[CANCER RESEARCH 56, 3859–3861, September 1, 1996]

Advances in Brief

Tyrphostin AG 1478 Preferentially Inhibits Human Glioma Cells Expressing Truncated Rather than Wild-Type Epidermal Growth Factor Receptors

Yuchun Han,[1] Cornelio Gacusana Caday, Anil Nanda, Webster K. Cavenee, and H-J. Su Huang

Brain Tumor and Stroke Research Laboratory, Department of Neurosurgery, Biomedical Research Institute, Louisiana State University Medical Center, Shreveport, Louisiana 71130 [Y. H., C. G. C., A. N.], and Ludwig Institute for Cancer Research, Department of Medicine [W. K. C., H-J. S. H.], and Center for Molecular Genetics [W. K. C.], University of California at San Diego, La Jolla, California 92093-0660

Abstract

The effects of a new epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, tyrphostin AG 1478, were tested on three related human glioma cell lines: U87MG, which expressed endogenous wild-type (wt) EGFR, and two retrovirally infected U87MG cell populations which overexpressed either wt (U87MG.wtEGFR) or truncated EGFR (U87MG.ΔEGFR). Although AG 1478 inhibited cell growth, DNA synthesis, EGFR tyrosine kinase activity, and receptor autophosphorylation of each cell line in a dose-dependent manner, it was significantly more potent in U87MG.ΔEGFR cells than in the other two cell lines. The increased inhibitory response of U87MG.ΔEGFR cells was due to a greater sensitivity of the constitutively autophosphorylated M_r 140,000 and 155,000 ΔEGFR species to AG 1478. These results suggest that AG 1478 is a relatively specific inhibitor of the ΔEGFR, and this finding may have important therapeutic implications since the ΔEGFR occurs frequently in glioblastomas and in breast, lung, and ovarian cancers.

Introduction

Overexpression and rearrangement of the *EGFR*[2] gene occurs frequently in human gliomas and is closely linked to their etiology (1–5). Among the *EGFR* gene mutations, an in-frame truncation of 801 bp in the extracellular domain of EGFR (hereafter referred to as truncated EGFR or ΔEGFR) is the most common form (5), and human glioma cells expressing this truncated EGFR were substantially more tumorigenic when transplanted into nude mice (6). Considering the poor response to therapy of human gliomas, especially glioblastomas, these findings have promoted the search for antiglioma inhibitors, specifically those directed against EGFR. In recent years, a series of tyrosine kinase inhibitors has been developed (7), and, although several studies have shown that some of these inhibitors are highly effective against human epidermoid carcinoma (8, 9) and glioma cell lines (10), no studies have been done to test such inhibitors on glioma cells with a naturally occurring tumor-specific *EGFR* gene mutation. Because of the high frequency of *EGFR* gene mutations in human gliomas and their cellular complexity (11), screening glioma growth inhibitors specific for mutated *EGFR* gene targets potentially offers a greater therapeutic advantage over testing those more general inhibitors that may also unavoidably target the normal EGFR. Here, we tested the effects of tyrphostin AG 1478, a new specific EGFR tyrosine kinase inhibitor (7, 12), on matched glioma cell lines with endogenous wt, overexpressed wt, or ΔEGFRs in conditions of serum starvation where the latter confers a growth advantage *in vitro*. We found that AG 1478 preferentially inhibited those glioma cells with ΔEGFR compared to those expressing endogenous wt EGFR or overexpressing exogenous wt EGFR.

Materials and Methods

Cell Lines and Reagents. Three related human glioma cell lines were used in the present study: U87MG (parental), U87MG.ΔEGFR, and U87MG.wtEGFR. The latter two cell lines were generated by retroviral transfer of the parental U87MG cell line with the indicated EGFR cDNA and sorting populations expressing similar levels of EGFR molecules with a cell sorter as described previously (6). Each of these cell lines also expresses wt EGFR from their endogenous genes. All cell lines were grown and maintained at 37°C in DMEM supplemented with 10% CCS (Hyclone Laboratories, Inc., Logan, UT). The tyrphostin AG 1478 initially used in this study was kindly provided by Dr. A. Levitzki (Hebrew University, Jerusalem, Israel), and later was purchased from Calbiochem-Novabiochem (San Diego, CA). A frozen stock of 100 mM AG 1478 in DMSO was freshly diluted into 95% ethanol, mixed with fresh culture medium to the desired concentrations, and then applied to the cells. The final concentration of ethanol was less than 1% (v/v).

