The Selective Tyrosine Kinase Inhibitor STI571 Inhibits Small Cell Lung Cancer Growth

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ABSTRACT
At least 70% of small cell lung cancers express the Kit receptor tyrosine kinase and its ligand, stem cell factor (SCF). Numerous lines of evidence have demonstrated that this coexpression constitutes a functional autocrine loop, suggesting that inhibitors of Kit tyrosine kinase activity could have therapeutic efficacy in this disease. STI571, formerly known as CGP 57148B, is a p.o. bioavailable 2-phenylaminopyrimide derivative that was designed as an Abl tyrosine kinase inhibitor, but also has efficacy against the platelet-derived growth factor receptor and Kit in vitro. Pretreatment of the H526 small cell lung cancer (SCLC) cell line with STI571 inhibited SCF-mediated Kit activation with an IC₅₀ of 0.1 μM as measured by inhibition of receptor tyrosine phosphorylation and 0.2 μM as measured by immune complex kinase assay. This paralleled the inhibition of SCF-mediated growth by STI571, which had an IC₅₀ of ~0.3 μM. Growth inhibition in SCF-containing medium was accompanied by induction of apoptosis. STI571 efficiently blocked SCF-mediated activation of mitogen-activated protein kinase and Akt, but did not affect insulin-like growth factor-1 or serum-mediated mitogen-activated protein kinase or Akt activation. Growth of five of six SCLC cell lines in medium containing 10% FCS was inhibited by STI571 with an IC₅₀ of ~5 μM. Growth inhibition in serum-containing medium appeared to be cytostatic in nature because no increase in apoptosis was observed. Despite this growth inhibition, STI571 failed to enhance the cytotoxicity of either carboplatinum or etoposide when coadministered. However, taken together with the minimal toxicity that this compound has shown in preclinical studies, these data suggest that STI571 could have a role in the treatment of SCLC, possibly to block or slow recurrence after chemotherapy-induced remissions.

INTRODUCTION
Protein tyrosine kinases play a critical role in intracellular signal transduction pathways leading to diverse cellular responses, such as proliferation, apoptosis, and differentiation (1, 2). Consequently, these enzymes have become primary targets for the development of novel therapeutics designed to block cancer cell proliferation, metastasis, angiogenesis, and promote apoptosis (3, 4). The strategy that has progressed farthest in clinical development is the use of monoclonal antibodies to target growth factor receptor tyrosine kinases (5). The use of small molecule tyrosine kinase inhibitors, however, could have significant theoretical advantages over monoclonal antibodies. Small molecule inhibitors could have better tissue penetration, could have activity against intracellular targets, and could be designed to have oral bioavailability. Their interaction with the target enzyme can be more easily studied through the use of X-ray crystallography and computer modeling, and their structure could be more easily engineered to give the drug more specificity and more favorable pharmacokinetic properties. Several lead compounds have shown promising activity against such targets as the EGFR (6, 7) and the vascular endothelial cell growth factor receptor (8).

The compound STI571 (formerly known as CGP 57148B) is a 2-phenylaminopyrimide derivative that was optimized for selective inhibition of the Bcr-Abl tyrosine kinase (9, 10), which is a fusion protein that results from the pathogenic 9:22 translocation that occurs in 95% of CML (11). The drug showed a cellular IC₅₀ for inhibition of Bcr-Abl kinase activity of ~0.25 μM and inhibited growth of cell lines engineered to be dependent on Bcr-Abl kinase activity at 1 μM (10, 12). In addition, STI571 also selectively inhibited the growth of continuous cell lines derived from CML patients, as well as primary CML progenitors in clonogenic assays, at low micromolar concentrations (10, 13, 14). Recently, it has been demonstrated that the drug, given at pharmacokinetically appropriate doses, can completely block the outgrowth of CML cells in nude mice xenografts and cause complete regression of the majority of established tumors with oral dosing without detectable systemic toxicity (15). Although STI571 appears to be a potentially exciting new treatment for CML patients not curable with allo-

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The abbreviations used are: EGFR, epidermal growth factor receptor; SCLC, small cell lung cancer; SCF, stem cell factor; CML, chronic myelogenous leukemia; IGF-1, insulin-like growth factor-1; PDGFR, platelet-derived growth factor receptor; IP, immunoprecipitation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; MAP, mitogen-activated protein.

