Isotype-specific Ras-GTP Levels Predict the Efficacy of Farnesyl Transferase Inhibitors against Human Astrocytomas Regardless of Ras Mutational Status

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ABSTRACT

Previous studies have demonstrated that astrocytomas express elevated levels of activated Ras-GTP despite the absence of activating Ras mutations. Farnesyl transferase inhibitors (FTIs) exert their antitumor effect in part through inhibition of Ras-mediated signaling. SCH66336 is a potent FTI presently undergoing clinical trials in patients with solid tumors. We evaluated the efficacy of SCH66336 against a panel of eight human astrocytoma cell lines and three human astrocytoma explant xenograft models in NOD-SCID mice. SCH66336 demonstrated variable antiproliferative effects against the cell lines, with IC_{50} ranging from 0.6 μM to 32.3 μM. Two of the three human glioblastoma multiforme (GBM) xenografts demonstrated substantial growth inhibition in response to SCH66336, with up to 69% growth inhibition after 21 days of treatment. Drug efficacy could be accurately predicted using a combination of the H-, K-, and N-isotype-specific Ras-GTP levels. These data indicate that the absence of Ras mutations does not preclude chemotherapeutic efficacy by FTIs, that Ras is likely a major target of FTIs regardless of Ras mutational status, and that isotype-specific Ras-GTP levels are a promising marker of drug efficacy.

INTRODUCTION

Activated GTP-bound Ras is a potent activator of intracellular signaling pathways, and its vital role is exemplified by the presence of oncogenic (activating) Ras mutations in approximately 25% of human malignancies (1). Inactive Ras-GDP is activated to the GTP-bound form by numerous upstream activators, and Ras-GTP is rapidly inactivated to Ras-GDP through an intrinsic GTPase activity catalyzed by p120-GAP and neurofibromin (2, 3). Oncogenic/activating Ras mutations lock Ras in its GTP-bound form (4), resulting in malignant transformation (5). Such activating mutations have not been identified in human astrocytomas, including the most malignant form, termed GBM (1, 6). However, these tumors are characterized by the overexpression of ligand-dependent - and independent growth factor receptors (7–13). We have demonstrated in previous studies (14–16) that receptor-induced Ras activation is a common feature of GBMs and their derived cell lines, regulating both proliferative and angiogenic signals.

FTIs represent a promising novel class of molecularly targeted chemotherapeutic agents. These drugs inhibit the critical initial step in the post-translational modification of Ras (17, 18) and other farnesylated proteins (19). This step, catalyzed by farnesyl protein transferase, involves the transfer of a 15-carbon trans,trans-farnesyl moiety from farnesyl PP to the cysteine residue on the CAAX (C = cysteine; A = aliphatic amino acid; X = any other amino acid) motif at the COOH-terminal of Ras. Although initially intended to inhibit the proliferation of tumors in which the presence of oncogenic/activating Ras mutations resulted in persistently elevated levels of Ras-GTP, there is growing evidence that such agents also inhibit the proliferation of human cancer cell lines lacking such mutations (20–22); e.g., we have demonstrated recently (22) that the FTI L-744,832 can inhibit the growth of established GBM cell lines by reducing cell cycle progression through both G_{1}-S and G_{2}-M, as well as by inducing apoptosis, even under anchorage-dependent conditions. Furthermore, L-744,832 potently inhibits the secretion of VEGF by these cells and, hence, may also demonstrate an antiangiogenic effect in vivo (22).

The FTI SCH66336 has recently completed Phase I clinical trials (23) with prior studies (24, 25) demonstrating promising effects against tumor cell lines in culture and in animal studies, although its efficacy against astrocytomas has not been reported previously. In the present paper, we demonstrate that SCH66336 decreases the viability of astrocytoma cells and inhibits the growth of human GBM explant xenografts in NOD-SCID mice. More importantly, we demonstrate that isotype-specific H-, K-, and N-Ras-GTP levels can accurately predict drug efficacy. These findings support the hypothesis that Ras is a major therapeutic target of FTIs even in tumors lacking oncogenic Ras mutations and that tumors with high levels of H-Ras-GTP are most sensitive to growth inhibition by these agents.