Cell Growth and DNA Synthesis Assays. The effects of AG 1478 on cell growth were examined using an Alamar Blue assay modified from the protocol provided by the manufacturer (Alamar Blue Biosciences, Inc., Sacramento, CA). In this assay, the presence of viable cells leads to the conversion of the oxidized (blue) form of the Alamar Blue to its reduced (red) form (13). Briefly, 2×10^4 cells were seeded in 96-well plates in DMEM/10% CCS and treated on the following day with different concentrations of AG 1478 before incubation for another 3 days. A 20-μl aliquot of Alamar Blue was added to each well, and its absorbance was determined using a Spectromax Scanning Microplate Reader (Molecular Devices, Sunnyvale, CA). The effects of AG 1478 are expressed as percentage of growth inhibition using untreated cells as the control (0% inhibition). Cellular DNA synthesis was determined using a [^{3}H]thymidine incorporation assay as described previously (14).

Immunoprecipitation and *in Vitro* ELISA EGFR Tyrosine Kinase Assay. Confluent cells were serum starved for 15 h and then lysed at 4°C for 30 min using lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP40, 1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 150 mM NaCl, 10 mM Na_3VO_4, 5 mM benzamidine, 1 mM PP_i, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml aprotinin. Cell lysates were immunoprecipitated with an anti-EGFR monoclonal antibody (catalogue no. E-3138; Sigma, St. Louis, MO) and protein G-Sepharose (catalogue no. P-3296; Sigma). The immunoprecipitated complex was washed three times with the lysis buffer, once with assay buffer [20 mM HEPES (pH 7.4), 2 mM $MnCl_2$, 100 μM Na_3VO_4, and 1 mM DTT], and finally diluted in enzyme dilution buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 0.1% BSA, and 1 mM DTT). EGFR tyrosine kinase activity was determined using an ELISA tyrosine kinase assay kit (Boehringer Mannheim, Indianapolis, IN). Briefly, 5 μg of each sample were preincubated with various concentrations of AG 1478 for 15 min at room temperature. The enzyme reaction was initiated by adding the sample to a mixture composed of a biotin-labeled substrate peptide, 5 mM ATP/50 mM $MgCl_2$, 5× concentrated assay buffer, and EGF (final concentration, 2 μg/ml) in a total volume of 100 μl. After a 10-min incubation, the reaction was terminated by adding 120 mM EDTA to the assay mixture. Fifty-μl aliquots of each sample were transferred to microtiter plate modules precoated with streptavidin and incubated for 30 min at 37°C. After three washes with PBS, peroxidase-labeled antiphosphotyrosine antibody was added to each well followed by peroxidase substrate. The phosphorylated substrate was measured at 405 nm using a Spectromax Scanning Microplate Reader. Controls and standards were

Received 6/6/96; accepted 7/16/96.

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[1] To whom requests for reprints should be addressed, at Department of Neurosurgery, Louisiana State University Medical Center, 1501 Kings Highway, Shreveport, LA 71130.

[2] The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; wt, wild type; CCS, cosmic calf serum.

