Effect of Herbimycin A on Tyrosine Kinase Receptors and Platelet Derived Growth Factor (PDGF)-Induced Signal Transduction

Yuko MURAKAMI, Hidesuke FUKAZAWA, Satoshi MIZUNO, and Yoshimasa UEHARA*

Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, 162-8640, Japan. Received May 13, 1998; accepted June 30, 1998

Herbimycin A is widely used as an inhibitor of Src family protein tyrosine kinases but is also reported to induce the downregulation of epidermal growth factor (EGF) receptor number in A431 cells without inhibiting its tyrosine kinase activity. To determine the specificity of the receptor downregulation, we examined its effect on a variety of cell lines which express different levels of EGF receptor and on other tyrosine kinase receptors. Long-term herbimycin A treatment decreased the amounts of all the tyrosine kinase receptors examined in a dose-dependent manner. It also reduced ligand-stimulated receptor autophosphorylation in accordance with the reduction in the receptor level. Herbimycin A inhibited platelet derived growth factor (PDGF)-induced tyrosine phosphorylation of cellular proteins and DNA synthesis in NIH3T3 cells but did not affect the serum-stimulated DNA synthesis. PDGF-induced tyrosine phosphorylation and activation of c-Src was inhibited but the protein level of c-Src was not reduced by herbimycin A. The reduced level of c-Src kinase activity correlated with the levels of both PDGF receptor and DNA synthesis. These results indicate that the herbimycin A treatment selectively downregulates receptor tyrosine kinases, independent of the number of receptors, and suggest that c-Src is to some degree involved in the selective inhibition of PDGF-induced mitogenesis by herbimycin A.

Key words EGF receptor; receptor tyrosine kinase; PDGF receptor; insulin receptor; herbimycin A

Signal transduction mediated by growth factors, cytokines, hormones and many other biological modulators is now a central part of research on the control of cell growth, development, differentiation and immunological response. Tyrosine kinases have been shown to play particularly crucial roles in signaling pathways in the cell; receptor tyrosine kinases are the integral parts and function as initial signaling molecules in response to ligand binding.^{1,2)} It is widely accepted that some tumors are caused by a disorder of the signal transduction of receptor tyrosine kinases. For instance, some squamous carcinoma cells have excess epidermal growth factor (EGF) receptors,³⁾ and in these cases paracrine and/or autocrine growth-stimulation by EGF is thought to induce the tumor progression. Expression of ErbB-2 in mammary carcinoma and K-sam in poorly differentiated gastric carcinoma are other examples in which an overexpression of the receptors correlates with poor prognosis.^{4,5)}

Herbimycin A has been extensively used as an inhibitor of the nonreceptor tyrosine kinases src, fps, yes, ros, erbB,⁶⁾ abl,⁷⁾ lck, fyn,⁸⁾ lyn,⁹⁾ etc. In the case of v-Src, the mechanism of inhibition by herbimycin A has been suggested to be direct inhibition of the kinase by binding to cysteine residues of v-Src, and an accelerated turnover of v-Src was observed.^{10,11)} As to EGF receptor tyrosine kinase, direct inhibition of EGF receptor kinase in A431 cells was not observed, but herbimycin A affected biosynthesis of the EGF receptor and reduced the receptor number.^{12,13)} We also demonstrated that the reduction was specific to the EGF receptor carrying the cytoplasmic kinase domain, and that neither truncated EGF receptor lacking the kinase domain nor transferrin receptor was affected by herbimycin A.¹³⁾

In the present study, we further examined the effect of herbimycin A on a more expanded range of receptor tyrosine kinases: EGF receptor, ErbB-2, PDGF receptor and insulin receptor, and also PDGF-stimulated mitogenic signal.

