The Farnesyl Transferase Inhibitor (FTI) SCH66336 (lonafarnib) Inhibits Rheb Farnesylation and mTOR Signaling

ROLE IN FTI ENHANCEMENT OF TAXANE AND TAMOXIFEN ANTI-TUMOR ACTIVITY*

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Lonafarnib (SCH66336) is a farnesyl transferase inhibitor (FTI) that inhibits the post-translational lipid modification of H-Ras and other farnesylated proteins. K- and N-Ras are also substrates of farnesyl transferase; however, upon treatment with FTIs, they are alternatively prenylated by geranylgeranyl transferase-1. Despite the failure to abrogate prenylation of K- and N-Ras, growth of many tumors in preclinical models is inhibited by FTIs. This suggests that the anti-proliferative action of FTIs is dependent on blocking the farnesylation of other proteins. Rheb (Ras homologue enriched in brain) is a farnesylated small GTPase that positively regulates mTOR (mammalian target of rapamycin) signaling. We found that Rheb and Rheb2 mRNA were elevated in various tumor cell lines relative to normal cells. Peptides derived from the carboxyl termini of human Rheb and Rheb2 are in vitro substrates for farnesyl transferase but not geranylgeranyl transferase-1. Rheb prenylation in cell culture was completely inhibited by SCH66336, indicating a lack of alternative prenylation. SCH66336 treatment also inhibited the phosphorylation of S6 ribosomal protein, a downstream target of Rheb, and mTOR signaling. SCH66336 did not inhibit S6 phosphorylation in cells expressing Rhee-CVL, a mutant construct of Rheb designed to be geranylgeranylated. Importantly, expression of Rhee-CVL also abrogated SCH66336 enhancement of tamoxifen- and docetaxel-induced apoptosis in MCF-7 breast cancer cells and ES-2 ovarian cancer cells, respectively. Further, inhibition of Rheb signaling by rapamycin treatment, small interfering RNA, or dominant negative Rheb enhanced tamoxifen- and docetaxel-induced apoptosis, similar to FTI treatment. These studies demonstrated that Rheb is modified by farnesylation, and it is not a substrate for alternative prenylation, and plays a role in SCH66336 enhancement of the anti-tumor response to other chemotherapeutics.

Rheb (Ras homologue enriched in brain) is a GTPase identified as a gene up-regulated in rat brain by synaptic activity and growth factors (1). It is ubiquitously expressed in human tissue and found to be up-regulated in transformed cells (2). For example, Rheb mRNA is higher in SV40-transformed MRC-5 V2 fibroblast as compared with the parental cell line. Tuberous sclerosis (TSC)\(^1\) complex TSC1-TSC2 (hamartin-tuberin) serves as a GTPase-activating protein for Rheb, and overexpression of TSC1-TSC2 results in a reduction in activated Rheb-GTP (3–6). Additionally, mutations in TSC1-TSC2 result in a loss of its tumor suppressor function. These mutations cause tuberous sclerosis, an autosomal dominant genetic disorder that occurs in 1 in 6000 people. TSC is characterized by benign tumors in the brain, heart, kidney, skin, and eyes, and common clinical symptoms of TSC include seizures, mental retardation, autism, and organ failure (7, 8).

TSC1-TSC2 complex is negatively regulated by Akt-mediated phosphorylation of TSC2, and as a result, Rheb activity is increased (9–13). The complex is positively regulated by LKB-stimulated AMP-activated protein kinase kinase-dependent phosphorylation of tuberin, and as a result, Rheb signaling is inhibited (14, 15). Mutations in the LKB tumor suppressor are associated with Peutz-Jeghers syndrome, an autosomal dominant inherited disorder that leads to gastrointestinal polyps and predisposes people to various tumors (16, 17).

Rheb positively regulates mTOR signaling, and overexpression of Rheb induces phosphorylation of mTOR substrates, S6 kinase, and 4EBP-1 (18, 19). Recently, Rheb was found to directly bind mTOR and, in a GTP-dependent manner, stimulate mTOR activity (20, 21). As a consequence, Rheb overexpression in Drosophila causes overgrowth of multiple tissues due to increase in cell size (22–24). Rheb is a farnesylated protein, and upon treatment with FTI-277, Rheb-induced phosphorylation of S6 kinase is inhibited (18, 25). Interestingly, the lack of Rheb results in increased arginine uptake in yeast, a phenotype also induced by mutation in farnesyl transferase (FPT) (26).

