand at a time. The radioactivity in the receptor bands was quantitated using the PhosphorImager. B, intact cells. Swiss 3T3 cells were cultured in 24-well plates to confluency and made quiescent by treatment with serum-free medium. The cells were incubated with AG1296 at the indicated concentrations for 6–8 h. Cells were then briefly stimulated with PDGF (100 ng/ml, 5 min at room temperature) or EGF (600 ng/ml, 2.5 min on ice) and extracted, and the extracts were subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies. The localization of the PDGF receptors (PDGFR) and EGF receptors (EGFR) is indicated.

either 5 ng/ml PDGF-AA or PDGF-BB or 10 ng/ml SCF, respectively, [<sup>3</sup>H]thymidine, and the inhibitors or DMSO. Incorporated [<sup>3</sup>H]thymidine was measured after 24 h. KDR expressing PAE cells were subjected to serum deprivation for 64 h. After 40 h of serum deprivation tyrphostins were added and during the last 20 h VEGF (2.5 ng/ml) was present. Two h prior to precipitation with ice-cold 10% TCA, [<sup>3</sup>H]thymidine was added. The samples were processed for liquid scintillation counting as described above.

**Cell Proliferation Assays.** Cells were seeded in 24-well plates (5000 cells/well) in DMEM/10% FCS. On the next day the medium was changed to DMEM/2% FCS with or without growth factors and tyrphostins were added as indicated. Three days later the cells were counted in a hemocytometer.

**Soft Agar Assays.** Cells (4000 cells/ml) were cultured in 0.3% agar in the presence of DMEM/10% FCS on a base layer of 0.5% agar in the presence or absence of tyrphostins. After 2 weeks the formed colonies were counted.

**RESULTS**

**Screening of Tyrphostins.** Tyrphostins from various structural families (Fig. 1) were tested for their potency to inhibit ligand-induced autophosphorylation of PDGF receptor and EGF receptor in Swiss 3T3 cell membranes which harbor both receptors. Membranes were incubated simultaneously with EGF, PDGF-BB, and [<sup>γ</sup>-<sup>32</sup>P]ATP in the absence or presence of the compounds to be tested. Autophosphorylation of the receptors was monitored by SDS-PAGE and autoradiography. In Fig. 2 the comparison of 6 tyrphostins, representing several families, is shown as an example. The compound AG34 had little effect on autophosphorylation of either of the receptors, AG785 inhibited EGF receptor autophosphorylation but had no effect on PDGF receptor autophosphorylation, and AG805 inhibited both receptor kinases with similar efficacy. Work on these three compounds was stopped at this point. Tyrphostins AG808, AG1295, and AG1296 abolished autophosphorylation of the PDGF receptor completely whereas the autophosphorylation of the EGF receptor was only slightly inhibited by AG808 or not at all affected by AG1295 and AG1296 (Fig. 2). The small group of compounds which scored positive in this screening were then tested for their ability to selectively inhibit PDGF receptor autophosphorylation and PDGF-stimulated DNA synthesis in intact cells. In these assays, only AG1295 and AG1296 scored positive and AG1152 partially positive (Table 1). AG1295 and AG1296 were further characterized (see below) but since the latter was slightly more potent (Table 1) only the data for AG1296 are presented. Both 1295 and 1296 belong to quinoxaline type tyrphostins, different from the previously characterized benzenemalononitrile tyrphostins (19, 20), represented by AG34 and the indole tyrphostins (21) represented by AG805 and AG808.

**Selective Inhibition of PDGF Receptor Kinase and PDGF-stimulated Cell Growth by AG1296 in Swiss 3T3 Cells.** Fig. 3A depicts the dose-response curve for inhibition of the PDGF receptor kinase in Swiss 3T3 cell membranes by AG1296. Whereas the

Table 2 Effect of the tyrphostin AG1296 on the DNA synthesis in porcine aortic endothelial cells expressing different human growth factor receptors

PAE cells expressing the respective receptors were treated with the tyrphostin and subsequently with the indicated ligand. Then DNA synthesis was measured by a [<sup>3</sup>H]thymidine incorporation assay.