MATERIALS AND METHODS

Materials Herbimycin A was isolated as described previously¹⁴⁾ and used from a 1.0 mg/ml stock solution dissolved in dimethyl sulfoxide. EGF (1.0 mg/ml) was purchased from Takara Shuzo Co. (Kyoto, Japan) and PDGF-BB (human) and α -casein were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Anti-(EGF receptor) mouse monoclonal antibody (OM-11-951) was purchased from Cambridge Research Biochemicals, Inc. (Wilmington, DE, U.S.A.), anti-(ErbB-2/c-neu) antibody (Ab-2) was from Oncogene Science, Inc. (Uniondale, NY, U.S.A.), and anti-(PDGF type A/B receptor) antibody and anti-phosphotyrosine mouse monoclonal antibody (4G10) were from Upstate Biotechnology, Inc. (U.B.I., Lake Placid, NY, U.S.A.). Anti-(insulin receptor β subunit) rabbit polyclonal antibody was from Transduction Laboratories (Lexington, KY, U.S.A.). Anti-(c-Src) rabbit polyclonal antibody (sc-18) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). L-[³⁵S] methionine (1200 Ci/mmol) and [³H]thymidine (64.7 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and γ -[³²P]ATP (3000 Ci/mmol) was from New England Nuclear Research Products (Wilmington, DE).

Cells and Cell Culture Human oral epidermoid carcinoma KB cells and normal mouse fibroblast NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DME) containing 10% calf serum (CS), and human cervix epithelioid carcinoma HeLa cells and human mouth squamous carcinoma cell line HSC-2 (obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan) were grown in minimal essential Eagle's medium (MEM) containing 10% CS. Human breast adenocarcinoma SK-BR-3 cells (obtained from American Type Culture Collection, Rockville, MD, U.S.A.) were grown in MEM containing 10% fetal calf serum (FCS). Human normal lung fibroblast WI-38 (obtained from the Japanese Cancer Research Resources Bank) were grown in Eagle's basal medium containing 10% FCS.

* To whom correspondence should be addressed.

Rat hepatoma H35 cells (obtained from Dr. O. Koshio, Asahi Seimei Co., Tokyo) were grown in MEM containing 6% FCS.

Immunoblotting Cells were washed with phosphate-buffered saline (PBS) and lysed with RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 100 kallikrein-inactivator (K.I.) units/ml aprotinin, 100 μ M Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride (PMSF), and 25 μ g/ml each of leupeptin, pepstatin, antipain) on ice, and centrifuged at 15000 rpm for 15 min to remove cell debris. The supernatant, containing a fixed amount of protein, was supplemented with the same volume of 2-fold concentrated electrophoresis sample buffer, and the mixture was boiled for 3 min and applied to 7.5 or 9% SDS-PAGE gels. Proteins in the gels were transferred to polyvinylidene difluoride (PVDF) membrane (Bio Rad or Immobilon, Millipore), blotted with antibodies and detected using ^{125}I -protein A (Amersham International, Amersham, Bucks., U.K.) or horseradish peroxidase-linked second antibody and ECL reagent (Amersham International).

Metabolic Labeling and Immunoprecipitation Cultured SK-BR-3 cells seeded at 1×10^5 cells/cm² and incubated for one day were washed with methionine-free MEM and replenished with a 4:1 mixture of the methionine-free MEM and normal MEM, 2% FCS, [^{35}S]methionine (60 μ Ci/ml, 5×10^{-8} M) and 0.3 or 1.0 μ g/ml of herbimycin A and incubated for 20 h.

Cells were lysed with RIPA buffer and cell debris was removed by centrifugation. The lysate was immunoprecipitated with anti-ErbB-2/c-neu antibody, followed by treatment with a second antibody (rabbit anti-mouse IgG, Organon Teknika Corp. -Cappel Products, West Chester, PA, U.S.A.). Immune complexes were collected with Protein A-Sepharose and washed with RIPA buffer. The immunoprecipitates were suspended in a sample buffer, then analyzed using SDS-PAGE. Bands which incorporated ^{35}S were visualized by fluorography or by BAS2000 (Fujix, Tokyo).