FPT catalyzes the transfer of a 15-carbon isoprenyl lipid from farnesyl diphosphate onto a cysteine residue of various protein substrates. FPT recognizes the carboxyl-terminal CAAX box of the substrate (C, cysteine; A, aliphatic amino acid; X, carboxyl-terminal amino acid). Proteins terminating in serine, methionine, glutamine, and alanine are recognized by FPT. Proteins ending in leucine are modified with a 20-carbon isoprenyl by a related enzyme, geranylgeranyl protein transferase-1 (GGPT-1) (27, 28). Farnesylated proteins include Ras, Pre-Lamin A, HDJ2, PTP-CAAX tyrosine phosphatases, CENP-E, and CENP-F (29–33). Due to the frequency of activating RAS mutations in human cancer, farnesyl transferase inhibitors were originally investigated as a way to antagonize

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\(^{1}\) The abbreviations used are: TSC, tuberous sclerosis complex; FPT, farnesyl transferase; FTI, farnesyl transferase inhibitor; GGPT, geranylgeranyl transferase; MAPK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; siRNA, small interfering RNA; RT, reverse transcription; Rheb, Ras homologue enriched in brain; mTOR, mammalian target of rapamycin.

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Ras function. SCH66336 is a selective, nonpeptidic CAAX-competitive inhibitor of FPT (FPT IC50, 1.9 nM; GGPT-1 IC50 >50,000 nM) (34). It has demonstrated broad anti-tumor activity in animal models and is undergoing clinical investigation in human cancer. Preclinically, SCH66336 and other FTIs enhance the anti-tumor activity of taxanes (35, 36).

Membrane localization of Ras requires its post-translation prenylation. Farnesylation is followed by proteolysis of the carboxyl AAX amino acids and carboxymethylation of the carboxyl-terminal farnesyl cysteine, rendering the carboxyl terminus more hydrophobic (37, 38). FTIs prevent H-Ras farnesylation, membrane localization, and reverse H-Ras-induced cellular transformation (39). However, K- and N-Ras are also in vitro substrates of GGPT-1, and in cells treated with FTIs, their isoprene content switches to geranylgeranyl (40, 41). Despite the failure to abrogate prenylation of K- and N-Ras, mouse mammary tumor virus (MMTV)-K-Ras and MMTV-N-Ras tumors are inhibited by FTIs (42, 43). This suggests that the anti-proliferative action of FTIs is dependent on blocking the farnesylation of other proteins in addition to the Ras family.

In the current studies, we found that both human Rheb and Rheb2 are in vitro substrates for FPT but not for GGPT-1. In cell culture, Rheb prenylation is completely inhibited by SCH66336. As a result, SCH66336 inhibits signaling downstream of Rheb and mTOR. This effect of FTIs is abrogated by the expression of Rheb-CSVL, a mutant engineered to be modified by geranylglyceranyl, which remains prenylated in the presence of SCH66336. This mutant also abrogates the ability of SCH66336 to enhance the apoptotic effect of both tamoxifen and docetaxel. Further, inhibition of Rheb signaling by rapamycin treatment, siRNA, or a dominant negative Rheb protein mimics the effect of SCH66336 treatment, enhancing the action of tamoxifen and docetaxel. These studies suggest that inhibition of Rheb farnesylation plays a role in the SCH66336 enhancement of other chemotherapeutics.

**MATERIALS AND METHODS**

**Materials—**SCH66336, SCH66337 (synthesized by Schering-Plough) (34), mevastatin, and 4-OH-tamoxifen (Sigma) were dissolved in MeSO2. Mevalonolactone, docetaxel, and estradiol (Sigma) were dissolved in ethanol. Heregulin (Sigma) was dissolved in phosphate-buffered saline. Rapamycin (Cell Signaling, Beverly, MA) was dissolved in dimethyl sulfoxide. All other reagents were from Sigma-Aldrich (St. Louis, MO) or Pfanstiehl (Galena, IL). Human tissues were from the Division of Pathology, Martin T. Rodbell Memorial Research Institute, National Institutes of Health (Bethesda, MD). Experimental procedures are described in detail in the Supplementary Methods and in the 50th anniversary issue of the Journal of Biological Chemistry, vol. 280, issue 42, November 14, 2005.

**Cell Culture—**Human cell lines (MCF-7, ES2 (American Type Culture Collection (ATCC, Manassas, VA), and MDA-435 (kindly provided by Dr. Janet Price, M. D. Anderson, Houston, TX) were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium:F-12. COS-7 (ATCC) cells were maintained in Dulbecco’s modified Eagle’s medium. The media were supplemented with 2 mM glutamine, 50 units/ml penicillin, 50 units/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Invitrogen) and incubated at 37 °C in 5% CO2.