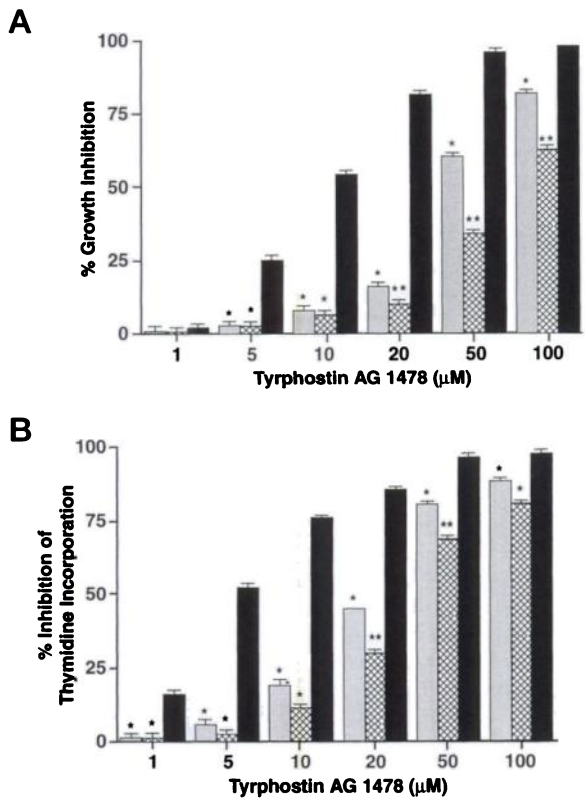

Fig. 1. Effects of AG 1478 on glioma cell growth and DNA synthesis. *A*, effects of AG 1478 on cell growth were analyzed using the Alamar Blue assay. The absorbance of Alamar Blue in AG 1478-treated cells was converted into percentage of inhibition to that of cells without AG 1478 treatment. *B*, effect of AG 1478 on DNA synthesis was assayed using [^{3}H]thymidine incorporation. The percentage of inhibition of AG 1478 is calculated as the ratio of radioactive counts incorporated cells treated with AG 1478 to those from untreated cells. Data represent means of four experiments. *Bars*, SE. U87MG, *gray column*; U87MG.wtEGFR, *hatched column*; U87MG.ΔEGFR, *black column*. *, statistically significant difference of U87MG cells or U87MG.wtEGFR cells *versus* U87MG.ΔEGFR cells; **, statistically significant difference of U87MG.wtEGFR cells *versus* U87MG and U87MG.ΔEGFR cells as analyzed by one-way ANOVA (Tukey's Studentized range test, $P < 0.01$).

performed at the same time in accordance with the manufacturer's instruction manual.

Immunoblotting. Confluent cells were serum starved for 15 h and lysed in lysis buffer. To test the effect of AG 1478 on the EGFR autophosphorylation, cells were treated with various concentrations of AG 1478 for 2 h, then exposed to 100 ng/ml of EGF (R&D, Minneapolis, MN) for 10 min at 37°C before cell lysis. Protein concentration of cell lysates was determined using the bicinchonic acid method (Pierce Chemical Co., Rockford, IL). Equal amounts of proteins were electrophoresed through SDS/7.5% polyacrylamide gels and transferred to nitrocellulose membranes (Micron Separations, Inc., Westboro, MA). Blots were probed with anti-EGFR antibody (catalogue no. E-3138; Sigma) or antiphosphotyrosine antibody (catalogue no. P3300; Sigma) treated with alkaline phosphatase-labeled secondary antibody (Bio-Rad, Hercules, CA), and the proteins were visualized using the ECL detection system (Bio-Rad).

Results and Discussion

Amplification and mutation of the *EGFR* gene has been implicated in the pathogenesis of a variety of human cancers, including those of brain, breast, lung, and ovary (5, 15). The most common and well-characterized type of mutation in human gliomas involves an in-frame truncation of 801 bp of the extracellular domain in the EGFR (1–5, 16). Since cell lines derived from primary tumors lose these mutated alleles when maintained in tissue culture (17), we constructed related U87MG human glioma cells, which overexpressed wt or truncated EGFR, to mimic the *in vivo* situation and to determine whether glioma cells with this defined EGFR mutation would respond differently to drug treatment. We had previously shown that cells expressing ΔEGFR had little difference in growth rate compared to parental cells when cultured in 10% or 2% serum (6). However, when grown under conditions of serum starvation, ΔEGFR cells continue to cycle for an extended time compared to the parental cells. Here, we serum starved cells for a short 16-h period before exposing cells to the various kinase inhibitors. This approach was used to screen a variety of inhibitors for their specificity for various *EGFR* gene mutations. Here, we tested the effects of tyrphostin AG 1478 on these glioma cell lines. The results showed that AG 1478 inhibited cell growth (Fig. 1*A*) and DNA synthesis (Fig. 1*B*) of each glioma cell line in a dose-dependent manner, but with different potencies (IC_{50} for growth inhibition of 8.7 ± 0.58 μM in U87MG.ΔEGFR, 48.4 ± 1.1 μM in U87MG.wtEGFR, and 34.6 ± 1.0 μM in U87MG cells and IC_{50} for DNA synthesis of 4.6 ± 0.8 μM in U87MG.ΔEGFR, 35.2 ± 2.1 μM in U87MG.wtEGFR, and 19.67 ± 1.6 μM in U87MG cells).