DNA Synthesis NIH3T3 cells were seeded at 1×10^5 cells per cm² and incubated for 2 d. The medium was changed to a base medium¹⁵⁾ containing 10% SH-FBS¹⁶⁾ and 1% FCS with or without herbimycin A. Twenty hours later, 30 ng/ml of PDGF-BB or 5% FCS and [^3H]thymidine (0.5 μ Ci/well, 3 μ M) were added, and the cells were again incubated. After an additional 24 h of incubation, cells were washed with PBS and extracted with cold 10% trichloroacetic acid (TCA). TCA-insoluble materials were dissolved in 0.4 N NaOH. Radioactivity was determined in a liquid scintillation counter. A parallel culture was prepared and stained with crystal violet, and the absorbance at 570 nm was measured to determine relative cell numbers. Assays were carried out in duplicate.

PDGF-Stimulated c-Src Kinase Activities NIH3T3 cells were seeded as described above. The medium was changed to the base medium containing 10% SH-FBS and 1% FCS with herbimycin A, and the cells again were incubated. After 20 h, the cells were treated with 30 ng/ml of PDGF-BB for the indicated times, then lysed with a lysis buffer. For c-Src kinase activity, the cell lysates with HNT buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 100 K.I. units/ml aprotinin, 100 μ M

Na_3VO_4 , 1 mM PMSF, and 25 μ g/ml each of leupeptin, pepstatin, antipain) were subjected to immunoprecipitation with anti-(c-Src) antibody and collected with Protein A-Sepharose. The immune complex was washed with HNT buffer, then 7.5 μ l of kinase buffer (20 mM HEPES, pH 7.4, 5 mM MgCl_2) containing α -casein (1 mg/ml) was added and a kinase reaction was started by adding 7.5 μ l of γ -[^{32}P]ATP (2 μ M, 10 μ Ci). The mixture was then incubated for 20 min at 25 °C, and the reaction was stopped with 5 μ l of 4-fold strength sample buffer. The reaction mixtures were analyzed by SDS-PAGE (by 9% gel) and the kinase activity of c-Src was determined by counting the radioactivity of ^{32}P in the bands of α -casein.

RESULTS

Effect of Herbimycin A on Levels of EGF Receptor, PDGF Receptor, Insulin Receptor, and ErbB-2 In our previous study, we examined the effect of herbimycin A on human epidermal carcinoma A431 cells, in which the EGF receptor was abnormally overexpressed (2×10^6 receptors/cell).¹²⁾ Here, we examined various types of cells expressing different levels of EGF receptor. The cell lines used were human normal fibroblast WI-38 cells (3×10^4 — 1×10^5), human carcinoma HeLa cells (4×10^4), human carcinoma KB cells (6×10^5),¹⁷⁾ and human carcinoma HSC-2 cells (8×10^6).³⁾ Cells were treated with various concentrations of herbimycin A for 20 h, and then EGF receptor was identified by immunoblotting with an anti-(EGF receptor) antibody and quantified with ^{125}I -protein A. Herbimycin A dose-dependently reduced the EGF receptor levels in all the cell lines examined (Fig. 1A and C).

We next examined the effect of herbimycin A on the PDGF receptor in NIH3T3 and WI-38 cells. The PDGF receptor level was also decreased dose-dependently by herbimycin A in these two cell lines (Fig. 1B and C).

To determine the effect of herbimycin A on an insulin receptor, we used rat hepatoma H35 cells and human KB cells expressing either a high (2×10^5) or low (5×10^4) level of insulin receptor, respectively.^{17,18)} The cells were treated with herbimycin A for 20 h and the insulin receptor was analyzed by immunoblotting with an anti-(insulin receptor β chain) antibody. Herbimycin A induced a dose-dependent reduction in insulin receptor β chain amount in the two cell lines (Fig. 1D).