**Transfections—**Human Rho proteins were expressed as fusion proteins containing HA tag at the amino terminus. The coding region was amplified by PCR from human brain cDNA (BD Biosciences) and subcloned into pHM6 vector (Roche Applied Science). HA-tagged Rheb-CSVL, Rheb-SYVM, Rheb-D60K, Rheb-D60V, and Rheb-D60V-CSVL were made by site-directed mutagenesis (Stratagene, La Jolla, CA).

**Protein Analysis—**COS-7 cells were electroporated with 20 μg of HA-Rheb and 5 μg of pMev transporter or MCF-7 pMev cells were transfected with 20 μg of HA-Rheb and 50 μl of polyclonal antibody (Roche Applied Science) and incubated in Amplify (Amersham Biosciences), dried, and viewed by autoradiography. Duplicate gels were scanned and quantified by densitometry.

**Immunochemistry—**Cells were fixed and processed for immunofluorescence microscopy at various time points. Confocal images were acquired on a Zeiss Axiovert 135 microscope equipped with a Zeiss PlanAchromat 63×/1.4 oil objective. Images were acquired in 1024 × 1024 pixel format with an emission range of 430 to 470 nm for DAPI, 490 to 520 nm for Alexa488, 530 to 570 nm for Alexa568, and 617 to 672 nm for rhodamine. The images were captured in a drift-matched grid using the Imaging Suite software (Zeiss) and analyzed using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD).

**RESULTS**

**Rheb mRNA Is Elevated in Tumor Cell Lines—**Rheb is ubiquitously expressed in human tissue. Rheb expression is elevated in SV40-transformed MRC-5 V2 fibroblasts, as compared with the parental cell line (2). We found that both Rheb and Rheb2 mRNAs were widely expressed with the highest expres-
sion in the brain. The two were overexpressed in most tumor cell lines, as compared with normal cell lines/tissue (Fig. 1). Rheb mRNA was analyzed by quantitative RT-PCR in 67 tumor cell lines and 10 normal cells for 8 different tissue types. For breast, lung, prostate, and skin, primary cells were used. For colon, brain, ovary, and pancreas, mRNA was obtained from normal tissue. Rheb mRNA expression was increased by at least 2-fold in various tumor cells: 2/9 breast, 7/12 lung, 2/4 prostate, 8/9 colon, 6/7 ovarian, 2/7 melanoma, and 7/7 pancreatic. Likewise, Rheb2 mRNA was increased by at least 2-fold in various tumor cells when compared with normal cells: 8/9 breast, 3/12 lung, 4/9 prostate, 4/7 ovarian, 7/7 melanoma, and 6/7 pancreatic. All colon, ovarian, melanoma, and pancreatic tumor cells in our panel had an elevated expression of Rheb, Rheb2, or both. Little or no overexpression of either was observed in glioma cell lines as compared with normal brain. These data suggest that, not only is activity of the Rheb pathway up-regulated in tumor cells by amplification and mutational activation of upstream signaling proteins, Rheb expression itself is elevated in many tumor cells.

Rheb Is Modified by Farnesylation—K- and N-Ras are substrates for GGPT-1, and therefore, upon cellular treatment with FTIs, the isoprene content of these Ras isoforms switches to geranylgeranyl (40). We wanted to determine whether human Rheb and Rheb2 were solely farnesylated or subject to alternative prenylation. Peptides derived from the carboxyl termini of Rheb (QGKSSCSVM) and Rheb2 (GQERRCHLM) were tested as in vitro substrates for FPT and GGPT-1. H-Ras peptide ending in CVLS served as a positive control for FPT, and a peptide ending in CAIL served as a positive control for GGPT-1. Following incubation of peptides with FPT or GGPT-1, incorporation of $^{3}H$-isoprene was measured using scintillation proximity assay. Both Rheb and Rheb2 were substrates for FPT but not for GGPT-1 (Fig. 2). Further, Rheb peptides bound FPT with greater affinity than the H-Ras peptide. The $K_{m}$ for Rheb and Rheb2 were 102 and 42 nM, respectively, compared with 528 nM for H-Ras. These data indicate human Rheb and Rheb2 are modified by farnesylation and are not substrates for GGPT-1.