Receptor	Ligand	IC <sub>50</sub> (μM) for inhibition of ligand-stimulated DNA synthesis
PDGF α-receptor	PDGF-AA	1.0
PDGF β-receptor	PDGF-BB	0.8
Kit-SCF receptor	SCF	1.8
KDR-VEGF receptor	VEGF	>10

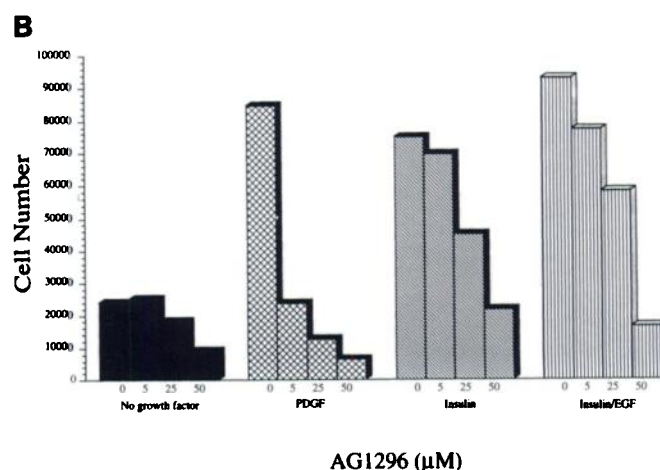
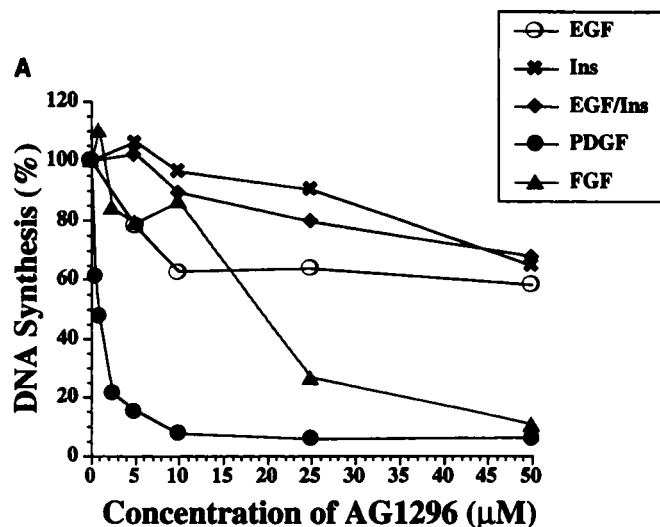


Fig. 4. Effect of AG1296 on growth of Swiss 3T3 cells stimulated by different growth factors. A, DNA synthesis. The treatment of cells by AG1296 was performed as described above and in "Materials and Methods." Then, growth factors (PDGF, 10 ng/ml; EGF, 10 ng/ml; insulin, 1 μg/ml; basic FGF, 10 ng/ml as indicated) were added together with [<sup>3</sup>H]thymidine for 20–24 h and TCA-precipitable radioactivity was measured. The stimulation of [<sup>3</sup>H]thymidine incorporation induced by PDGF, EGF, EGF plus insulin (*Ins*), insulin, and basic FGF was 180-, 5-, 225-, 40-, and 88-fold over background, respectively, and was set at 100%. Typical curves as obtained in at least 3 independent experiments are shown. B, cell proliferation. Swiss 3T3 cells were treated with different concentrations of AG1296 in DMEM supplemented with 2% FCS in the absence or presence of PDGF (10 ng/ml), insulin (1 μg/ml), or insulin plus EGF (10 ng/ml). Cell numbers were determined after 3 days. Control values are: no growth factor, 24,000 cells/well; PDGF, 84,500 cells/well; insulin, 74,600 cells/well; insulin/EGF, 92,800 cells/well.