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geneic bone marrow transplantation (16), it could also be potentially useful for the treatment of other malignancies. This is attributable to the fact that although STI571 is highly selective for Bcr-Abl and c-Abl versus most other tyrosine kinases, it does inhibit the PDGFR with similar efficacy to Bcr-Abl (12). Preliminary evidence also suggests that the drug can also inhibit the Kit receptor tyrosine kinase in vitro.4 This cross-inhibition is likely secondary to a conserved structure of the ATP-binding pocket, the site of drug interaction, among these kinases. Thus, we hypothesized that STI571 might also be effective against malignancies in which Kit activation plays an important role.

The Kit growth factor receptor tyrosine kinase is in the same subclass as the receptors for platelet-derived growth factor and colony-stimulating factor-1 (17). Kit’s ligand is SCF (alternatively named mast cell growth factor or steel factor), a hematopoietic growth factor that, in conjunction with other hematopoietic growth factors, supports the proliferation and differentiation of multiple hematopoietic cell lineages from early precursors (18). In addition to hematopoietic cells, Kit is also normally expressed in melanocytes and germ cells, as well as in a variety of hematological malignancies and solid tumors, including SCLC. In fact, >70% of SCLC cell lines and tumor specimens coexpress Kit and its ligand, SCF (19–21), leading to the hypothesis that this coexpression constitutes an autocrine growth loop. Evidence supporting a SCF/Kit autocrine loop includes the observation that growth and chemotaxis of selected SCLC cell lines is stimulated by exogenous SCF (22, 23). In addition, we demonstrated that reconstitution of SCF and Kit coexpression in a SCLC cell line led to enhanced growth factor independence, and inhibition of Kit activation by transfection of a dominant-negative c-kit gene resulted in a loss of growth factor independence (23, 24). Furthermore, the quinoxaline tyrphostin AG 1296, an inhibitor of Kit and the PDGFR, completely blocked SCF-dependent growth of the H526 cell line and promoted apoptosis within 48 h (23). Treatment of a representative panel of SCLC cell lines grown in FCS with an optimal concentration of AG 1296 led to growth inhibition averaging ~50% in five of six responding cell lines. The use of AG 1296, however, is limited by its poor solubility in aqueous solution. The purpose of the present study was to determine whether the more soluble and p.o. bioavailable STI571 would inhibit SCF-dependent Kit activation and growth of SCLC cell lines.

**MATERIALS AND METHODS**

**Cell Growth.** SCLC cell lines were grown in RPMI 1640 medium supplemented with 2 mM l-glutamine, with (complete medium) or without 10% fetal bovine serum (Life Technologies, Gaithersburg, MD); when grown in the absence of serum, 0.1% BSA (Sigma, St. Louis, MO) was added to the medium. Where indicated, serum-free medium was supplemented with recombinant SCF (Intergen, Purchase, NY) or IGF-1 (R&D, Minneapolis, MN) at the indicated concentrations. The H146, H209, H432, H510, and H526 cell lines have been previously characterized by Carney et al. (25). The WBA cell line was grown from the involved bone marrow aspirate of an untreated patient, and it coexpresses SCF and Kit, as well as N-myc (26). Cells were stimulated with SCF and IGF-1 after preincubation in serum-free medium overnight. Cell growth was measured using the MTT (Sigma) colorimetric dye reduction method, an assay shown to correlate very well with viable SCLC cell number under the conditions used (26). Duplicate plates containing eight replicate wells per assay condition were seeded at a density of 1 × 10^4 cells in 0.1 ml of medium, and data were expressed as the change in absorbance at 540 nm over 72 h, relative to initial values obtained 3 h after plating. STI571 was solubilized in DMSO; final concentration of DMSO in all cultures, including controls, was 0.1%. Stock solutions of carboplatinum (kindly provided by Bristol Myers Squib, Princeton, NJ) and etoposide (Calbiochem, San Diego, CA) were also made up at 1000-fold the desired final concentration in H_2O and DMSO, respectively.