SCH66336 Inhibits Rheb Farnesylation—Next, we wanted to determine whether Rheb was modified by farnesylation in vivo. Cells were transfected with a cDNA encoding the mevalonate transporter and treated with mevastatin to increase labeling of cellular isoprene pools with $^{3}H$-mevalonolactone. Mevalonolactone is a precursor for both geranylgeranyl diphosphate and farnesyl diphosphate. COS-7 cells were electroporated with cDNA encoding HA-Rheb and labeled with $^{3}H$-mevalonolactone. In untreated cells, immunoprecipitated HA-Rheb incor-
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porated [3H]isoprene (Fig. 3A). SCH66336 treatment completely inhibited this modification at 1000 nM, with an IC50 between 10 and 100 nM. Although there was a 15% decline in HA-Rheb at 1000 nM, at 100 nM, there was no decline in HA-Rheb and a >50% decline in [3H]isoprene. SCH66336 is a selective FPTase inhibitor that has no effect on GGPT-1 at the concentrations used. Incorporation of [3H]mevalonolactone into Rheb would not be blocked by SCH66336 if Rheb was alternatively prenylated by GGPT-1. These data show that Rheb is a substrate for farnesylation and is not alternatively prenylated in FTI-treated cells. Additionally, upon treatment with SCH66336, HA-Rheb underwent a gel mobility shift on SDS-PAGE (Fig. 3A), similar to the shift seen when unprenylated HDJ-2 and H-Ras accumulate upon FTI treatment.

A point mutation in Rheb changing the CAAX box from CSVL to CSVM was predicted to convert it into a substrate for GGPT-1, whereas mutation of the modified cysteine residue to serine (SSVM) will block all prenylation of Rheb. We found that prenylation of HA-Rheb-CSVL in cells was not inhibited by SCH66336, consistent with Rheb-CSVL geranylgeranylation (Fig. 3B). As expected, HA-Rheb-SSVM did not incorporate [3H]isoprene (Fig. 3B). HA-Rheb-SSVM migrated on SDS-PAGE with similar mobility to that of HA-Rheb in FTI-treated cells, whereas HA-Rheb-CSVL migrated between untreated and treated HA-Rheb (Fig. 3B).

We expanded this finding to human cancer cells. MCF-7 cells that stably expressed mevalonate transporter were labeled with [3H]mevalonolactone. Similar to the COS-7 finding, HA-Rheb was labeled with [3H]isoprene, and this modification was completely inhibited by SCH66336 (Fig. 3C).

SCH66336 Inhibits S6 Phosphorylation—Rheb has been shown to promote phosphorylation of S6 kinase, a downstream target of mTOR signaling. Rheb overexpression in 293T cells stimulates phosphorylation of S6 kinase, and this is inhibited by FTI-277 (18). We found that SCH66336 inhibited phosphorylation of S6 ribosomal protein in the human breast cancer cell line MCF-7 (Fig. 4A). Several experiments with 1 μM SCH66336 resulted in an average 70% reduction in S6 phosphorylation. Interestingly, concentrations required to inhibit S6 phosphorylation were similar to those that blocked Rheb farnesylation. Additionally, heregulin-stimulated phosphorylation of S6 in MCF-7 cells was blocked by SCH66336 (Fig. 4B). Similar inhibition of S6 phosphorylation was observed in the human ovarian cancer cell line ES2 (data not shown). In contrast, stimulation of Akt and MAPK phosphorylation in MCF-7 cells was unaffected by SCH66336 treatment. Surprisingly, basal phosphorylation of MAPK was slightly increased in FTI-treated MCF-7 cells.

Ectopic expression of HA-Rheb-CSVL, a mutant designed to be modified by geranylgeranylation, abrogated SCH66336 inhibition of S6 phosphorylation (Fig. 4C). MCF-7 and ES-2 cells were treated with 1 μM SCH66336 for 24 h. In both cell types, SCH66336 induced a complete gel shift of HA-Rheb. In vector- and HA-Rheb MCF-7- and ES2-transfected cells, SCH66336 reduced levels of S6 phosphorylation by ~80%. However, in HA-Rheb-CSVL-transfected MCF-7 cells levels of S6 phosphorylation were unaffected in the presence of 1 μM SCH66336. In HA-Rheb-CSVL-transfected ES2 cells, 1 μM SCH66336 resulted in only a 15% decline in S6 phosphorylation. These data suggest that inhibition of Rheb farnesylation is responsible for SCH66336-induced reduction in S6 phosphorylation.

