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The farnesyl protein transferase inhibitor SCH66336 synergizes with taxanes in vitro and enhances their antitumor activity in vivo

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Abstract *Purpose*: SCH66336 is an orally active, farnesyl protein transferase inhibitor. SCH66336 inhibits ras farnesylation in tumor cells and suppresses tumor growth in human xenograft and transgenic mouse cancer models in vivo. The taxanes, paclitaxel (Taxol) and docetaxel (Taxotere) block cell mitosis by enhancing polymerization of tubulin monomers into stabilized microtubule bundles, resulting in apoptosis. We hypothesized that anticancer combination therapy with SCH66336 and taxanes would be more efficacious than single drug therapy. *Methods*: We tested the efficacy of SCH66336 and taxanes when used in combination against tumor cell proliferation in vitro, against NCI-H460 human lung tumor xenografts in nude mice, and against mammary tumors in wap-ras transgenic mice. *Results*: SCH66336 synergized with paclitaxel in 10 out of 11 tumor cells lines originating from breast, colon, lung, ovary, prostate, and pancreas. SCH66336 also synergized with docetaxel in four out of five cell lines tested. In the NCI-H460 lung cancer xenograft model, oral SCH66336 (20 mg/kg twice daily for 14 days) and intraperitoneal paclitaxel (5 mg/kg once daily for 4 days) caused a tumor growth inhibition of 56% by day 7 and 65% by day 14 compared to paclitaxel alone. Male transgenic mice of the *wap-ras/F* substrain [FVB/N-TgN(WapHRAS)69LlnYSJL] spontaneously develop mammary tumors at 6–9 weeks of age which have been previously shown to be resistant to paclitaxel. Paclitaxel resistance was confirmed in the present study, while SCH66336 inhibited growth of these tumors. Most importantly, SCH66336 was able to sensitize *wap-ras/F* mammary tumors to paclitaxel chemotherapy. *Conclusion*: Clinical investigation of combination therapy using SCH66336 and taxanes in cancer patients is warranted. Further, SCH66336 may be useful for sensitizing paclitaxel-resistant tumors to taxane treatment.

Key words Farnesyl protein transferase inhibitor · Paclitaxel · Docetaxel · Ras transgenic mice

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Introduction

Oncogenic mutations in the *ras* gene are prevalent in human cancer, including up to 50% of colon cancers and more than 90% of pancreatic carcinomas [1]. In normal cells, RAS switches between an inactive GDP-bound and an active GTP-bound state which can initiate several intracellular signaling pathways [11]. RAS signaling is terminated by hydrolysis of GTP to GDP in a reaction that is stimulated by guanosine triphosphatase-activating proteins. As a consequence of specific mutational events in the *ras* sequence, oncogenic RAS proteins have a greatly reduced capacity to hydrolyze GTP. This leads to constitutive activation of downstream signaling pathways resulting in unregulated cellular proliferation [1, 19]. Three *ras* genes encode four ras protein isoforms (*H-ras*, *N-ras*, *K-ras4A*, and *K-ras4B*) with *K-ras4A* and *K-ras4B* being splice variants of the same gene transcript [19]. Although the functional differences between the four isoforms remain unknown, oncogenic mutations of different isoforms predominate in different tumors [2]. *H-ras* mutations are generally found in carcinomas of the bladder, kidney and thyroid. *N-ras* mutations are found in myeloid and lymphoid cancers, liver carcinoma and melanoma. *K-ras* mutations predominate in colon, lung and pancreatic carcinomas.

Many lines of evidence suggest that antitumor activity can be achieved by interfering with the function of

oncogenic RAS proteins [5, 6, 29, 36]. Signal transduction by RAS is dependent on its plasma membrane localization. This localization is supported by a series of post-translational modifications, the first of which is farnesylation of a Cys residue near the C-terminus of RAS proteins. This reaction is catalyzed by farnesyl protein transferase (FPT). RAS prenylation is critical for proper membrane localization and function [9, 12, 34]. Therefore, FPT inhibition is a potential mechanism for interfering with RAS-driven tumor growth.