MATERIALS AND METHODS

Determination of Drug Efficacy against Human Astrocytoma Cell Lines. SCH6636 was obtained from Schering-Plough Research Institute (a gift of Dr. W. Robert Bishop, Kenilworth, NJ). SCH6636 is a novel trihalobenzocycloheptapyridine FTI (M, 638.8) modified from agents identified through a random drug screening program (26). U373 and U-343C32.6 cells were a gift of R. Westernmark (Uppsala, Sweden); U118 cells were a gift of C. David James (Mayo Clinic, Rochester, MN); U138 and U87 cells were obtained from the American Type Culture Collection (Rockville, MD); and U251, SF763, and SF767 cells were a gift of Dolores Dougherty (University of California-San Francisco Brain Tumor Research Center). All of the cells were grown at 37 °C in a 5% CO_{2} incubator in DMEM supplemented with 10% calf serum. To measure cell viability, 1000 cells were plated in 96-well plates and treated with SCH66336 (1 μM-100 μM) for 10 days, with the number of viable cells determined using the Cell Titer 96 Aqueous One Solution kit (Promega, Madison, WI; Ref. 27). Control cells were grown in medium alone or in medium supplemented with the vehicle (0.1% v/v DMSO). Dose-response curves were determined by modeling a log-normal dose-response relationship, with the IC_{50} defined as the dose at which the number of cells in the treatment well was 50% of that in control wells (Table 1).

Determination of Drug Efficacy against Human Explant Xenograft Models. NOD/SCID mice (NOD/LtSz-Prkdc scid/J mice; Jackson Laboratory, Bar Harbor, ME) between 6–12 weeks of age were used to propagate human GBM explants obtained at craniotomy. Preliminary studies determined that the tumors grew more predictably in these mice than in BALB/c nude mice or SCID beige mice (data not shown). Pathologically verified GBM operative specimens were cut into approximately 2 × 2 × 2-mm fragments, and four to five pieces were implanted into the s.c. space of the right flank of each mouse.
with 20% HP based on twice-weekly mouse weights. Vehicle-treated mice were provided the regression model: IC\textsubscript{50} (predicted) = 2.0 - 225.8[relative H-Ras\textsubscript{GTP}] + 169.8[relative K-Ras\textsubscript{GTP}] + 103.8[relative N-Ras\textsubscript{GTP}].

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Three such xenografts (XEN01, XEN05, and XEN08) were used for this study, with IHC analysis of tumors from each passage confirming the maintenance of histological and molecular characteristics similar to the original human GBM tumor.

SCH66336 was dissolved in 20% w/v HP\textsubscript{BID} and dosed at 50 mg/kg p.o. bid by oral gavage (volume, 100 \mu l), with the administered dose adjusted based on twice-weekly mouse weights. Vehicle-treated mice were provided with 20% HP\textsubscript{BID} by twice-daily oral gavage. After the tumor volume (V = \frac{a^2h}{2}, where a < h) had reached approximately 200–250 mm\textsuperscript{3}, mice were randomized to receive either SCH66336 or HP\textsubscript{BID}. The study was continued for 21 days, with twice-weekly mouse weight and tumor volume determination. Mice were sacrificed at the conclusion of the study, with portions of the tumor either flash-frozen in liquid nitrogen (to measure levels of Ras\textsubscript{GTP}) or fixed in 10% buffered formalin (IHC analyses). Percentage growth inhibition \{\frac{(\text{volume in drug group at day } 0) - (\text{volume in vehicle group at day } 21)}{\text{volume in drug group at day } 0}\} was calculated to determine the effect of SCH66336 on tumor growth (Fig. 1). The percentage growth inhibition was calculated twice weekly throughout the study, and the mean of these individual calculations was used to estimate the overall mean percentage growth inhibition. The growth fraction [1 – (percentage growth inhibition/100)] was calculated for use in the regression analyses.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded tissue sections from vehicle and drug-treated tumors were subjected to standard IHC analysis (Fig. 2). Primary antibodies were used to detect Ki-67 (polyclonal rabbit antibody #A0047; DAKO, Carpinteria, CA; used at 1:400), VEGF (polyclonal rabbit antibody #PU360-UP; BioGenex, San Ramon, CA; used at 1:400), and Factor VIII (rabbit polyclonal antibody #A0082; DAKO; used at 1:1250). Secondary antibody was a goat antimouse antibody (Zymed) used at 1:200, and antigens were detected using the avidin-biotin complex method (Vector Laboratories) and diaminobenzidine substrate. Apoptotic nuclei were detected using the TUNEL assay (28), and detection was performed according to the manufacturer’s suggestions (In Situ Cell Death Detection kit-Po, Boehringer Mannheim).