Inhibition of Rheb Function Enhances the Effects of Tamoxifen—SCH66336 enhances the anti-tumor response to both tas-

FIG. 2. Rheb is modified by farnesylation. The indicated amount of peptides from the carboxyl termini of Rheb, Rheb2, H-Ras, and lamin B (CAAX containing a methionine to leucine substitution at the X position) were tested as substrates for FPT and GGPT-1 enzymes in the presence of [3H]FPP or [3H]GGPP. Incorporation of [3H]isoprene was detected using scintillation proximity assay. The average of four experiments is shown with S.E.

FIG. 3. SCH66336 inhibits Rheb farnesylation. A and B, COS-7 cells were electroporated with HA-Rheb and mevalonate (Mev) transporter. C, MCF-7/pMev cells were transfected with HA-Rheb. A–C, cells were labeled with 100 μCi/ml [3H]mevalonolactone for 18 h in the presence of 20 μM mevastatin and varying concentrations of SCH66336. Rheb was immunoprecipitated with anti-HA beads. Gels were viewed by autoradiography or immunoblotted for HA. Experiments were repeated in both COS-7 and MCF-7 cells, and a representative experiment is displayed. WT, wild type; DMSO, MeS0.
moxifen and taxanes in tumor xenograft models (36). In cell culture, we found that 24-h treatment with 1 \mu{M} SCH66336 or 10 \mu{M} tamoxifen reduced the levels of S6 phosphorylation in MCF-7 cells (Fig. 5A). Combination treatment completely blocked S6 phosphorylation. Similar results were observed in both vector- and HA-Rheb-transfected cells. Expression of HA-Rheb-CSVL rescued the loss of S6 phosphorylation in response to FTI alone or in response to the combination. SCH66336 equally inhibited farnesylation of the co-chaperone protein HDJ2 in both control and Rheb-transfected cells.

SCH66336 treatment augmented tamoxifen-induced apoptosis, as demonstrated by accumulation of the p85 PARP fragment in MCF-7 cells (Fig. 5A). Similar results were seen in control and HA-Rheb-transfected cells. SCH66336 and tamoxifen induced a 1.5- and 2.0-fold increase in p85 PARP fragment, respectively.

The combination of these agents enhanced this effect and induced a 3.5-fold increase. This enhancement of tamoxifen-induced apoptosis was abrogated by expression of geranylgeranylated HA-Rheb-CSVL. In these cells, SCH66336 induced a 1.3-fold increase in p85 PARP fragment. Tamoxifen alone or in combination with SCH66336 induced a 2.0- and 2.1-fold increase, respectively. These data suggest that SCH66336 enhancement of tamoxifen-induced pro-apoptotic effects is a consequence of inhibiting Rheb farnesylation.

We hypothesized that inhibition of Rheb signaling may augment the effects of tamoxifen. To test this hypothesis, other measures of inhibiting Rheb function were tested. We first treated cells with increasing concentrations of rapamycin to block mTOR signaling downstream of Rheb. MCF-7 cells were treated with rapamycin with or without 10 \mu{M} tamoxifen for 24 h. Rapamycin, similar to SCH66336, enhanced tamoxifen induced PARP cleavage and loss of S6 phosphorylation (Fig. 5B). Tamoxifen and rapamycin (0.1 nM) alone induced a 50 and 80% reduction in S6 phosphorylation, respectively. The

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Fig. 4. SCH66336 inhibits S6 phosphorylation. Levels of total and phosphorylated S6, Akt, and MAPK were determined by Western blot analysis. Experiments were repeated a minimum of three times, and a representative experiment is shown. A, MCF-7 cells were treated with SCH66336 in the presence of 0.1% serum for 24 h. B, MCF-7 cells were treated with 1 \mu{M} SCH66336 for 72 h, of which during the last 24 h, the cells were starved. The cells were then stimulated with 10 ng/ml heregulin (HRG) for 10 min. C, cells were transfected with HA-Rheb and treated with 1 \mu{M} SCH66336 in the presence of 0.1% serum for 24 h. WT, wild type.

Fig. 5. Inhibition of Rheb function enhances the effects of tamoxifen. MCF-7 cells were drug-treated in the presence of 1 nM estradiol in estrogen-depleted medium containing 10% dialyzed serum for 24 h. Cell lysates were immunoblotted for the p85 PARP fragment, P-S6, S6, HDJ2, and HA. Experiments were repeated three times, and a representative experiment is shown. A, cells transfected with HA-Rheb were treated with Me2SO, 1 \mu{M} SCH66336, 10 \mu{M} 4-OH-tamoxifen, or both. B, cells were treated with increasing concentrations of rapamycin with or without 10 \mu{M} 4-OH-tamoxifen. C, HA-Rheb-transfected cells were treated with 0, 5, and 10 \mu{M} 4-OH-tamoxifen. WT, wild type.