compound inhibited the PDGF receptor autophosphorylation with an IC<sub>50</sub> of 0.3–0.5 μM (Fig. 3A), the EGF receptor kinase in the same membranes was not inhibited up to the concentration of 100 μM (Fig. 3A and data not shown). Similar results were obtained comparing the potency of AG1296 to inhibit PDGF receptor autophosphorylation and EGF receptor autophosphorylation in intact cells (Fig. 3B; Table 1): PDGF receptor autophosphorylation was strongly inhibited at submicromolar concentration of AG1296; whereas EGF receptor autophosphorylation was not blocked even at 100 μM. AG1296 was also highly inhibitory towards PDGF-induced mitogenesis but had a minimal effect on mitogenesis mediated by EGF, insulin, or a combination of these factors (Fig. 4A). The inhibition of PDGF-stimulated DNA synthesis by AG1296 was fully reversible (Fig. 5). The mitogenic effect of basic FGF on Swiss 3T3 cells was clearly inhibited by AG1296 (Fig. 4A), albeit with an IC<sub>50</sub> of 12.3 ± 3.1 μM (mean of 5 experiments ± SD) which is 1 order of magnitude higher than that for

PDGF-induced DNA synthesis ( $1.5 \pm 0.7 \mu\text{M}$ , mean of 5 experiments in direct comparison). AG1296 also potently inhibited PDGF-induced cell growth ( $\text{IC}_{50}$   $3.2 \mu\text{M}$ ) (Fig. 4B) at a much lower concentration range than that needed for the inhibition of cell growth induced by EGF, insulin, or a combination of both growth factors ( $\text{IC}_{50} > 25 \mu\text{M}$ ). At higher concentrations of AG1296, however, a general inhibition of cell division was observed.

**Effect of AG1296 on Tyrosine Kinase Receptors Expressed in Porcine Aortic Endothelial Cells.** To further explore the potency and specificity of AG1296, its effects on various tyrosine kinase receptors expressed in porcine aortic endothelial cells were analyzed. AG1296 was found to inhibit autophosphorylation of both human PDGF  $\alpha$ - and PDGF  $\beta$ -receptors (Fig. 6) in intact cells and to inhibit equipotently PDGF  $\alpha$ - and PDGF  $\beta$ -receptor-dependent DNA synthesis in cells transfected with either receptor (Table 2). We then examined the effect of tyrphostins on the SCF receptor Kit (31), a close relative of the PDGF receptor, expressed in PAE cells. As shown in Table 2, this receptor is as sensitive to AG1296 as are the PDGF receptors. KDR, another receptor tyrosine kinase which is structurally related to the receptors for PDGF, is expressed on endothelial cells and functions as a receptor for VEGF (32–36). KDR was found to be virtually insensitive to AG1296, at both the level of receptor autophosphorylation (Fig. 6) and VEGF-stimulated DNA synthesis (Table 2).

**AG1296 Reverses Cell Transformation Induced by *sis* but Not by *src*.** *sis*-transfected fibroblasts acquire a transformed phenotype characterized by the loss of density-dependent growth arrest, the ability to form tumors in nude mice, and the ability to grow in semisolid agar due to activation of PDGF receptors (37, 38). When *sis*-transformed cells were treated with the tyrphostin AG1296, we observed a dose-dependent change from a non-contact-inhibited phenotype to a flattened, contact-inhibited morphology similar to that of nontransformed NIH 3T3 cells (Fig. 7). This effect is already detectable at  $10 \mu\text{M}$  AG1296 and the reversion of the transformed phenotype is complete at  $50 \mu\text{M}$ . AG1296 also potently inhibits the growth of the *sis*-transformed NIH 3T3 cells in