**Assays for Apoptosis and Tritiated Thymidine Incorporation.** DNA fragmentation was analyzed using the TUNEL technique (27), which was performed with the In Situ Cell Death Detection Kit (Boehringer-Mannheim, Indianapolis, IN) using the protocol supplied by the manufacturer. Fluorescence was quantitated using a Becton Dickinson FACScan flow cytometer with the FACScan research software package; nuclear labeling was confirmed by fluorescence microscopy. Caspase-3 activity was determined using the CaspACE assay system (Promega, Madison, WI). Briefly, after a 48-h incubation under the indicated conditions, cell pellets were resuspended in cell lysis buffer at a concentration of 10^6 cells/ml. To achieve cell lysis, cells were frozen at −80°C overnight, thawed, and incubated on ice for 15 min. Lysates were cleared by centrifugation at 15,000 × g for 20 min in the cold, and 300 μg of supernatant protein were used for the colorimetric assay exactly as described in the manufacturer’s protocol. Tritiated thymidine incorporation was determined after plating 2 × 10^5 cells in eight replicate wells of a microplate dish containing 0.1 ml of medium to which 1 μCi of tritiated thymidine (DuPont NEN, Boston, MA) had been added. Cells were then transferred to glass fiber filter plates using an automated cell harvester (Packard Instruments, Meridian CT), extensively washed with H_2O, and counted using a liquid scintillation counter.

**IP, Immune Complex Kinase Assay, and Western Blotting.** H526 cells were lysed in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP40, 1.5 mM MgCl_2, 1 mM EGTA, 10% glycerol, 0.2 mM NaVO_4, 100 μg/ml phenylmethylsulfonfluoride, 1 μg/ml aprotonin, and 10 μg/ml leupeptin using a Dounce homogenizer with a tight-fitting pestle; protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL). The lysate, containing 1–1.5 mg of protein, was centrifuged for 10 min at 10,000 × g to obtain a soluble postnuclear supernatant. IP was initiated by the addition of 10 μg of monoclonal anti-Kit antibody (K45: NeoMarkers, Fremont, CA), followed by incubation for 2 h at 4°C and by an additional 2 h in the presence of Protein A+G agarose. The IP was washed four times in lysis buffer and then once in PBS. The pellet was aspirated to dryness, and 30 μl of kinase buffer (20 mM 1,4-piperazinediethanesulfonic acid, 10 mM MnCl_2) containing 0.1 μM [γ-^32P]ATP (3000 Ci/mmole; DuPont NEN) was added. The kinase reaction was carried out for 10 min at room temperature and terminated by the addition of an equal volume

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4 E. Buchdunger, unpublished observations.
of 2× SDS sample loading buffer; the reaction was then resolved on a 10% polyacrylamide gel and analyzed by autoradiography. Western blotting was performed using standard procedures, with detection using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). Staining was accomplished using the following antibodies: anti-Kit, 3D6 monoclonal (Boehringer-Mannheim); antiphosphotyrosine, PY20 monoclonal (Transduction Labs, Lexington, KY); anti-activated MAP kinase polyclonal (Promega); anti-phospho-Akt Thr308, Ser473, and pan-Akt polyclonals (New England Biolabs, Beverly, MA).

RESULTS

STI571 Blocks SCF-mediated Kit Activation. As is the case for other receptors in its class, ligand binding induces receptor dimerization, which in turn leads to autophosphorylation and an enhancement of the specific activity of the Kit kinase domain (18). The H526 SCLC cell line expresses high levels of Kit, relatively low levels of SCF, and can grow in serum-free medium containing recombinant SCF (as the sole growth factor), which rapidly induces tyrosine phosphorylation and an increase in the specific enzymatic activity of Kit (23). To determine the effect of STI571 on receptor activation, H526 cells made quiescent by overnight incubation in serum-free medium were pretreated with increasing concentrations of drug or DMSO vehicle for 30 min followed by the addition of SCF. Five min after the addition of SCF, cells were divided into two equal portions, and the degree of Kit activation was determined by assessing the extent of in vivo autophosphorylation by immunoblotting in one portion and in the other portion by directly measuring kinase activity in an IP-kinase assay. As illustrated in Fig. 1, both the kinase assay and the assessment of in vivo tyrosine phosphorylation revealed an IC50 of 0.2 and 0.1 μM, respectively, in good agreement with previous studies on the highly related PDGFR, as well as the originally intended target, Bcr-Abl (9, 12). The residual kinase activity seen at high drug concentrations may be attributable to a small degree of binding reversibility during the IP and kinase reaction, both performed in the absence of additional drug.