Prenylation of Ras proteins is complex. In vitro, both K- and N-RAS proteins can serve as substrates for a related protein prenyl transferase, geranylgeranyl protein transferase-1 (GGPT-1) [10, 43]. Although this reaction occurs with a lower catalytic efficiency than the farnesylation of these proteins, geranylgeranylation of K- and N-RAS proteins has been observed in cells treated with FPT inhibitors (FTIs) [32, 42]. In contrast, the H-RAS protein is not a substrate for GGPT-1 in vitro or in cells treated with FTIs. Despite this alternative prenylation, FTIs demonstrate in vitro and in vivo antitumor efficacy in a variety of preclinical cancer models [13, 14, 18, 22, 37]. Therefore, the observed activity of FTIs may, in some cases, be due to the inhibition of farnesylation of proteins in addition to or other than RAS.

SCH66336 is an orally active, potent, and selective inhibitor of the FPT enzyme [17, 27]. This novel therapeutic agent has activity against a wide variety of human tumor xenografts and also causes regression of tumors in *wap-H-ras* transgenic mice. Enhanced antitumor activity has been reported in preclinical cancer models when SCH66336 is combined with cyclophosphamide, 5-fluorouracil, vincristine, and p53 gene therapy [17, 26]. In the studies reported here, we examined the efficacy of SCH66336 in combination with the taxanes, paclitaxel (Taxol) and docetaxel (Taxotere). Taxanes inhibit cell replication by enhancing polymerization of tubulin monomers into stabilized microtubule bundles that are unable to reorganize into the proper structures for mitosis [8, 15, 33]. This results in cell cycle blockage in mitosis and apoptosis, or cell lysis, all of which may be p53-independent [4, 35, 41].

Materials and methods

Cell lines

All the human tumor cell lines were purchased from ATCC (Rockville, Md.). MDA-MB-231 human breast adenocarcinoma cells and PANC-1 human pancreatic epithelioid carcinoma cells were cultured in 90% Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, N.Y.) with 10% fetal bovine serum (FBS; GIBCO). DU-145 human prostate carcinoma cells and PA-1 human ovarian teratocarcinoma cells were cultured in 90% Eagle's MEM plus 10% FBS. MIAPaCa2 human pancreatic carcinoma cells were cultured in DMEM with 10% FBS and 2.5% horse serum (HS; GIBCO). LNCap human prostate adenocarcinoma cells, DLD-1 human colorectal adenocarcinoma cells, and NCI-H460 human lung large-cell carcinoma cells were cultured in RPMI-1640 (GIBCO) with 10% FBS. AsPC-1 human pancreatic adenocarcinoma cells were cultured in RPMI-1640 (GIBCO) with 20% FBS. MDA-MB-468 human breast adenocarcinoma cells were cultured in Leibovitz's L-15 medium plus 10% FBS. All the cells were cultured at 37 °C in an atmosphere containing 5% CO_2, except MDA-MB-468 cells which were maintained at 37 °C without CO_2.

In vitro drug interaction studies

SCH66336, (+)4-{2-[4-(8-chloro-3,10-dibromo-6,11-dihydro-5H-benzocyclohepa{1,2-b}pyridin-11-yl)-1-piperidinyl]-2-oxoethyl}-1-piperidinecarboxamide, was synthesized by Schering-Plough and its structure has been published [17, 27]. Paclitaxel (Taxol) was purchased from Calbiochem. Docetaxel (Taxotere, Rhone-Poulenc Rorer) was purchased from Drug Fair (Westfield, N.J.). Paclitaxel and docetaxel were dissolved in absolute ethanol to 10 mg/ml, then diluted in culture medium immediately before use. SCH66336, 100 m*M* in DMSO, was diluted with culture medium for in vitro studies. Tumor cells were seeded into culture wells of 96-well plates and allowed to attach for 3 h. The cells were incubated with paclitaxel or vehicle for 4 h, washed, then SCH66336 or vehicle was added and the incubation continued for 7 days. Multiple dose response curves were generated for each drug alone and in combination, from zero response to maximal response, for each individual cell line. Cell proliferation was measured using the MTT assay [21]. Briefly, 25 µl 5 mg/ml MTT vital dye [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well and allowed to incubate for 3–4 h at 37 °C in an atmosphere containing 5% CO_2. Then, 100 µl 10% SDS was added to each well and the incubation was continued overnight. Fluorescence in each well was quantitated using a Molecular Devices microtiter plate reader.