**Ras\textsubscript{GTP} and Isotype-specific Ras\textsubscript{GTP} Assays.** A previously described (14, 29, 30) luciferase-based enzymatic technique was used to determine levels of activated Ras\textsubscript{GTP} in both cells and in flash-frozen tissue specimens. This protocol was modified to determine isotype-specific Ras\textsubscript{GTP} levels, with an additional immunoprecipitation step using a polyclonal antibody specific for H-Ras (sc-520) and monoclonal antibodies specific for K-Ras (sc-30) and N-Ras (sc-31; Santa Cruz Biotechnology, Santa Cruz, CA; Ref. 31). Lysates were immunoprecipitated with two of these isofrom-specific antibodies for 1 h at 4°C, before subsequent immunoprecipitation with the neutralizing pan-anti-Ras Y13-259 antibody (Oncogene Science), which locks Ras in either its GDP- or GTP-bound state; e.g., H-Ras-specific Ras\textsubscript{GTP} levels were determined from the supernatant of lysates precipitated with anti-K-Ras and anti-N-Ras antibodies. The affinity and specificity of the Ras isotype-specific antibodies were confirmed using lysates of cells overexpressing individual isotypes (a gift of Santa Cruz Biotechnology) and in experiments using astrocytoma cell and tumor lysates (Fig. 3). Total Ras\textsubscript{GTP}, as well as H-, K-, and N-Ras-specific Ras\textsubscript{GTP} levels were determined in duplicate from each

<p>| Table 1 Total and isofrom-specific Ras\textsubscript{GTP} levels in astrocytoma cells and control rodent fibroblasts |
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Fig. 1. Effect of SCH66336 on the growth of xenograft models was evaluated in NOD/SCID mice bearing s.c. flank XEN01, XEN05, or XEN08 GBM xenografts. Mice were dosed twice daily by oral gavage with either 100 \mu l of 20% w/v HP\textsubscript{BID} or 50 mg/kg SCH66336 in a total volume of 100 \mu l. 100 \mu l of 20% w/v HP\textsubscript{BID} demonstrated relatively little antitumor effect against XEN01 xenografts, with a mean tumor growth inhibition of 19.4 ± 11.2% (\textit{a}). In contrast, SCH66336 demonstrates significant antitumor effect against both XEN05 and XEN08 xenografts, with a mean growth inhibition of 68.7 ± 9.3% for XEN05 (\textit{b}) and of 63.8 ± 5.0% for XEN08 (\textit{c}).
Fig. 2. a, photomicrographs of sections of xenograft GBM tumors (XEN08 model) from the s.c. space of NOD/SCID mice. Top panels are from vehicle-treated mice (A and B), whereas the bottom panels are from mice treated with 50 mg/kg p.o. bid SCH66336 (C and D). Vehicle-treated tumors demonstrate large regions of central necrosis (H&E staining; B, star; original magnification, ×100), with a virtual absence of TUNEL-positive cells within the solid portions of the tumor (A, arrow; original magnification, ×400). SCH66336-treated tumors were much smaller and demonstrate smaller regions of central necrosis but frequent regions of geographic necrosis throughout the tumor. Clusters of TUNEL-positive cells were seen throughout the drug-treated tumors (C; original magnification, ×200). A number of blood vessels in drug-treated tumors reveal TUNEL-positive cells within the vessel wall, suggesting an antiangiogenic effect of SCH66336 (D; original magnification, ×400), a feature that was not observed in the vehicle-treated tumors. b, photomicrographs of sections of xenograft GBM tumors (model XEN08) from the s.c. space of NOD/SCID mice. Left panels are from vehicle-treated mice, whereas the right panels are from mice treated with 50 mg/kg p.o. bid SCH66336 for 20 days. Sections were stained with H&E and probed for Ki-67, VEGF, and Factor VIII. Drug-treated tumors demonstrate an abnormal cytoarchitecture compared with vehicle-treated tumors (H&E). Both drug-treated and vehicle-treated tumors demonstrate the presence of Factor VIII-positive structures. These structures failed to form mature vascularized blood vessels in SCH66336-treated tumors (H&E). Vehicle-treated tumors demonstrate robust VEGF staining, even in solid tumor remote from the central necrosis, whereas drug-treated tumors demonstrate weak VEGF staining, even surrounding areas of necrosis. The Ki-67 labeling index appears uniformly high in vehicle-treated tumors, whereas in SCH66336-treated tumors, most of the tumor demonstrates very low or absent Ki-67 labeling. Original magnification, ×100 for H&E and Factor VIII; 200 × for Ki-67 and VEGF.
...with this effect, the mean tumor weight of SCH66336-treated XEN05 (n = 17; 9 vehicle, 8 SCH66336), with a mean growth inhibition of 63.8 ± 5.0% (P < 0.0001; Fig. 1c). Drug-treated XEN08 tumors weighed 1.04 ± 0.19 g at harvest, compared with 1.88 ± 0.23 g for vehicle-treated tumors (a 44.9% reduction; P = 0.0136). HP/BCD alone did not affect the growth of these tumors, in comparison with mice given 100 μl of drinking water twice-daily by oral gavage (data not shown; n = 19; P = 0.88).