STI571 Selectively Inhibits SCF-mediated Growth. To determine the effect of STI571 on SCF-mediated growth, H526 cells were incubated in serum-free medium containing SCF, and increasing concentrations of drug and growth were measured at the end of 3 days using the MTT dye reduction assay. IGF-1-mediated growth was also assayed in the same fashion to determine the specificity of the drug. As illustrated in Fig. 2, SCF-mediated growth was inhibited in a dose-dependent fashion. Increasing concentrations of STI571 were added 30 min before the growth factors. Cell growth was assessed using the MTT colorimetric dye reduction method, which involved measuring the change in absorbance over 72 h. Data are representative of three individual experiments; bars, SD from the mean of eight replicate wells.

Fig. 1 STI571 efficiently inhibited SCF-mediated Kit activation. H526 cells were serum-starved overnight and then either pretreated with DMSO vehicle or increasing concentrations STI571 for 30 min. The cells were then either left unstimulated or stimulated with 100 ng/ml SCF for 5 min, as indicated. Kit was then immunoprecipitated, and the degree of SCF-stimulated activation was analyzed by immune complex kinase assay and autoradiography (top panel) or antiphosphotyrosine Western blotting (middle panel). The Western blot was stripped and restained for Kit (lower panel). The lane labeled IgG represents a mock IP using nonimmune mouse IgG. IC50s were calculated by densitometry; data are representative of a minimum of two replicates of each assay.

Fig. 2 STI571 selectively inhibited SCF-mediated growth. H526 cells were incubated in serum-free medium overnight and then left unstimulated (NS) or stimulated with saturating concentrations of SCF (100 ng/ml) or IGF-1 (20 ng/ml). These values represent twice the average growth factor concentration needed to produce a maximal growth response. Increasing concentrations of STI571 were added 30 min before the growth factors. Cell growth was assessed using the MTT colorimetric dye reduction method, which involved measuring the change in absorbance over 72 h. Data are representative of three individual experiments; bars, SD from the mean of eight replicate wells.
found in serum. To determine whether the drug could inhibit growth under these circumstances, a panel of six representative cell lines was incubated in serum-containing medium for 72 h in the presence of increasing concentrations of STI571, and growth was measured by MTT assay; data on H510 were not illustrated because no significant growth inhibition was observed. The data were expressed as the percentage of growth at the indicated STI571 concentration relative to the DMSO vehicle control. Data are representative of three individual experiments; bars, SD from the mean of eight replicate wells.

Surprisingly, despite the decreased growth of cells treated with STI571 noted by MTT assay, no increase in the appearance of morphologically apoptotic cells over controls was observed in any of the treated cell lines, suggesting a cytostatic mechanism of action. This was opposed to the cell death observed when cells were treated while growing in medium containing SCF as the sole growth factor (Fig. 2). Because it is important to understand whether the drug mediates its effect through cytotoxic or cytostatic mechanisms, we directly assayed for the induction of apoptosis. First, we sought to confirm that the cell death we observed in serum-free medium containing SCF was attributable to apoptosis. H526 cells were exposed to STI571 or DMSO vehicle for 48 h in SCF medium, and apoptosis was assayed using a flow cytometric TUNEL assay. Fig. 4 illustrates the marked increase in terminal deoxynucleotidyl transferase labeling of cells treated with STI571, indicative of DNA

Fig. 3  STI571 inhibited growth of SCLC in serum-containing medium. Six representative cell lines were incubated in serum-containing medium for 72 h in the presence of increasing concentrations of STI571, and growth was measured by MTT assay; data on H510 were not illustrated because no significant growth inhibition was observed. The data were expressed as the percentage of growth at the indicated STI571 concentration relative to the DMSO vehicle control. Data are representative of three individual experiments; bars, SD from the mean of eight replicate wells.