We also investigated the effect of herbimycin A on ErbB-2/c-Neu, a tyrosine kinase receptor which is structurally similar to EGF receptor (ErbB-1), but whose ligand has not been identified. The human breast adenocarcinoma SK-BR-3 cells expressing a high level of ErbB-2/c-Neu⁴⁾ were labeled with [^{35}S]methionine for 20 h in the presence or absence of herbimycin A (0.3 and 1.0 μ g/ml). Analysis by immunoprecipitation with anti-ErbB-2/c-neu antibody and by SDS-PAGE showed the ^{35}S radioactivity of the 185 kDa ErbB-2 band to be greatly reduced by herbimycin A (Fig. 1E). Thus it was indicated that herbimycin A dose-dependently reduced protein amounts of all the receptor tyrosine kinases, and this effect was independent of the number of receptors. Since herbimycin A did not significantly reduce the amount of the bulk protein,¹⁴⁾ nor did it inhibit the biosynthesis of transferin receptors or truncated EGF receptors,¹³⁾ the reduction was

Fig. 1. Effect of Herbimycin A on Protein Amount of EGF Receptor (A), PDGF Receptor (B), Insulin Receptor (D), ErbB-2 (E) and Dose-Response Curves (C) of (A) and (B)

Cells were treated with the indicated concentrations of herbimycin A for 20 h. (A) Cell lysates containing fixed protein amounts (HSC-2: 25 μg, KB: 60 μg, HeLa: 40 μg, WI-38: 30 μg) were analyzed for EGF receptor by immunoblotting with anti-EGF receptor antibody and ¹²⁵I-protein A. (B) Cell lysates containing 30 μg (WI-38) and 50 μg (NIH3T3) protein were analyzed by immunoblotting with anti-PDGFR antibody. The receptor levels were determined by counting the radioactivity of ¹²⁵I-protein A bound to the receptor antibody. (C) Dose-dependent decrease in EGF receptor and PDGF receptor. (D) Cell lysates containing 50 μg protein (KB, H35) were analyzed by immunoblotting with anti-insulin receptor β chain antibody and ECL. (E) SK-BR-3 cells were labeled with [³⁵S]methionine as described in Materials and Methods for 20 h with herbimycin A. Cell lysates containing 100 μg protein were immunoprecipitated with anti-ErbB-2 antibody and analyzed.

thought to be specific to these receptor protein kinases.

Effect of Herbimycin A on a Ligand-Induced Receptor Autophosphorylation As herbimycin A reduced the receptor levels, we next examined ligand-induced receptor autophosphorylation. EGF receptor autophosphorylation in WI-38 cells induced by EGF was decreased by herbimycin A, in parallel with the reduction of EGF receptor (Fig. 2 vs. Fig. 1A). The autophosphorylation of PDGF receptor in NIH3T3 cells was also reduced (Fig. 2) corresponding to the reduction in PDGF receptor level (Fig. 1B). Thus, the levels of autophosphorylation of these two receptors were proportional to the levels of receptor proteins.

Effect of Herbimycin A on PDGF-Stimulated DNA Synthesis and Tyrosine Phosphorylation of Cellular Proteins Herbimycin A reduction of the PDGF-induced au-

tophosphorylation of the tyrosine kinase receptor caused us to examine PDGF-induced DNA synthesis. NIH3T3 cells were treated with herbimycin A for 20 h and stimulated with PDGF. DNA synthesis was measured by [³H]thymidine uptake for the next 24 h. Herbimycin A reduced DNA synthesis in a dose-dependent manner (Fig. 3). This reduction correlated with the reduction in PDGF receptor levels (Fig. 1B and 1C), and reflected the decrease in the autophosphorylation of PDGF receptor (Fig. 2). In contrast, FCS-induced DNA synthesis was slightly stimulated by herbimycin A (Fig. 3).

Next, overall PDGF-stimulated tyrosine phosphorylation in NIH3T3 cells was analyzed by immunoblotting with anti-phosphotyrosine antibody. In the control cells, tyrosine phosphorylation of 185, 135, 64—62, 53 and 32 kDa proteins was

Fig. 2. Effect of Herbimycin A on Tyrosine Phosphorylation in EGF (Left) and PDGF (Right) Receptors

Cells were pretreated with herbimycin A under the same condition as described in Fig. 1 and stimulated for 30 min with 50 ng/ml of EGF (WI-38) or 30 ng/ml of PDGF (NIH3T3). Cell lysates containing 20 μg (WI-38) or 30 μg (NIH3T3) protein were analyzed by immunoblotting with anti-phosphotyrosine antibody and ¹²⁵I-protein A. Tyrosine phosphorylation of EGF or PDGF receptor was measured by the radioactivity of ¹²⁵I-protein A, and the values under the lanes represent the percentages of control.