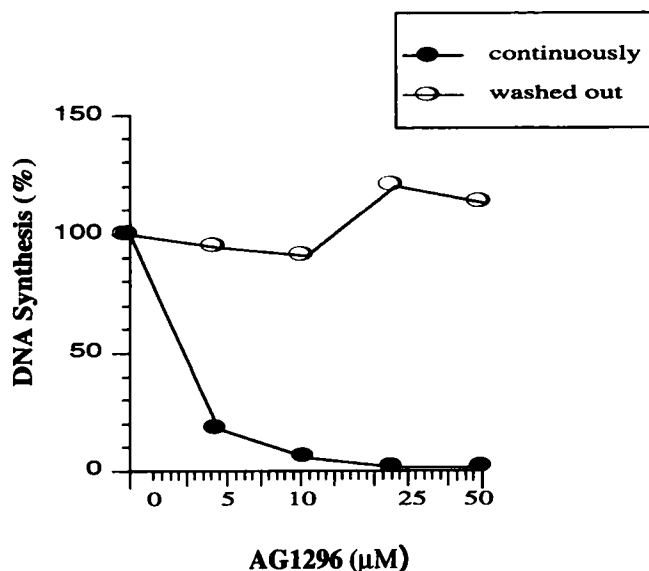


Fig. 5. Reversibility of the inhibition of DNA synthesis by tyrphostin AG1296. Swiss 3T3 cells were treated for 8 h with different concentrations of AG1296. Then the inhibitor was washed out or kept in the samples as indicated. [ $^3\text{H}$ ]thymidine and PDGF were added and stimulation of DNA synthesis was measured as described in the legend to Fig. 4. Stimulation of DNA synthesis by PDGF in the control cultures (100%) was 67-fold. One of two experiments with identical results is shown.

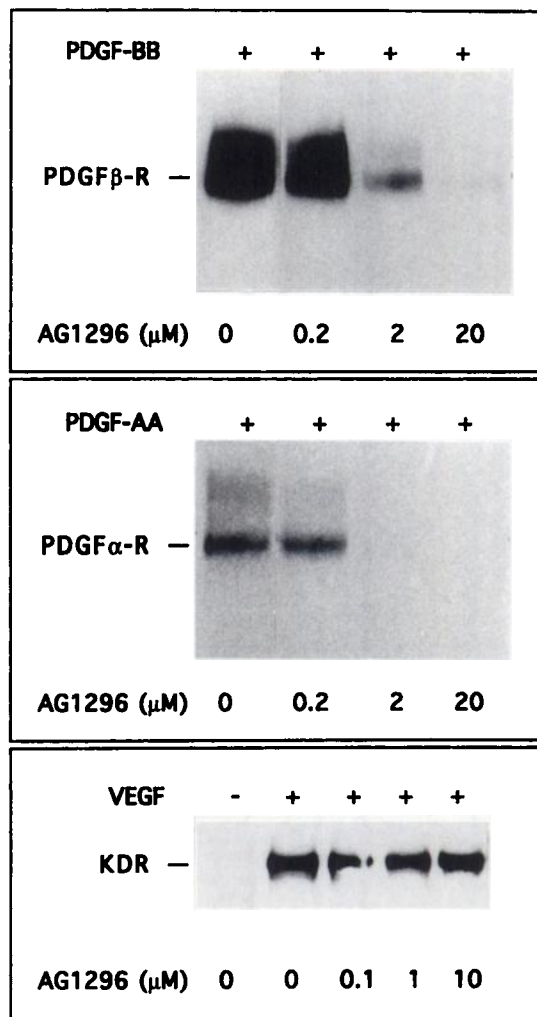


Fig. 6. Effect of tyrphostin AG1296 on the autophosphorylation of different human growth factor receptors expressed in porcine aortic endothelial cells. PAE cells expressing the different receptors were serum deprived, treated with various concentrations of AG1296, and stimulated briefly at  $37^\circ\text{C}$  with PDGF-AA, PDGF-BB, or VEGF as indicated. Thereafter, the cells were extracted and the receptors were partially purified (for details see "Materials and Methods") and subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies.

semisolid agar, with approximately 80% inhibition of colony formation at  $5 \mu\text{M}$  (Table 3).

We then examined whether the *sis*-antagonizing activity of AG1296 could be attributed to the inhibition of PDGF receptor kinase in the cells harboring the oncogene. To this end, *sis*-transformed cells were treated with different concentrations of the tyrphostin. The extent of tyrosine phosphorylation of the endogenous PDGF receptors was then evaluated by immunoblotting with anti-phosphotyrosine antibodies either in whole cell lysates or after immunoprecipitation of the PDGF receptors with receptor-specific antibodies (Fig. 8). In contrast to normal Swiss 3T3 cells (Fig. 3), activated PDGF receptors were detectable in *sis*-transformed NIH 3T3 cells in the absence of exogenous PDGF. When the *sis*-transformed NIH 3T3 cells were treated with AG1296, the autophosphorylation of the PDGF receptors was nearly abolished (Fig. 8). The dose dependence of the inhibition of PDGF receptor autophosphorylation in *sis*-transfected cells by AG1296 corresponds to the dose-dependent reversal of the transformed morphology of the cells (not shown).