Cell proliferation data from drug interaction studies were analyzed using the Thin Plate Spline methodology [28]. Briefly, the response surface (response, dose of A, dose of B) was fitted using a spatial regression model related to thin plate splines. The response is the logit transformation of the percent of cell proliferation [logit = log(percent/100–percent)]. In the model, the dose of A, the dose of B, and the interaction of A and B were the fixed effects. A Gaussian spatial covariance was used for smoothing. The *P*-value for synergism was given by the *P*-value for the interaction term in the model. Computations were performed using the Statistical Analysis System procedure MIXED (SAS/STAT Software, changes and enhancements through release 6.12; SAS Institute, Cary, N.C.). After the spatial regression model fit, the isobole was calculated and graphed using the Statistical Analysis System procedure GCONTOUR (SAS/GRAPH Software, version 6, 1st edn, vol 2. SAS Institute). The smooth response surface was graphed by the procedure G3D.

In vivo efficacy studies

Nude mice (Crl:NU/NU-*nu*BR) were purchased from Charles River Laboratories (Wilmington, Mass.). Line 69-2F *wap-ras*/*F* transgenic mice [FVB/N-TgN(WapHRAS)69LlnYSJL] [24, 25] were from the SPRI breeding colony (also available from the Jackson Laboratory Induced Mutant Resource). All mice were maintained in a VAF-barrier facility. Animal procedures were performed in accordance with the rules set forth in the N.I.H. Guide for the Care and Use of Laboratory Animals and approved by the SPRI Animal Care and Use Committee. SCH66336 was sonicated until dissolved in 20% hydroxyl-propyl-betacyclodextrin (20% HPβCD). Paclitaxel was dissolved in 100% ethanol, vortexed into Cremophor EL (1/1 v/v; Sigma Chemical Co., St. Louis, Mo.), and diluted into PBS immediately prior to use. Tumor growth was quantitated by measuring tumors in three dimensions. Tumor volumes were calculated as (length × width × height)/2. The statistical significance of tumor growth inhibition in the combination treatment group compared to single-drug treatment on each day was analyzed using Student's *t*-test.

Table 1 Analysis of in vitro drug interactions between SCH66336 and paclitaxel ($P = 0.05$ indicates synergy or antagonism, $P > 0.05$ indicates no drug interaction, i.e. additive effects)

Cell line	Tumor type	p53 protein	Ras mutation	Isobole analysis
MDA-MB-468	Human breast	Mutant	Wild-type	Synergy ($P = 0.0094$)
MDA-MB-231	Human breast	Mutant	Mutant	Antagonism ($P = 0.0093$)
DLD-1	Human colorectal	Mutant	K-ras	Synergy ($P = 0.0592$)
NCI-H460	Human lung	Wild-type	K-ras	Synergy ($P = 0.0309$)
PA-1	Human ovarian	Wild-type	K-ras	Synergy ($P = 0.0122$)
DU-145	Human prostate	Mutant	Wild-type	Synergy ($P = 0.0238$)
LNCaP	Human prostate	Wild-type	Wild-type	Synergy ($P = 0.0021$)
AsPC-1	Human pancreatic	Null	K-ras	Synergy ($P = 0.0328$)
BxPC-3	Human pancreatic	Mutant	Wild-type	Synergy ($P = 0.0185$)
MIAPaCa2	Human pancreatic	Mutant	K-ras	Synergy ($P = 0.0002$)
PANC-1	Human pancreatic	Mutant	K-ras	Synergy ($p = 0.0011$)

For the NCI-H460 tumor xenograft study, each female nude mouse was inoculated subcutaneously with 3×10^6 NCI-H460 cells on day 0. Mice were treated orally with 0.2 ml vehicle or 20 mg/kg SCH66336 twice a day (at 7:30 a.m. and 7:30 p.m.) from days 4 to 14. Intraperitoneal paclitaxel (5 mg/kg) or vehicle was given once a day on days 4 to 7.