Fig. 3. Effect of Herbimycin A on PDGF- and FCS-Induced DNA Synthesis of NIH3T3 Cells

Cells, incubated with the indicated concentrations of herbimycin A for 20 h, were further incubated with [³H]thymidine for 24 h to monitor DNA synthesis in the presence of PDGF or FCS. Values represent percentages of [³H]thymidine uptake per relative cell number.

Fig. 5. Effect of Herbimycin A on PDGF-Stimulated Kinase Activity and Protein Amount of c-Src in NIH3T3 Cells

Cells were treated with the indicated concentration of herbimycin A for 20 h and then stimulated with or without PDGF (30 ng/ml) for 5 min. One portion of cell lysates (50 μg) was immunoprecipitated with anti-Src antibody, and applied to a kinase assay *in vitro* (upper panel); another portion (25 μg) was immunoblotted with anti-Src antibody and detected by ECL (lower panel).

Fig. 4. Effect of Herbimycin A on PDGF-Stimulated Tyrosine-Phosphorylation of PDGF Receptor and Overall Cellular Protein Molecules of NIH3T3 Cells

Cells were pretreated with herbimycin A (0.3 μg/ml) for 20 h and stimulated with PDGF (30 ng/ml), then harvested at 0, 2.5, 10, 30 and 60 min after PDGF addition. Cell lysates (10 μg) were immunoblotted with anti-phosphotyrosine antibody and detected by ECL.

detected after 2.5 min of PDGF stimulation (Fig. 4). The 185 kDa PDGF receptor, as well as other protein bands, were tyrosine phosphorylated most intensely after 10 min. In herbimycin A treated cells, tyrosine phosphorylation of the 185 kDa PDGF receptor and other protein bands decreased about 50% after 10 min, while the maximal level of tyrosine phosphorylation was at 2.5 min; after 10 min it fell to the basal level. Thus, the decrease in tyrosine-phosphorylation of PDGF receptor and other cellular proteins paralleled the decrease in PDGF receptor level.

To learn whether c-Src is involved in the PDGF signal transduction pathway, we analyzed the PDGF-induced activation of c-Src kinase in NIH3T3 cells, since it was reported that the Src family tyrosine kinases are activated in association with PDGF receptor.^{19,20} PDGF rapidly induced the acti-

vation of c-Src kinase as judged using casein as a substrate. Herbimycin A inhibited the activation of c-Src to levels similar to the PDGF receptor in a concentration dependent manner. Autophosphorylation of the PDGF receptor in association with c-Src was similarly decreased. Herbimycin A, however, did not reduce the c-Src protein level at all (Fig. 5).

DISCUSSION

Various tyrosine kinase inhibitors with different inhibitory potencies for different tyrosine kinases have been identified.^{21–23} We have shown that herbimycin A has a differential effect on p60^{v-src} non-receptor tyrosine kinase and EGF receptor tyrosine kinase: it directly inactivates the kinase activity of p60^{v-src},¹⁰ but it induces degradation of the EGF receptor precursor without any direct effect on kinase activity of the mature EGF receptor.¹³