To further examine the preferential inhibitory effects of AG 1478 on ΔEGFR, we assessed the tyrosine kinase activities and autophosphorylation status of the receptors. As shown in Fig. 2, AG 1478 preferentially inhibited the tyrosine kinase activity of the ΔEGFR compared to endogenous or overexpressed exogenous wt EGFR. The M_r 170,000 EGFR was expressed in U87MG and overexpressed in U87MG.wtEGFR (Fig. 3*A*). U87MG.ΔEGFR cells expressed high levels of smaller EGFR species, between M_r 140,000 and 155,000, in addition to the endogenous M_r 170,000 wt EGFR (Fig. 3*A*). Stimulation of serum-starved U87MG.ΔEGFR cells with EGF failed to induce further tyrosine phosphorylation of the M_r 140,000–155,000 ΔEGFR, whereas the endogenous M_r 170,000 wt receptors responded appropriately to EGF (Fig. 3*B*). The autophosphorylation per EGFR molecule was approximately 10-fold lower for ΔEGFR compared to wt EGFR activated by ligand, as 10-fold less endogenous wt EGFR in the same cells (U87MG.ΔEGFR) resulted in similar levels of phosphorylation (Fig. 3*B*). The constitutive autophosphorylation of the M_r 140,000–155,000 EGFR was inhibited by AG 1478 in a dose-dependent manner and at lower concentrations compared with the ligand-activated endogenous M_r 170,000 EGFR in the same cells (Fig. 3*B*) or in different cells (Fig. 3*C*), consistent with its effects on cellular growth. Moreover, AG1478 did not cause a decrease in the number of receptors over the concentrations used here (determined by probing the same Western blots with anti-EGFR and antiphosphotyrosine antibodies; data not shown) but rather inhibited their autophosphorylation. In contrast, autophosphorylation of the wt M_r 170,000 EGFR in U87MG,

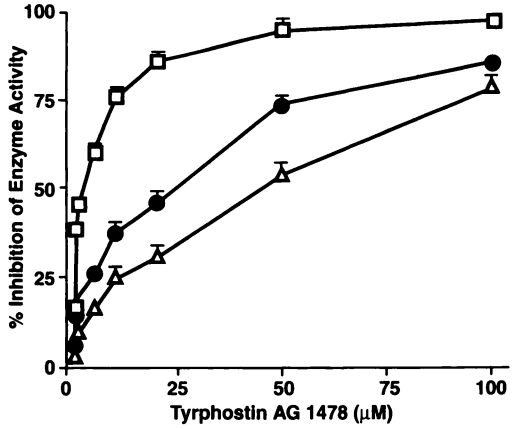

Fig. 2. Effect of AG 1478 on tyrosine kinase activity of various forms of EGFRs. The tyrosine kinase activities in response to AG 1478 treatment were analyzed using an ELISA as described in "Materials and Methods" and converted to percentage of inhibition over controls. Data represent means of three experiments. *Bars*, SE. U87MG, ●; U87MG.wtEGFR,△; U87MG.ΔEGFR, □.