Male *wap-ras/F* transgenic mice with palpable tumors were randomized into four treatment groups. Group 1 was dosed with vehicles. Group 2 was dosed with 20 mg/kg SCH66336 orally twice daily for 3 weeks. Group 3 was dosed with 5 mg/kg paclitaxel intraperitoneally once daily on days 4 to 7. Group 4 was dosed with 20 mg/kg SCH66336 twice daily for 3 weeks plus 5 mg/kg paclitaxel on days 4 to 7.

Results

Drug interaction studies in vitro

SCH66336 synergized with paclitaxel to inhibit the proliferation of every tumor cell line tested, except MDA-MB-231. These results were independent of p53 mutational status, ras mutational status, or tissue of origin. The results of the in vitro drug interaction assays are summarized in Table 1, and representative Isobole curves are shown in Fig. 1. SCH66336 also synergized with docetaxel to inhibit the proliferation of every tumor cell line tested, except the MDA-MB-231 line where there was no drug interaction (Table 2).

Efficacy in vivo

In the NCI-H460 lung cancer xenograft model ($p53^{wt}$, $K\text{-}ras^{mut}$), treatment with oral SCH66336 alone or intraperitoneal paclitaxel alone had caused tumor growth inhibitions of 52% and 61%, respectively, by the end of the study (Fig. 2). Combination treatment resulted in 86% inhibition of tumor growth and was more effective than therapy with either single agent ($P < 0.05$). Relative to paclitaxel alone, combination therapy had inhibited tumor growth 56% by day 7 and 65% by day 14.

In line 69 *wap-ras* transgenic mice, an activated H-ras oncogene is carried on the Y chromosome [23]. Male mice of the *wap-ras/F* substrain [FVB/N-TgN(WapHRAS)69LlnYSJL] spontaneously develop mammary tumors between 6 and 9 weeks of age [25]. These tumors have been previously shown to be resistant to paclitaxel therapy [30] and this finding was confirmed in the present study (Fig. 3). Oral treatment with SCH66336 by itself resulted in nearly complete inhibition of tumor growth ($P = 0.05$) and more importantly, was able to sensitize the tumors to paclitaxel chemotherapy. Tumors in mice treated with both drugs underwent regression over the first 8 days of treatment. The combination of SCH66336 and paclitaxel was significantly more effective than SCH66336 alone ($P = 0.06$ for days 7 to 21).

Discussion

The FPT inhibitor SCH66336 has activity against a wide variety of human tumor xenografts and causes regression of tumors in $H\text{-}ras^{mut}$ transgenic mice [17, 27]. Regression of the *H-ras* transgenic tumors is attributable to increased apoptosis and a decreased mitotic index [17]. SCH66336 also has enhanced antitumor activity when combined with cyclophosphamide, 5-fluorouracil, vincristine, or p53 tumor suppressor gene therapy [17, 26]. Synergy (or antagonism) between two therapeutic agents is an in vitro empirical phenomenon, in which the observed effect of the combination is more (or less) than that which would be predicted from the effects of each agent working alone. Although in vitro synergy is not directly provable in the clinical setting, it does predict a favorable outcome when the two agents are combined. By contrast, overt antagonism warns of future problems. Sophisticated statistical modeling techniques were used to evaluate the presence of synergistic, additive, or antagonistic efficacy between SCH66336 and several other anticancer agents. Combination therapy using SCH66336 and SCH58500, a recombinant adenovirus expressing p53, has synergistic or additive efficacy against tumor cell proliferation [26]. Treatment with the three-agent combination SCH66336, paclitaxel, and SCH58500 had overall additive efficacy in DU-145 prostate tumor cells, due to the pronounced synergy observed for each two-drug combination. Both intraperitoneal and subcutaneous DU-145 tumor xenograft models had enhanced sensitivity to combination therapy with oral SCH66336 and intratumoral SCH58500, as compared to either drug alone. In addition, mammary tumors in *wap-ras/F* transgenic mice rapidly regressed within 4 days of the start of combination therapy with