In this study we examined the effect of herbimycin A on distinct types of tyrosine kinase receptors, PDGF receptor, insulin receptor and ErbB-2, and demonstrated that it caused a dose-dependent reduction in the amount of these receptors. The data suggested that receptor tyrosine kinases in general were susceptible to this effect of herbimycin A. We previously demonstrated that herbimycin A acted on EGF receptor precursors during biosynthesis, but that neither the biosynthesis of transferrin receptor nor truncated EGF receptor lacking the cytoplasmic kinase domain was affected.¹³ Thus, there may be a common target in the biosynthesis of tyrosine kinase receptors which are susceptible to herbimycin A. The mechanism of degradation is not clear, but it is apparently *via* ubiquitin-dependent degradation by proteasome in the endoplasmic reticulum.^{24,25} Since the finding that herbimycin A dissociated v-Src from the complex with heat shock protein 90 (hsp90),²⁶ studies have reported that herbimycin A and its derivatives affect proteins that complex with hsp90, including Raf-1, a serine/threonine kinase,²⁷ and steroid hormone receptor²⁸; it has further been suggested that benzoquinone ansamycins act on hsp90, causing the dissociation and degradation of associated proteins. Recently the binding site of geldanamycin, a derivative of herbimycin A, on hsp90 was determined by X-ray analysis.^{29,30} Although there is a report that herbimycin A derivatives bind to GRP94, a member of the hsp90 family which causes the depletion of ErbB-2,^{31–33} the effects of herbimycin A itself on the associations of EGF receptor, PDGF receptor and insulin receptor with hsp90 remain to be examined.

To determine whether herbimycin A inhibits the mitogenic signals of these growth factors, we analyzed its effect on the signal transduction of PDGF receptor in NIH3T3 cells. Herbimycin A decreased the autophosphorylation of PDGF receptor and caused the reduction of PDGF-induced overall tyrosine phosphorylation of cellular proteins and DNA synthesis. These effects were proportional to the reduced levels of PDGF receptor and its autophosphorylation. This result suggested that the mitogenic signal by PDGF is dependent on the level of PDGF receptor.

We finally showed that the c-Src kinase activity was also reduced by herbimycin A to levels equivalent to those of the PDGF receptor (Fig. 5). Moreover, the autophosphorylation of PDGF receptor that was associated with c-Src was similarly reduced, indicating that the amount of active c-Src that

was associated with PDGF receptor was determined by the receptor number. Signals for DNA synthesis mediated by receptor activation are transmitted *via* various cellular substrates and/or adaptor proteins. For PDGF signaling, PLC- γ , ras-GAP, PI-3 kinase, Shc, Src, Syp (SHPTP-2), and others¹ have been identified, although the relationship between these molecules and identification of those essential for mitogenic response has not yet been clearly elucidated. Since the down-regulation of PDGF receptor by herbimycin A caused a reduction in c-Src kinase activation as well as overall tyrosine phosphorylation, c-Src is at least to some degree involved in the mitogenic signals that are relayed from the PDGF receptor and it thereby contributes to the selective inhibition of PDGF-induced mitogenesis by herbimycin A. We cannot, however, exclude the possibility that c-Src was inactivated directly by herbimycin A.