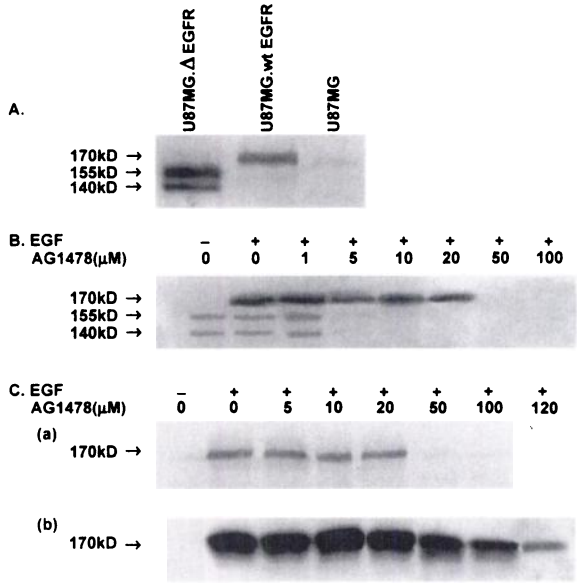

Fig. 3. EGFR expression and effects of AG 1478 on tyrosine phosphorylation of EGFRs determined using Western blotting. *A*, equal amounts of cell lysates were subjected to 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with anti-human EGFR monoclonal antibody. *B* and *C*, serum-starved U87MG.ΔEGFR cells (*B*), U87MG cells (*C*, *a*), and U87MG.wtEGFR cells (*C*, *b*) were exposed to various concentrations (0–120 μM) of AG 1478 for 2 h and treated (+) or not treated (−) with EGF for 10 min before cell lysis. Western blots prepared as in *A* were probed with antiphosphotyrosine monoclonal antibody. wt EGFR, M_r 170,000; ΔEGFR, M_r 140,000–155,000. *kD*, kilodaltons.

U87MG.wtEGFR, and U87MG.ΔEGFR cells was induced by exogenous EGF (Fig. 3, *B* and *C*) and inhibited only when the concentration of AG 1478 was higher than 20 μM for U87MG and U87MG.ΔEGFR cells. However, even at 120 μM, AG1478 still failed to completely inhibit the ligand-induced tyrosine phosphorylation of a similar number of wt EGFR in U87MG.wtEGFR cells (Fig. 3*C*, *b*). Taken together, these results indicate that tyrphostin AG 1478 preferentially inhibits glioma cells with truncated EGFR (M_r 140,000–155,000) as compared to those with endogenous or overexpressed exogenous wt M_r 170,000 EGFR.

Using different tumor cell lines, Osherov and Levitzki (12) and Fry *et al.* (9) have provided evidence that AG 1478 and its analogue, PD 153035, specifically inhibited EGFR tyrosine kinase activities. In the current study, AG 1478 did inhibit the wt M_r 170,000 EGFR, but at a concentration much higher than that required to inhibit the truncated EGFR. Since the three glioma cell populations used in the present study differ only in the form of EGFR expressed, these data suggest that the specific forms of EGFR may exhibit different structural or biochemical properties to account for their altered interaction with AG 1478. Although the precise mechanism for this is not known, our results suggest that specific forms of a tyrosine kinase receptor (*e.g.*, EGFR) may have different affinities and sensitivities to various tyrosine kinase inhibitors. This notion was further supported by our recent observation that ethyl-2,5-dihydroxycinnamate, another new EGFR tyrosine kinase inhibitor, which showed the reverse specificity of AG 1478, preferentially inhibited gliomas with amplified wt EGFR compared to those with the truncated EGFR.[3]

The finding that tyrphostin AG 1478 preferentially inhibited glioma cells with ΔEGFR may hold significant potential for glioma therapy. Several lines of evidence have suggested that truncation mutations of the *EGFR* gene contribute to the high malignancy and invasiveness of human glioblastomas: (*a*) human glioma cells with ΔEGFR showed significantly enhanced tumorigenicity *in vivo* compared with cells with amplified wt EGFR (6); (*b*) cells that expressed ΔEGFR showed a dramatic increase in DNA synthesis in the absence of serum (16) and enhanced transforming activity (5); (*c*) *EGFR* gene truncation has been frequently demonstrated not only in gliomas but also in other cancers, such as those of the breast, lung, and gynecological system (5, 15); and (*d*) ΔEGFR is tumor specific and not present in normal tissue (5), thus making it a unique target for cancer therapy. Additional studies using animal models will provide valuable data concerning the possibility of clinical applications. Since *EGFR* gene truncation resulted in enhanced DNA synthesis, the increased susceptibility of ΔEGFR to AG 1478 may also be used as a valuable tool to study signal transduction pathways mediated by the truncated EGFR in the oncogenesis of these tumors.

Tyrphostin AG 1478 represents the first inhibitor that preferentially inhibits glioma cells with a common truncated form of the EGFR. This preferential inhibitory effect of AG 1478 was due to more selective inhibition of autophosphorylation of the M_r 140,000–155,000 ΔEGFR compared to the M_r 170,000 wt form of the receptor. Our data emphasize that screening and developing anticancer inhibitors should take advantage of specific gene mutations. Inhibitors targeting well-defined gene mutations found in human cancers may offer a greater therapeutic advantage than inhibitors with broader specificities which might also inhibit the wt form of the target gene product found in normal cells.