III. Analysis of SCH6636-treated Tumors. Formalin-fixed, paraffin-embedded sections from each GBM xenograft were subjected to IHC analysis. Vehicle-treated tumors grew very large, with extensive central necrosis (Fig. 2a, part B), a feature not found in the much smaller SCH6636-treated tumors. In contrast, the presence of TUNEL-positive tumor cells was unusual in vehicle-treated tumors (Fig. 2a, Part A) but was a common feature of SCH6636-treated GBMs (Fig. 2a, Part C). Individual blood vessels in drug-treated tumors revealed the presence of TUNEL-positive endothelial cells, suggesting an antiangiogenic effect that contributed to the overall decreased tumor growth in SCH6636-treated animals (Fig. 2a, part D). Apoptosis was not a feature of the tumor-associated blood vessels in the vehicle-treated GBMs (data not shown).

Viable regions of vehicle-treated tumors demonstrated extensive Ki-67 and bromodeoxyuridine labeling, many vascularized and Factor VIII-positive blood vessels, and robust VEGF expression by the tumor cells. In contrast, few components of drug-treated tumors were viable, with generally absent Ki-67 and bromodeoxyuridine labeling, a disorganized histological architecture, and weak VEGF expression (Fig. 2b). Both vehicle- and drug-treated tumors contained numerous Factor VIII-positive vascular structures. However, vehicle-treated tumors were well vascularized, with numerous red blood cells seen in H&E sections from these tumors. In contrast, drug-treated tumors lacked such evidence of vascularization (Fig. 2b).