To further explore the specificity of the inhibition of *sis* transformation by tyrphostin AG1296, we investigated the possible

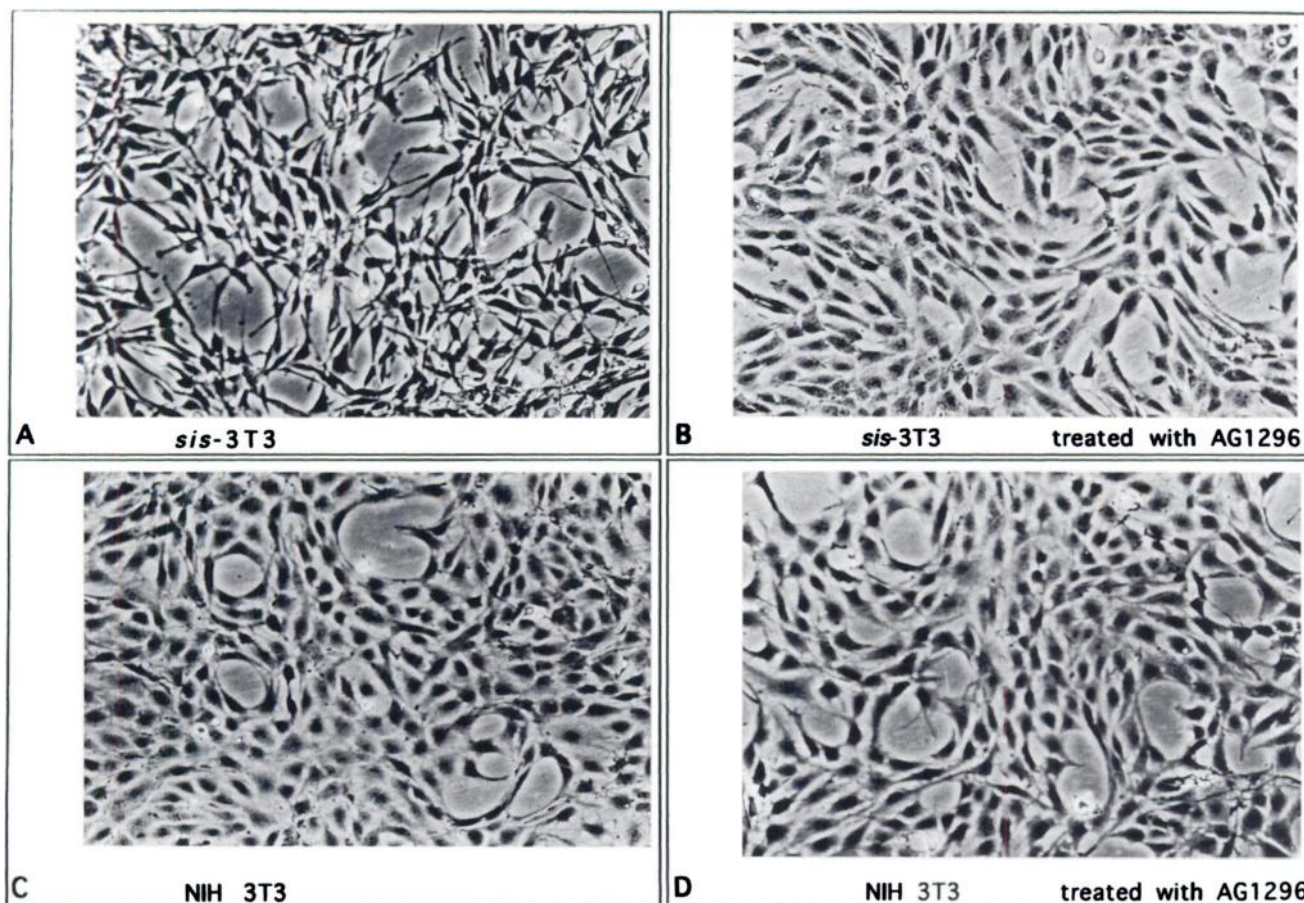


Fig. 7. Effect of tyrphostin AG1296 on the morphology of *sis*-transformed cells. *sis*-transformed NIH 3T3 cells were treated with DMSO alone (A) or with 50  $\mu\text{M}$  AG1296 (B) for 72 h in DMEM supplemented with 10% FCS. Normal NIH 3T3 cells were also treated with DMSO (C) or AG1296 (D). Microphotographs were taken with a phase contrast microscope.  $\times 300$ .