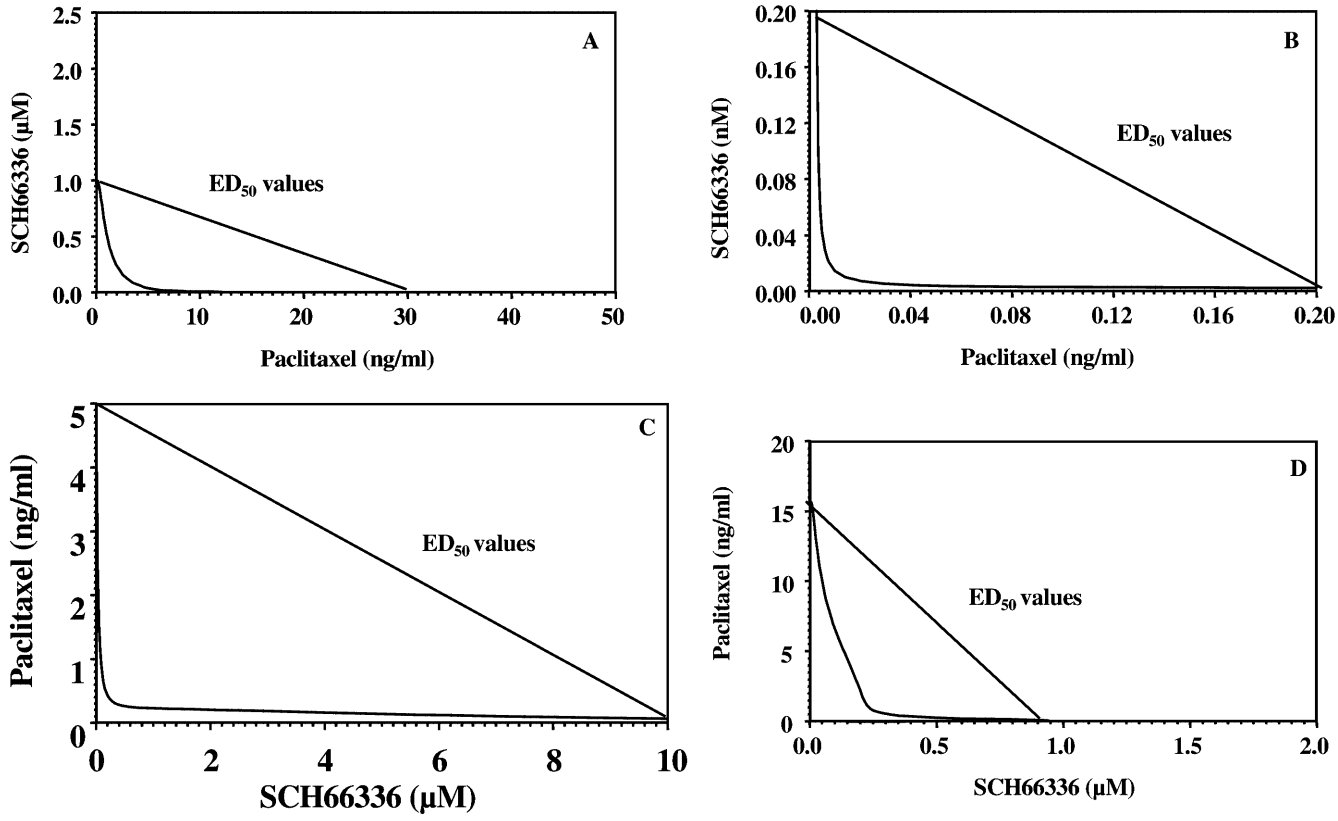

Fig. 1A–D Representative isoboles for tumor cells with differing p53 and K-ras status treated with SCH66336 and paclitaxel. **A** NCI-H460 large-cell lung tumor cells, $p53^{wt}$ and K-ras^{mut}. **B** LNCaP prostate tumor cells, $p53^{wt}$ and K-ras^{wt}. **C** BxPC-3 pancreatic tumor cells, $p53^{mut}$ and K-ras^{wt}. **D** MIAPaCa2 pancreatic tumor cells, $p53^{mut}$ and K-ras^{mut}. Tumor cells were treated with paclitaxel for 4 h followed by treatment with SCH66336 as detailed in Materials and methods. Cell proliferation was quantitated 7 days later

SCH66336 and SCH58500, but continued to grow for several more days under single-drug treatment regimens.