Prediction of Drug Efficacy from Isotype-specific Ras-GTP Levels. Total and isotype-specific Ras-GTP levels were measured in all of the eight human malignant astrocytoma cell lines (Table 1). Total Ras-GTP levels did not help predict drug efficacy in the cell lines. Furthermore, H-Ras, K-Ras, and N-Ras isotype-specific Ras-GTP levels were relatively poor individual predictors of drug efficacy. However, forward stepwise multiple regression analysis demonstrated that the overall model comprised of the combination of H-, K-, and N-Ras-GTP levels was highly predictive of the IC50 (R2 = 0.9255; F = 16.5587; P = 0.0102). As depicted in Table 1, the “predicted IC50” from this regression analysis and derived from the overall model closely approximates the “actual IC50” of the astrocytoma cell lines. As shown in Fig. 4, a–d (top), increasing K- and N-Ras-GTP levels predict for greater drug resistance, whereas increasing H-Ras-GTP levels predict for greater drug efficacy.
Similar analyses were carried out for the human GBM xenograft models in NOD-SCID mice, with total and isotype-specific Ras-GTP levels measured from two tumors (denoted as A and B) from each of the three xenograft models (XEN01, XEN05, and XEN08; Table 2). To aid in the comparison of the cell line and xenograft data, IC₅₀ was approximated in the xenografts by expressing drug efficacy as a growth fraction. The growth fraction was defined as the mean tumor volume increase over the course of the experiment in SCH66336-treated tumors in comparison with the mean volume increase in vehicle-treated GBMs was given a score of 1.0; thus, a growth fraction of either total or any isotype-specific Ras-GTP levels predicted correlated in the cell lines (Fig. 3, a–d) and xenograft data (Fig. 4, a–h). To approximate the concept of an IC₅₀, in which higher levels imply greater drug resistance, the model for the xenografts predicts the growth fraction. The growth fraction is defined as the growth of drug-treated tumors in comparison with vehicle-treated tumors, with vehicle-treated tumors given a value of 1.0. The strongest drug efficacy was observed for the combination of H-, K-, and N-Ras GTP levels. Predicted and actual IC₅₀ levels were closely correlated in the cell lines (Fig. 3, a and Table 1), as were predicted, and actual growth fractions in the xenograft tumors (Fig. 4, and Table 2). The horizontal dashed line in each graph represents the mean of all of the values. Leverage plots (b–d and f–h) depict the effect on residuals if that variable was removed from the model (49). These leverage plots demonstrate that increasing H-Ras-GTP levels correlate with increased drug sensitivity and reduced IC₅₀ growth fraction (b and f), whereas increasing K- or N-Ras-GTP levels correlate with increased drug resistance and increased IC₅₀ growth fraction (c, g, and h). Dashed curves around the regression line indicate the 95% confidence interval for each regression line.

DISCUSSION

FTIs represent one of the first classes of antineoplastic agents designed through rational drug design. The high prevalence of activating Ras mutations in human cancers (1) initially suggested that approximately 25% of human malignancies may demonstrate sensitivity to such agents (32). However, two critical observations have made this prediction more complex. First, the presence or absence of Ras mutations does not appear to be the sole determinant of FTI efficacy, at least in cell lines (21). Second, H-Ras-transformed cells appear to be more sensitive to these agents than those transformed by either K-Ras or N-Ras (20, 31, 33, 34).

The first observation has cast doubts on whether Ras is a major therapeutic target of FTIs in vivo. This is because in addition to Ras, approximately 0.5% of all of the cellular proteins undergo farnesylation (19), including nuclear laminin A and B, skeletal muscle phosphorylase kinase, and retinal proteins, as well as other members of the Ras superfamily such as Rap2 proteins and RhoB (17, 35–37). Of these, RhoB in particular has been identified as an important putative antineoplastic target of FTIs (38–41). The second observation was initially disappointing in terms of human cancers, because K-Ras mutations occur more frequently in human malignancies than H-Ras mutations (1). Two factors appear to explain this observation (42). First, K-Ras4B has a 10–20-fold greater affinity for farnesyl protein transferase, the target of FTIs, necessitating much higher levels to inhibit the post-translational processing of this Ras isotype (43). Second, N-Ras, K-Ras4A, and K-Ras4B can also act as substrates for geranylgeranyl protein transferase-1 (43), which catalyzes the addition of a 20-carbon geranylgeranyl isoprenyl group to the cysteine residue of the CAAX motif at the COOH terminal of proteins such as Ras. Under physiological conditions, these Ras isotypes undergo farnesylation almost exclusively, but when treated with FTIs, K- and N-Ras, but not H-Ras, can be efficiently geranylgeranylated.
measured by a RhoB-specific antibody on Western blot analysis were cated in transformation and appears to be a major therapeutic target of xenografts, because this farnesylated small G-protein has been impli-

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ined with low levels of K-Ras consistent with the known pharmacodynamics of FTIs, as discussed a large amount of the aberrant growth-promoting signals was being

mice. Total Ras of the three human GBM explant xenografts grown in NOD-SCID Furthermore, SCH66336 was effective in inhibiting the growth of two

xenografts (resistant) and XEN08 (sensitive) xenografts express similar levels of (89)

ACKNOWLEDGMENTS

We thank Dr. W. Robert Bishop (Schering-Plough Research Institute, Kenilworth, NJ) for providing the SCH66336 agent.

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