Fig. 4  STI571 induced apoptosis of cells incubated in serum-free medium containing SCF but not of cells incubated in medium containing 10% FCS. A, H526 cells were incubated in serum-free medium containing 100 ng/ml SCF in the presence of either 5 µM STI571 (dotted curve) or DMSO vehicle (solid curve). After 48 h, a TUNEL assay was performed using fluorescently tagged dUTP and analyzed by flow cytometry. Similar results were obtained using 1 and 10 µM STI571. B, H526, H146, and H209 cells were incubated in medium containing 10% FCS and 10 µM STI571, 25 µM etoposide, or DMSO vehicle. After 48 h, caspase-3 activity was analyzed colorimetrically using 500 µg of cellular lysate; specific activity was calculated by comparison to p-nitroaniline standards. Bars, SD of duplicate determinations. Data are representative of three independent experiments.
STI571 Blocks Both SCF-Mediated MAP Kinase and Akt Activation. To confirm that STI571 blocked downstream signal transduction from Kit and to develop some insight into the reasons for its cytotoxic effect on cells grown in SCF versus its cytostatic effect on cells grown in serum, we studied the effect of the drug on activation of MAP kinase and Akt, which lies downstream of phosphorylidyinositol-3 kinase. Activation of these signal transduction proteins was studied by Western blotting using phospho-specific antibodies. As illustrated in Fig. 6, the addition of SCF to quiescent H526 cells resulted in a potent activation of both MAP kinase and Akt that, as expected, was completely inhibited by pretreatment with 10 μM STI571. IGF-1 produced a weak activation of MAP kinase and a potent activation of Akt, which were unaffected by the presence of STI571. The addition of 10% FCS to quiescent cells produced an intermediate activation of MAP kinase, which was unaffected by the presence of the drug; no FCS-induced activation of Akt was seen in three replicate experiments. These experiments suggest that persistent activation of MAP kinase could be partially responsible for the lack of apoptosis of STI571-treated H526 cells when grown in FCS.

STI571 Fails to Synergize with Carboplatinum. It has been demonstrated that treatment of tumors expressing epidermal growth factor family receptors with blocking antibodies sensitizes the tumor cells to the effects of cis-platinum both in vitro and in vivo (28–30). This observation could indicate a specific property of epidermal growth factor family receptors, of the antibodies used, or a general phenomenon relating to the interaction between the chemotherapeutic agent and blockade of necessary receptor tyrosine kinases. Because platinum compounds are an integral part of present chemotherapeutic regimens for SCLC (31, 32), we decided to determine whether STI571 would sensitize SCLC cell lines to the cytotoxic effects of carboplatinum, which was chosen because of its better aqueous solubility compared to cis-platinum. Cells were incubated in the continuous presence of increasing concentrations of carboplatinum in the presence of 5 μM STI571. The 5-μM concentration was chosen because it was the average IC_{50} as well as a potentially achievable serum concentration based on preliminary results of the ongoing Phase I trial (33). The degree of growth inhibition relative to control cells incubated in the presence of carboplatinum alone was determined after 72 h by MTT assay. Three cell lines were chosen for study based on their varying sensitivity to carboplatinum, with IC_{50}s ranging from <0.1 μg/ml to 5 μg/ml, and their varying sensitivity to STI571. As illustrated in Fig. 7, STI571 failed to synergize with the cytotoxic effects of carboplatinum in any of these three cell lines; a fourth cell line (WBA) also showed no synergy (data not shown). On the contrary, in experiments using H526, there appeared to be a mild but reproducible protective effect in the presence of the kinase inhibitor, especially at high carboplatinum concentrations. In addition to carboplatinum, etoposide, the other integral component of SCLC chemotherapy regimens, was also tested in combination with STI571; no enhanced cytotoxicity over that produced by etoposide (1–10 μM) alone could be demonstrated for the combination using H526, WBA, or H432 cell lines (data not shown).