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REFERENCES

- 1) van der Geer P., Hunter T., *Annu. Rev. Cell Biol.*, **10**, 251–337 (1994).
- 2) Resh M. D., *Biochem. Biophys. Acta*, **1155**, 307–322 (1993).
- 3) Yamamoto T., Kamata N., Kawano H., Shimizu S., Kuroki T., Toyoshima K., Rikimaru K., Nomura N., Ishizaki R., Pastan I., Gamou S., Shimizu N., *Cancer Res.*, **46**, 414–416 (1986).
- 4) Kraus M. H., Popescu N. C., Amsbaugh S. C., King C. R., *EMBO J.*, **6**, 605–610 (1987).
- 5) Hattori Y., Odagiri H., Nakatani H., Miyagawa K., Naito K., Sakamoto H., Katoh O., Yoshida T., Sugimura T., Terada M., *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5983–5987 (1990).
- 6) Uehara Y., Murakami Y., Mizuno S., Kawai S., *Virology*, **164**, 294–298 (1988).
- 7) Okabe M., Uehara Y., Miyagishima T., Itaya T., Tanaka M., Kunieda Y., Kurosawa M., Miyazaki T., *Blood*, **80**, 1330–1338 (1992).
- 8) June C. H., Fletcher M. C., Ledbetter J. A., Schieven G. L., Siegel J. N., Phillips A. F., Samelson L. E., *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 7722–7726 (1990).
- 9) Mizuguchi J., Yamanashi Y., Tamura T., Nariuchi H., Gyotoku Y., Fukazawa H., Uehara Y., Yamamoto T., *J. Immunol.*, **148**, 689–694 (1992).
- 10) Uehara Y., Murakami Y., Sugimoto Y., Mizuno S., *Cancer Res.*, **49**, 780–785 (1989).
- 11) Fukazawa H., Uehara Y., Murakami Y., Mizuno S., Hamada M., Takeuchi T., *FEBS Lett.*, **340**, 155–158 (1994).
- 12) Murakami Y., Fukazawa H., Mizuno S., Uehara Y., *Biochem. J.*, **301**, 57–62 (1994).
- 13) Murakami Y., Mizuno S., Uehara Y., *Biochem. J.*, **301**, 63–68 (1994).
- 14) Uehara Y., Hori M., Takeuchi T., Umezawa H., *Jpn. J. Cancer Res. (Gann)*, **76**, 672–675 (1985).
- 15) Li P., Fukazawa H., Yamamoto C., Mizuno S., Tanaka K., Hori M., Yaginuma S., Saito T., Uehara Y., *Oncogene*, **8**, 1731–1735 (1993).
- 16) van Zoelen E. J. J., van Oostwaard T. M. J., van der SAAG P. T., de Laat S. W., *J. Cell. Phys.*, **123**, 151–160 (1985).
- 17) Kadowaki T., Koyasu S., Nishida E., Sakai H., Takaku H., Yahara I., Kasuga M., *J. Biol. Chem.*, **261**, 16141–16147 (1986).
- 18) Shimizu Y., Shimizu N., *J. Biol. Chem.*, **261**, 7342–7346 (1986).
- 19) Gould K. L., Hunter T., *Mol. Cell. Biol.*, **8**, 3345–3356 (1988).
- 20) Kypta R. M., Goldberg Y., Ulug E. T., Courtneidge S. A., *Cell*, **62**, 481–492 (1990).
- 21) Levitzki A., Gazit A., *Science*, **267**, 1782–1788 (1995).
- 22) Kovalenko M., Gazit A., Bohmer A., Rorsman C., Ronnstrand L., Heldein C., Waltenberger J., Bohmer F., Levitzki A., *Cancer Res.*, **54**,

- 6106—6114 (1994).
- 23) Buchdunger E., Trinks U., Mett H., Regenass U., Muller M., Meyer T., McGlynn E., Pinna L. A., Traxler P., Lydon N. B., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 2334—2338 (1994).
- 24) Sepp-Lorenzino L., Ma Z., Lebowitz D., Vinitzky A., Rosen N., *J. Biol. Chem.*, **270**, 16580—16587 (1995).
- 25) Mimnaugh E., Chavany C., Neckers L., *J. Biol. Chem.*, **271**, 22796—22801 (1996).
- 26) Whitesell L., Mimnaugh E. G., de Costa B., Myers C. E., Neckers L. M., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 8324—8328 (1994).
- 27) Schulte T. W., Blagosklonny M. V., Ingui C., Neckers L., *J. Biol. Chem.*, **270**, 24585—24588 (1995).
- 28) Segnitz B., Gehring U., *J. Biol. Chem.*, **272**, 18694—18701 (1997).
- 29) Stebbins C. E., Russo A., Schneider C., Rosen N., Hartl F. U., Pavletich N. P., *Cell*, **89**, 239—250 (1997).
- 30) Prodromou C., Roe S., O'Brien R., Ladbury J. E., Piper P. W., Pearl L. H., *Cell*, **90**, 65—75 (1997).
- 31) Miller P., Schnur R. C., Barbacci E., Moyer M. P., Moyer J. D., *Biochem. Biophys. Res. Commun.*, **201**, 1313—1319 (1994).
- 32) Miller P., DiOrio C., Moyer M., Schnur R. C., Bruskin A., Cullen W., Moyer, J. D., *Cancer Res.*, **54**, 2724—2730 (1994).
- 33) Chavany C., Mimnaugh E., Miller P., Bitton R., Nguyen P., Trepel J., Whitesell L., Schnur R., Moyer J. D., Neckers L., *J. Biol. Chem.*, **271**, 4974—4977 (1996).