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combination of these two agents induced an almost complete loss. Further, the p85 PARP fragment was barely detectable in tamoxifen and rapamycin (0.1 μM) single agent-treated cells. In cells treated with the combination, there was a 2.5-fold increase in the p85 PARP fragment. This was further enhanced to 7.0-fold in the presence of 1 nM rapamycin, a concentration that alone induced a 1.8-fold increase.

Dominant negative mutant Rheb-D60K lacks the ability to bind both GTP and GDP, whereas the Rheb-D60V dominant negative preferentially binds GDP (19). We found that, in MCF-7 cells, expression of either of these two dominant negative proteins reduced basal levels of phosphorylated S6 (Fig. 5C). Expression of HA-Rheb-D60K and HA-Rheb-D60V induced a 70 and 50% reduction in S6 phosphorylation, respectively. When treated with tamoxifen for 24 h, cells expressing dominant negative Rheb were more sensitive to tamoxifen-induced PARP cleavage and loss of phosphorylated S6 (Fig. 5F). Tamoxifen (10 μM) treatment resulted in a 75% reduction in S6 phosphorylation. This was enhanced to an almost complete loss of S6 phosphorylation in the presence of dominant negative Rheb. The p85 PARP fragment was barely detected in vector- and HA-Rheb-transfected cells treated with 5 μM tamoxifen but was easily detected in cells expressing dominant negative Rheb. Further, p85 PARP accumulation was enhanced by >2-fold in cells expressing dominant negative Rheb as compared with vector- and HA-Rheb-transfected cells. These data show rapamycin treatment or expression of dominant negative HA-Rheb proteins mimicked SCH66336 with respect to enhancing tamoxifen-induced effects.

**Inhibition of Rheb Function Enhances the Effects of Docetaxel**—Similar to the results with tamoxifen, we demonstrated that SCH66336 enhances the consequences of docetaxel treatment, including induction of caspase-3 activity. We sought to determine the effects of HA-Rheb-CSVL, dominant negative HA-Rheb, and rapamycin on the response to FTI in combination with docetaxel. ES2 ovarian cancer cells were transfected with vector, HA-Rheb, or HA-Rheb-CSVL, and caspase-3 activity was measured (Fig. 6A). The cells were treated with Me2SO, 1 μM SCH66336, 10 nM docetaxel, or both for 4 h followed by Me2SO or SCH66336 for the remainder of the 48-h treatment. Single agent SCH66336 treatment had minor effects on caspase-3 activity (1.3-fold increase). Treatment of cells with docetaxel caused a 2-fold increase in caspase-3 activity. Combination therapy resulted in a 3.4-fold induction in caspase activity. A similar pattern was seen in cells transfected with wild-type HA-Rheb. Ectopic expression of HA-Rheb-CSVL abrogated SCH66336 enhancement of docetaxel-induced caspase activity. In these cells, docetaxel and combination treatment induced a 1.8- and 2-fold increase in caspase activity, respectively. Similar results were observed in MDA-435 breast cancer cells (data not shown).

To determine whether SCH66336 enhancement of docetaxel-induced caspase activity was a result of inhibiting Rheb, we blocked Rheb signaling by rapamycin treatment, expression of dominant negative HA-Rheb-D60V, or siRNA. Cells were treated with vehicle or 100 nM rapamycin with or without 10 nM docetaxel for 4 h followed by vehicle or rapamycin alone for the remainder of the 48-h treatment. Docetaxel induced a 3.3-fold increase in caspase-3 activity in rapamycin-treated cells, compared with a 2.2-fold increase in vehicle-treated cells (Fig. 6B). Similar to rapamycin treatment, ectopic expression of HA-Rheb-D60V enhanced docetaxel-induced caspase activity. Docetaxel induced a 3.5-fold increase in HA-Rheb-D60V cells, as compared with a 2.2- and 2.3-fold increase in vector- and HA-Rheb-transfected cells. Further, a HA-Rheb-D60V Rheb mutant terminating in CSVL also functioned as a dominant negative, and docetaxel induced a 3.1-fold increase in cells expressing this protein (Fig. 6B).