Here we report that SCH66336 also synergized with paclitaxel to inhibit the proliferation of 10 out of 11 tumor cell lines originating from six different tissues. Only in MDA-MB-231 cells was antagonism observed, for reasons which are currently unknown. SCH66336 also synergized with docetaxel to inhibit the proliferation of four out of five tumor cell lines originating from three different tissues. Synergy between SCH66336 and taxanes was observed in tumor cell lines expressing wild-type or mutant RAS and wild-type, null, or mutant p53.

The enhanced efficacy of combination therapy with SCH66336 and paclitaxel was also observed in vivo. In the NCI-H460 lung cancer xenograft model, oral SCH66336 and intraperitoneal paclitaxel had caused tumor growth inhibition of 56% by day 7 and 65% by day 14 relative to the effects of paclitaxel alone. These benefits of combination therapy were further illustrated using an activated *H-ras* transgenic tumor model. Male transgenic mice of the *wap-ras/F* substrain [FVB/N-TgN(WapHRAS)69LlnYSJL] spontaneously develop mammary tumors at 6–9 weeks of age which have been previously shown to be resistant to paclitaxel [25, 30]. Paclitaxel resistance was confirmed in the present study, while SCH66336 inhibited growth of these tumors. Most importantly, SCH66336 was able to sensitize *wap-ras/F* mammary tumors to paclitaxel chemotherapy, an effect which is not observed when p53 gene therapy is combined with paclitaxel [26].

Moasser et al. have observed in vitro synergy between the peptidomimetic FTI, L-744832, and paclitaxel [20]. We report here on a different FTI chemical class which showed synergy with both paclitaxel and docet-

Table 2 Analysis of in vitro drug interactions between SCH66336 and docetaxel ($P = 0.05$ indicates synergy or antagonism, $P > 0.05$ indicates no drug interaction, i.e. additive effects)

Cell line	Tumor type	p53 protein	Ras mutation	Isobole analysis
MDA-MB-468	Human breast	Mutant	Wild-type	Synergy ($P = 0.0019$)
MDA-MB-231	Human breast	Mutant	Mutant	Additive ($P = 0.4008$)
NCI-H460	Human lung	Wild-type	K-ras	Synergy ($P = 0.0127$)
MIAPaCa2	Human pancreatic	Mutant	K-ras	Synergy ($P = 0.0336$)
PANC-1	Human pancreatic	Mutant	K-ras	Synergy ($P = 0.0194$)

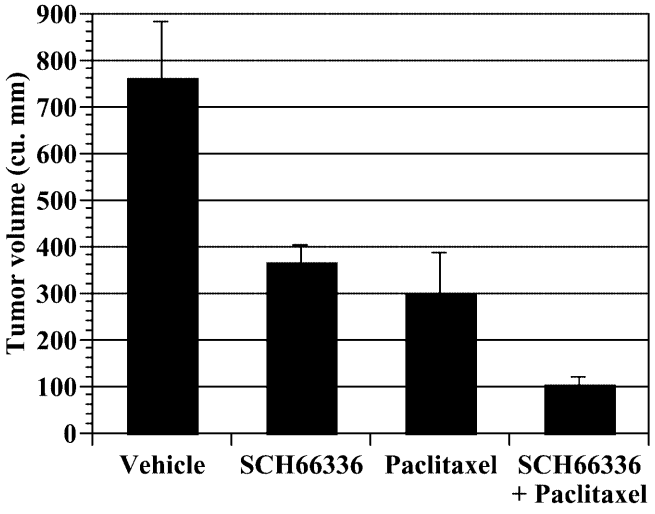

Fig. 2 Efficacy of SCH66336 and paclitaxel in the NCI-H460 lung tumor model ($p53^{wt}$, K-ras^{mut}). Tumor volumes ±SEM for day 14 are shown

axel. Our data also demonstrate that the combination of a nonpeptidomimetic FTI and paclitaxel has enhanced efficacy in vivo, which had only been shown previously with a peptidomimetic (FTI-2148) in a single A-549 tumor xenograft study [38]. These data suggest a drug interaction mechanism(s) dependent upon inhibition of FPT activity rather than on a specific FTI chemical structure. However, the previous finding of synergy between the peptidomimetic L-744832 and paclitaxel in MDA-MB-231 cells [20] contrasts with the antagonism we observed between SCH66336 and paclitaxel in the same cell line. In addition, the observation of additivity between SCH66336 and docetaxel suggests the possibility of different mechanisms for the interactions between SCH66336 and the two taxanes, although synergy was observed for the majority of cell lines.