[3] Unpublished data.

References

1. Sugawa, N., Ekstrand, A. J., James, C. D., and Collins, V. P. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. Proc. Natl. Acad. Sci. USA, *87:* 8602–8606, 1990.
2. Ekstrand, A. J., James, C. D., Cavenee, W. K., Seliger, B., Pettersson, R. F., and Collins, V. P. Genes for epidermal growth factor receptor, transforming growth factor, and epidermal growth factor and their expression in human gliomas *in vivo*. Cancer Res., *51:* 2164–2172, 1991.
3. Ekstrand, A. J., Sugawa, N., James, C. D., and Collins, V. P. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. Proc. Natl. Acad. Sci. USA, *89:* 4309–4313, 1992.
4. Wong, A. J., Ruppert, J. M., Bigner, S. H., Grzeschik, C. H., Humphrey, P. A., Bigner, D. D., and Vogelstein, B. Structural alterations of the epidermal growth factor receptor gene in human gliomas. Proc. Natl. Acad. Sci. USA, *89:* 2965–2969, 1992.
5. Moscatello, D. K., Holgado-Madruga, M., Godwin, A. K., Ramirez, G., Gunn, G., Zoltick, P. W., Biegel, J. A., Hayes, R. L., and Wong, A. J. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer Res., *55:* 5536–5539, 1995.
6. Nishikawa, R., Ji, X-D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavenee, W. K., and Huang, H-J. S. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. Proc. Natl. Acad. Sci. USA, *91:* 7727–7731, 1994.
7. Levitzki, A., and Gazit, A. Tyrosine kinase inhibition: an approach to drug development. Science (Washington DC), *267:* 1782–1788, 1995.
8. Yaish, P., Gazit, A., Gilon, C., and Levitzki, A. Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. Science (Washington DC), *242:* 933–935, 1988.
9. Fry, D. W., Kraker, A. J., McMichael, A., Ambroson, L. A., Nelson, J. M., Leopold, W. R., Connors, R. W., and Bridges, A. J. A specific inhibitor of the epidermal growth factor receptor tyrosine kinase. Science (Washington DC), *265:* 1093–1095, 1994.
10. Miyaji, K., Tani, E., Shindo, H., Nakano, A., and Tokunaga, T. Effects of tyrphostin on cell growth and tyrosine kinase activity of epidermal growth factor receptor in human gliomas. J. Neurosurg., *81:* 411–419, 1994.
11. Westphal, M., and Herrmann, H-D. Growth factor biology and oncogene activation in human gliomas and their implications for specific therapeutic concepts. Neurosurgery, *25:* 681–694, 1989.
12. Osherov, N., and Levitzki, A. Epidermal-growth-factor-dependent activation of the Src-family kinases. Eur. J. Biochem., *225:* 1047–1053, 1994.
13. Fields, R. D., and Lancaster, M. V. Dual-attribute continuous monitoring of cell proliferation/cytotoxicity. Am. Biotechnol. Lab., *11:* 48–50, 1993.
14. Kovalenko, M., Gazit, A., Böhmer, A., Rorsman, C., Rönnstrand, L., Heldin, C-H., Waltenberger, J., Böhmer, F-D., and Levitzki, A. Selective platelet-derived growth factor receptor kinase blockers reverse *cis*-transformation. Cancer Res., *54:* 6106–6114, 1994.
15. Garcia de Palazzo, I. E., Adams, G. P., Sundareshan, P., Wong, A. J., Testa, J. R., Bigner D. D., and Weiner, L. M. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. Cancer Res., *53:* 3217–3220, 1993.
16. Ekstrand, A. J., Longo, N., Hamid, M. L., Olson, J. J., Liu, L., Collins, V. P., and James, C. D. Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with *EGFR* gene amplification. Oncogene, *9:* 2313–2320, 1994.
17. Filmus, J., Pollak, M. N., Cairncross, J. G., and Buick, R. N. Amplified, overexpressed and rearranged epidermal growth factor receptor gene in a human astrocytoma cell line. Biochem. Biophys. Res. Commun., *131:* 207–215, 1985.

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Cancer Res 1996;56:3859-3861.