Rheb siRNA also enhanced docetaxel-induced caspase activity. In control siRNA-transfected cells, docetaxel induced a 2.3-fold increase. Docetaxel induced a 2.2- and 2.8-fold increase in cells transfected with either Rheb or Rheb2 siRNA, respectively (Fig. 6B). Rheb siRNA reduced Rheb mRNA levels by 86% as determined by quantitative RT-PCR, whereas Rheb2 siRNA reduced Rheb2 mRNA levels by 62% (data not shown). Each specific siRNA had no effect on mRNA expression of the other family member. In cells transfected with both Rheb siRNA plus Rheb2 siRNA, Rheb and Rheb2 mRNA were reduced by 90 and 79%, respectively. As commercially available Rheb antibody detects endogenous Rheb poorly, we could not confirm knockdown of the Rheb protein. We therefore transfected cells with HA-Rheb with or without Rheb siRNA and found HA-Rheb was depleted completely in the presence of the siRNA (data not shown). We found that docetaxel induced a 4.8-fold increase in caspase activity in these cells, suggesting that both siRNA against Rheb and Rheb2 was needed to sensitize cells to docetaxel-induced caspase activity (Fig. 6B).

**DISCUSSION**

SCH66336 (lonafarnib) is a farnesyl transferase inhibitor (FTI) that blocks post-translational processing of farnesylated proteins. This enzyme catalyzes the attachment of farnesyl pyrophosphate to cysteine residues in the C-terminus of several proteins, including cyclin-dependent kinases, protein kinase C, and small GTPases of the Ras family. SCH66336 has been shown to inhibit cell proliferation in vitro and in vivo, and to induce apoptosis in various cancer cell lines. It has been proposed that SCH66336 mimics the effects of a dominant negative Rheb mutant, by preventing the farnesylation of Rheb and thus blocking its signaling activity. In this study, we investigate the potential of SCH66336 to enhance the effects of docetaxel and tamoxifen, two drugs currently used in the treatment of breast cancer. We found that SCH66336 enhances docetaxel-induced caspase activity and decreases protein expression of the downstream target of Rheb, p85 PARP. This effect was more pronounced in cells expressing dominant negative Rheb, suggesting that SCH66336 enhances the consequences of docetaxel treatment, including induction of caspase-3 activity.

SCH66336 was also shown to inhibit mTOR signaling, which is a key player in the control of cell growth and survival. This was demonstrated by the ability of SCH66336 to block the phosphorylation of S6, a downstream target of mTOR. SCH66336 also enhanced the effects of tamoxifen, a drug used to treat estrogen receptor-positive breast cancer. This enhancement was more pronounced in cells expressing dominant negative Rheb, suggesting that SCH66336 enhances the consequences of tamoxifen treatment, including induction of caspase-3 activity.

In conclusion, SCH66336 enhances the effects of docetaxel and tamoxifen by inhibiting mTOR signaling and enhancing the downstream effects of these drugs. These results suggest that SCH66336 may have potential as a combinational therapy in breast cancer treatment.

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proteins including H-Ras. Upon treatment of cells with FTIs, some farnesylated proteins, including K- and N-Ras, become alternatively prenylated by geranylgeranyl transferase-1 (40, 45). Despite the failure to abrogate prenylation of K- and N-Ras, growth of many tumors in preclinical models is inhibited by FTIs. This suggests the anti-proliferative action of FTIs is dependent on blocking farnesylation of proteins in addition to the Ras family. In this report, we investigated the role of farnesylation of Ras (Ras homologue-enriched in brain), a small GTPase that positively regulates mTOR signaling. Ras overexpression induces phosphorylation of mTOR substrates, an effect blocked by treatment with FTI-277, suggesting that Ras function is regulated by farnesylation (18). Clark et al. (25) previously reported that GST-Rheb is labeled by farnesyl diphosphate (but not geranylgeranyl diphosphate) when incubated with rabbit reticulocyte lysates.

We demonstrated that human Rheb and Rheb2 are farnesylated and are not substrates for alternative prenylation. In vitro, peptides derived from the carboxyl termini of Rheb and Rheb2 were substrates for FPT but not GGPT-1. In cell culture, Rheb protein was labeled by mevalonolactone and was thus a substrate for prenylation. This modification was completely blocked by SCH66336, a selective FPT inhibitor, indicating that, similar to H-Ras, Rheb and Rheb2 are not alternatively prenylated in FTI-treated cells. Post-translational processing of both Rheb family members is inhibited by SCH66336.