effects of the compound on NIH 3T3 cells transfected with an oncogenic variant of *c-src* (chicken *src* F527 mutant). Similar to *sis*-transfected cells, these cells show an irregular growth pattern and grow in semisolid agar (39, 40). No morphological changes of the *src*-transformed cells could be seen up to a 50  $\mu\text{M}$  dose of the compound AG1296 nor could inhibition of any tyrosine-phosphorylated protein be detected by immunoblotting of whole cell lysates (not shown). To directly test the effect of AG1296 on the Src tyrosine kinase, the p60<sup>c-src(F527)</sup> protein was precipitated from cell lysates and the kinase activity was assayed. As shown in Fig. 9, little inhibition of the Src kinase could be detected (25% inhibition at 50  $\mu\text{M}$  AG1296). Independent experiments show that AG34 and its homologue, which have no effect in the PDGF receptor system (Fig. 2), reverse Src F527 induced transformation and block Src kinase activity (41).

Table 3 Inhibition of colony formation of *sis*-transformed cells in semisolid agar by tyrphostin AG1296

Cells were seeded in semisolid agar in the absence or presence of the indicated concentrations of tyrphostins. The final concentration of DMSO was 0.2%. After 2 weeks of culture, colonies were scored visually. Mean values of duplicate assays in a typical experiment are given.

Treatment	No. of colonies	%
None	212	100
DMSO	211	100
5 $\mu\text{M}$	21	10
10 $\mu\text{M}$	3	1.4
25 $\mu\text{M}$	1	0.5
50 $\mu\text{M}$	0.5	0.2

## DISCUSSION

Low molecular weight tyrosine kinase inhibitors of the tyrphostin type have previously been reported to inhibit PDGF-induced mitogenesis in human bone marrow fibroblasts (21) and in rabbit vascular smooth muscle cells (20). In this study we describe a novel class of tyrphostin derivatives which are more potent and specific in inhibiting PDGF receptor tyrosine kinase, both in normal and *sis*-transformed murine fibroblasts and in porcine aortic endothelial cells expressing human PDGF  $\alpha$ - or  $\beta$ -receptors.

The new class of tyrphostins, represented by AG1296 and AG1295, is much superior to the previously described compounds due to their high potency and excellent selectivity. This is demonstrated by their effect on PDGF-induced cell growth in Swiss 3T3 cells as compared with their effects on cell growth driven by EGF, insulin, a combination of EGF and insulin, or basic FGF. Furthermore, in contrast to the previously described tyrphostins, these compounds have only a negligible effect on p60<sup>c-src(F527)</sup> kinase activity and on *src*-transformed cells. The IC<sub>50</sub> for PDGF receptor kinase inhibition in intact cells is slightly lower than the value measured for DNA synthesis (Table 1). This is probably due to the necessarily different experimental conditions; still the selectivity of AG1295 and AG1296 is clear. Close correlation of the inhibition of PDGF receptor tyrosine kinase activity in intact cells and of the DNA synthesis induced by PDGF in both Swiss 3T3 cells and PDGF receptor expressing PAE cells by AG1296 demonstrates that inhibition of the receptor kinase plays a primary role in the inhibition of PDGF-induced growth of both cell lines. Much higher concentrations of AG1296 were required to partially