DISCUSSION

We have demonstrated that STI571 efficiently inhibits SCF-mediated Kit activation (in serum-free medium) at concentrations similar to those that inhibit both Bcr-Abl and the PDGFR in cellular assays, with an IC_{50} in the 0.1–0.5 μM range (9, 12). This observation likely reflects the related nature of the ATP-binding sites of these three kinases because the drug is competitive for ATP binding (9). The inhibition of SCF-mediated SCLC growth closely parallels inhibition of Kit kinase activity, with complete inhibition of H526 growth occurring at 1 μM. The drug was clearly selective because the same degree of growth inhibition was not obtained in IGF-1-containing medium until a 10-fold higher concentration was attained.

The selectivity seen in serum-free medium supplemented
with single growth factors was apparently reduced in serum-containing medium. The IC$_{50}$ for H146, which does not express Kit, was $5 \mu M$, which was approximately the average for all of the sensitive cell lines combined. This observation suggests two conclusions. The first is that the contribution of Kit activation to growth of SCLC cell lines in medium containing 10% FCS is likely to be relatively limited because the IC$_{50}$ in serum is far higher than the drug concentration needed to completely block growth mediated by saturating concentrations of SCF. A potentially confounding factor affecting this conclusion could be binding or inactivation of the drug by some component of serum. We feel that this is not likely to be an important factor because the growth inhibition of H526 in serum relatively closely paralleled the growth inhibition seen in serum-free medium containing IGF-1, suggesting that drug inactivation by serum does not significantly contribute to the higher IC$_{50}$. Response to the drug was fairly consistent, despite distinct biological properties of the cell lines used (25) and despite variation in the sensitivity to carboplatinum (Fig. 7) and etoposide. It is worth pointing out, however, that there appears to be some correlation between the degree of endogenous SCF expression and the sensitivity to STI571. Both the H432 and H209 cell lines express high levels of both SCF and Kit (20) and are the most sensitive to drug in serum, whereas H526, which expresses very high levels of Kit and little SCF, is the least sensitive. However, expression levels of SCF cannot explain the growth inhibition of H146 because it expresses no Kit. Therefore, it is likely that there is an additional drug target that is partially responsible for SCLC growth inhibition by STI571, especially at drug concentrations that exceed 2.5 $\mu M$. The identity of this hypothetical target is not known at this time, but given the in vitro selectivity of this compound for a conserved ATP-binding pocket, it is possible that the additional target is a tyrosine kinase related to the PDGFR family or c-ABL. The fact that the relative sensitivity of a panel of cell lines to STI571 parallels their sensitivity to the quinoxaline tyrophostin AG1296 (23), an inhibitor of PDGFR and Kit but not Abl (34), suggests that unidentified drug target may be more likely related to the PDGFR family. In addition, the similar pattern of growth inhibition observed using two tyrosine kinase inhibitors having dissimilar chemical structures suggests that the inhibition is attributable to their ability to inhibit Kit and related kinases. One important question that arises is whether the existence of additional targets of STI571 affects its potential as a therapeutic agent for SCLC. Because the toxicity of the drug at concentrations up to 10 $\mu M$ for nontransformed cells in culture was nonexistent (10, 12) or limited (9, 13) and limited animal studies produced no detectable toxicity (15), it is possible that the additional drug target is selectively activated in SCLC. The existence of an additional drug target may be an advantage because it provides the potential for inhibition of the 25–30% of SCLCs, represented by the H146 cell line, which do not express Kit. If further in vivo toxicity studies confirm little toxicity at concentrations that inhibit SCLC growth, there could be an exploitable therapeutic index even if the effect of STI571 cannot entirely be attributable to inhibition of Kit.

SCLC is generally thought of as a highly chemotherapy-responsive disease, with a variety of multidrug combinations producing response rates of $>80\%$, with a third or more being complete responses (31, 32). Despite this high response rate, virtually all extensive stage patients and 75–80% of limited stage patients (treated with combined modality therapy) suc-
Fig. 7 STI571 did not synergize with the cytotoxic effects of carboplatinum. H432, H209, and H526 cells were incubated in serum-containing medium with increasing concentrations of carboplatinum in the presence or absence of 5 μM STI571. Growth was measured by MTT assay over a period of 72 h; an increase in absorbance of 100% indicates a doubling of viable cell number, and a decrease of 100% indicates complete death of the initial cell population. Data are representative of at least two individual experiments; bars, SD from the mean of eight replicate wells.

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