As a result of inhibiting Rheb farnesylation, SCH66336 blocks S6 phosphorylation. FTI L-744,832 was previously reported to mimic rapamycin by inhibiting DNA synthesis and S6 kinase activation in response to serum. Law et al. (46) further demonstrated that a rapamycin-resistant mutant of S6 kinase is resistant to FTI-induced dephosphorylation. The mechanism whereby FTI inhibits S6 kinase activation was undefined in these studies. Recently, it was found that Rheb overexpression induces phosphorylation of mTOR substrates S6 kinase and 4E-BP1. Rheb-induced phosphorylation of S6 is blocked by rapamycin, FTI-277, or its upstream regulator TSC (3, 18, 19). Similar to Law et al., we showed that SCH66336 blocked heregulin-induced phosphorylation of S6, although having no effect on the activation of Akt and MAPK. Further, basal phosphorylation of S6 was reduced in SCH66336-treated cells. These effects were abrogated by the expression of HA-Rheb-CSVL, a mutant Rho that is geranylgeranylated and thus not affected by SCH66336. These data indicate that inhibition of Rheb farnesylation is responsible for FTI-induced reduction in S6 phosphorylation.

SCH66336 synergizes with various chemotherapy agents, including taxanes and cisplatin, to enhance their anti-tumor activity (47, 48). We found that single agent FTI treatment has little effect on apoptosis, but FTI treatment enhances tamoxifen- and taxane-induced apoptosis. Rapamycin was recently reported to synergistically interact in combination with paclitaxel and carboplatin (49). Further, rapamycin restores sensitivity to Akt-induced tamoxifen-resistant cells (50). Our data suggest that FTI enhancement of tamoxifen- and taxane-induced apoptosis is a consequence of the inhibition of Ras signaling. Expression of HA-Rheb-CSVL abrogated SCH66336 enhancement of both tamoxifen- and docetaxel-induced apoptosis. Further, inhibition of Ras signaling by rapamycin treatment or expression of dominant negative Rho proteins mimicked FTI treatment and sensitized cells to tamoxifen- and docetaxel-induced apoptosis. We found that Rheb-Asp-60 mutations created dominant negative proteins, supporting the findings of Tabancay et al. (19) and conflicting with the findings of Li et al. (51). Rheb siRNA also enhanced docetaxel-induced caspase activity. In cells transfected with siRNA against Rheb and Rheb2, docetaxel induced a 4.8-fold increase in caspase activity, as compared with a 2.2-fold increase in control-transfected cells. Cells transfected with siRNA against either Rheb or Rheb2 alone did not display enhanced sensitivity to doce- taxel, indicating that inhibition of both family members is needed.

We have reproduced these experiments using structurally distinct FTIs, including R115777 (Tipifarnib) and BMS-214662 (data not shown). We also tested SCH66337, an enantiomer of SCH66336 that does not inhibit FPT in vitro and fails to induce a HDJ-2 mobility shift in treated cells. We found that the FTIs (but not SCH66337) inhibited Rheb farnesylation, as detected by a mobility shift on SDS-PAGE. Similar to SCH66336, R115777 and BMS-214662 (but not SCH66337) enhanced apoptosis in response to both tamoxifen and docetaxel.

Rheb may represent one of the critical elusive targets of farnesyl transferase inhibitors. We found that Rheb and Rheb2 expression were elevated in tumor cell lines relative to their normal counterparts. Rheb plays a key role in cell growth in that it activates signaling via the mTOR pathway. The Rheb GTPase-activating protein hamartin-tuberin complex is negatively regulated by Akt-mediated phosphorylation of tuberin, resulting in increased Rheb activity (9). Akt signaling is elevated in tumors by Akt amplification, phosphatidylinositol 3-kinase amplification, loss of heterozygosity of phosphatase and tensin homologue, HER2 amplification, and EGFR-activating mutations (52–59). Our data show that Rheb activity may also be increased in tumor cells by elevated mRNA expression.

Our data demonstrated that Rheb is overexpressed in tumor cells, is modified by farnesylation, is not a substrate for alternative prenylation, and plays a role in SCH66336 enhancement of other chemotherapeutics. To date, FTIs have demonstrated efficacy in hematologic malignancies (60) but have shown limited single agent activity in solid tumors. Ongoing clinical trials with lonafarnib and other FTIs in combination with taxanes and hormonal therapies in solid tumors are in progress. Inhibition of other farnesylated proteins in addition to Rheb may contribute to the anti-tumor effects of FTIs. Candidate proteins include RhoB and the centromere-associated proteins CENP-E and CENP-F (33, 61, 62). Defining the relative importance of these various proteins in the anti-tumor activity of FTIs requires further study. Our current results suggest that Rheb may be one of the critical FTI targets and that inhibition of Rheb farnesylation by FTIs may play a critical role in enhancing the anti-tumor effect of docetaxel and tamoxifen.

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