inhibit DNA synthesis and cell proliferation induced by other growth factors and even then no effect could be measured on their respective receptor kinases. This finding suggests that other intracellular targets, possibly the tyrosine kinases involved in intracellular signaling pathways used by multiple growth factors, might be susceptible to inhibition by AG1296 at higher concentrations of the tyrphostin. No signs of cytotoxicity of AG1296 were observed and the inhibition of PDGF-stimulated DNA synthesis was fully reversible. Among the various pharmacophores examined, the quinoxaline moiety was found to be the most selective and potent (Table 1). The benzenemalononitrile pharmacophore and indole used previously (20, 21), although efficient, are less effective (Table 1). The quinoxaline structure of these tyrphostins serves as a pharmaceutical lead for developing agents for treatment of the disorders involving pathological PDGF-induced cell proliferation. The oncogenic potential of the compounds is indicated by their ability to revert the transformed phenotype of *sis*-transfected fibroblasts, as judged by cell flattening (Fig. 7) and inhibition of soft agar growth (Table 3). In the *sis*-transfected cells the inhibition of receptor kinase activity (Fig. 8) and in turn the reversion of the transformed phenotype required higher concentrations of the tyrphostin compared to the concentrations needed to inhibit the kinase in Swiss 3T3 cells and PDGF receptor expressing PAE cells. The higher concentrations might be necessary partially because the assays with *sis* cells had to be performed in the presence of serum and partially because the majority of PDGF receptors in the *sis* cells is not at the cell surface (42, 43) and might be thus less readily accessible for the inhibitors. Another potentially interesting target for this novel class of PDGF receptor kinase blockers might be the pathological proliferation and cell migration in relation to atherosclerosis and restenosis (8). In this respect it is of particular interest that compound AG1296 inhibited potently the PDGF receptor kinase but had virtually no effect on the kinase activity of the VEGF receptor KDR. Since PDGF receptors are predominantly expressed in smooth muscle cells (8) whereas the expression of KDR is restricted to the endothelium

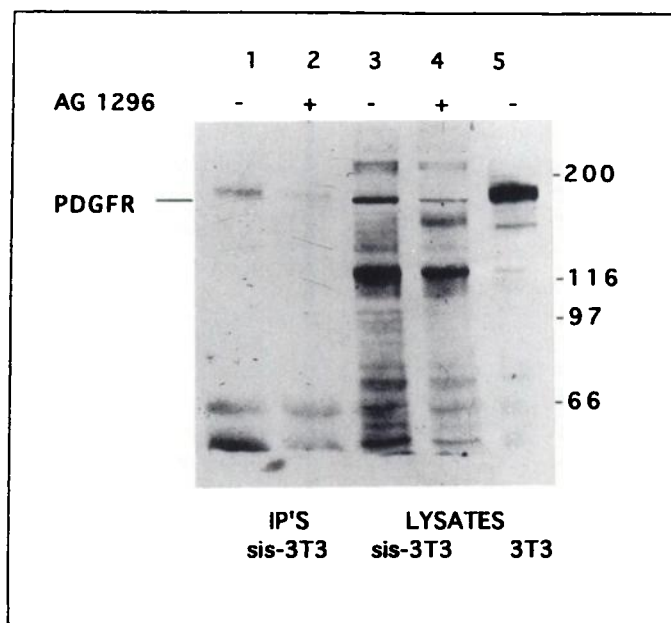


Fig. 8. Effect of tyrphostin AG1296 on autophosphorylation of the PDGF receptor in *sis*-transformed cells. *sis*-transformed NIH 3T3 cells were treated for 48 h with DMSO (Lanes 1 and 3) or 50  $\mu$ M AG1296 (Lanes 2 and 4). Cell extracts were either directly analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibodies (Lanes 3 and 4) or subjected to immunoprecipitation with anti-PDGF receptor antibody (Lanes 1 and 2) prior to SDS-PAGE and immunoblotting. For comparison, a cell extract of PDGF-stimulated Swiss 3T3 cells was also analyzed (Lane 5).

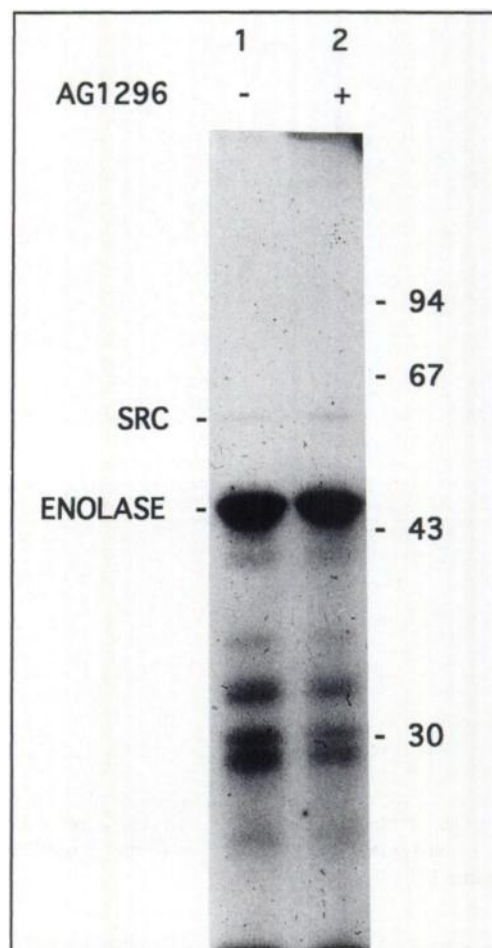


Fig. 9. Effect of tyrphostin AG1296 on the activity of Src-kinase against an exogenous substrate. p60<sup>c-Src</sup>(F327) was immunoprecipitated from *src*-transformed cells, and the immunoprecipitates then treated with 50  $\mu$ M AG1296 (Lane 2) or DMSO (Lane 1). The activity of the kinase was assayed with acid-treated enolase as exogenous substrate in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. The samples were analyzed by SDS-PAGE and autoradiography.

(35), one would expect a selective inhibition of smooth muscle cell proliferation and migration by AG1296 whereas endothelial cell function may remain unaltered in the presence of the compound. AG1296 or related substances therefore represent candidate drugs for the prevention of restenosis, *i.e.*, the migration (44) and rapid proliferation (45) of smooth muscle cells secondary to percutaneous angioplasty resulting in the narrowing of the arterial lumen and limiting the intermediate and long term success of the treatment of atherosclerotic lesions (46).

There is no effect of compound AG1296 on PDGF binding.<sup>7</sup> The cellular uptake of AG1296 is rapid, since incubation of the cells for 2 min with 50  $\mu$ M compound is sufficient to block the PDGF receptor kinase completely.<sup>8</sup> This is similar to previously reported behavior of tyrphostins (21, 47). For other tyrphostins an inhibition of the target kinases based on competition with the protein substrate has already been demonstrated (46, 47). The exact mode of PDGF receptor kinase inhibition has not been investigated but the mechanism of EGF receptor kinase and insulin receptor kinase inhibition has been studied in detail (48–51). In these studies it has been shown that some of the blockers are competitive *versus* the substrate and ATP and others are competitive *vis-à-vis* the substrate and noncompetitive with respect to ATP. From the high selectivity observed for the quinoxalines repre-

<sup>7</sup> C. Rorsman *et al.*, data not shown.

<sup>8</sup> A. Böhmer *et al.*, data not shown.

sented by AG1295 and AG1296, it is likely that their mode of interacting with tyrosine kinases is similar. We observed that receptors closely related to the PDGF receptor were more sensitive to inhibition by AG1296 than more divergent receptor tyrosine kinases. Therefore PDGF  $\alpha$ - and  $\beta$ -receptors as well as the closely related Kit/SCF receptor exhibited equal inhibition by AG1296. The more distantly related FGF receptor 1 was more than 1 order of magnitude less sensitive to AG1296 and the EGF receptor, the insulin receptor, and the VEGF receptor KDR which is structurally related to the PDGF receptors were completely refractory to the action of AG1296. Thus, despite some overall similarity between the PDGF receptor and the KDR kinase, they differ in their sensitivity towards kinase inhibitors and one can therefore speculate that both receptors might also differ in their substrate specificity. Work is under way to characterize the mechanism of action of compound AG1296 in detail. The relative sensitivity of the Kit/SCF receptor may cause leukopenia in *in vivo* experiments, although preliminary experiments did not reveal leukopenia in mice when 20 mg/kg were injected daily (data not shown). Nevertheless, we are now attempting to design derivatives of AG1295 and AG1296 which differentiate between Kit/SCF receptor and PDGF receptor and still retain the high selectivity toward the latter.

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