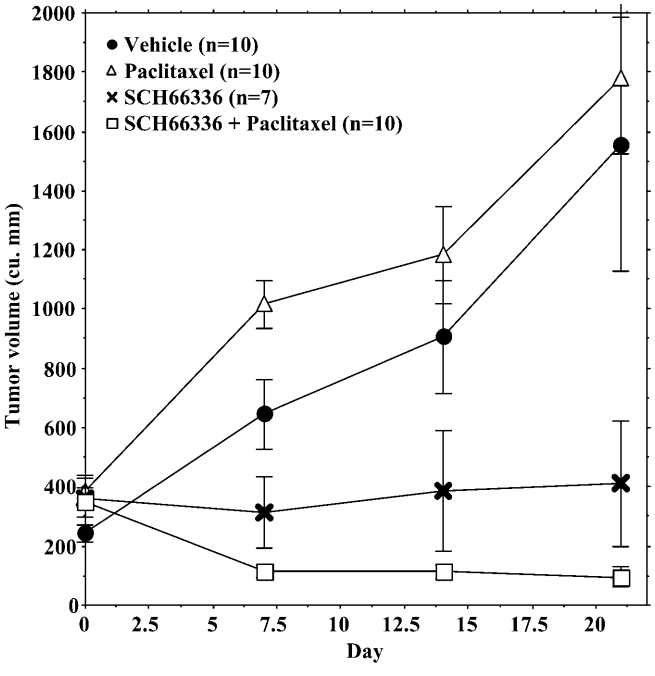

Fig. 3 Efficacy of SCH66336 and paclitaxel against paclitaxel-resistant *wap-ras/F* transgenic mouse mammary tumors (H-ras^{mut})

Many lines of evidence point toward multiple farnesylated protein targets when transferase activity is suppressed. For example, the FTIs, SCH66336 and SCH66177, inhibit the membrane association of H-RAS, but not K- or N-RAS in human tumor cell lines (Ashar et al., submitted for publication). In addition, these drugs cause human tumor cells with an activated *H-ras* to accumulate in the G0/G1 phase of the cell cycle, while tumor cells with an activated *K-ras* or wild-type ras tend to accumulate in the G2/M phase. These results are independent of p53 status. None of the cell lines used for our isobole analyses carry an activated *H-ras*. Therefore, the synergistic activities of SCH66336 and paclitaxel observed in our panel of cell lines might be partially explained by the observation that FTI treatment leads to accumulation of K-ras^{mut} tumor cells in the G2/M phase of the cell cycle (Ashar et al., submitted for publication) when paclitaxel activity is most effective [4]. Conversely, inhibition of FPT might enhance the mitotic block induced by paclitaxel [20].

There are several reports in the literature that taxanes may affect protein prenylation or Ras processing and trafficking. Danesi et al. [3] have reported that nanomolar concentrations of paclitaxel inhibit protein isoprenylation in PC-3 human prostate tumor cells. In addition, they have reported that the antiproliferative and proapoptotic effects of paclitaxel in this cell line can be partially overcome by the addition of farnesyl pyrophosphate or geranylgeranyl pyrophosphate to the cell culture medium. The biochemical basis for these effects have not been elucidated and isoprenyl pyrophosphates only results in a small shift in the cellular sensitivity to the biological effects of the paclitaxel. Another link between taxane treatment and prenyl protein processing comes from the work of Thissen et al. [39] who have reported that treatment of mouse NIH-3T3 fibroblasts with paclitaxel (3 μM) results in a mislocalization of K-RAS protein, although H-RAS localization to the plasma membrane is unaffected. These data suggest that protein prenylation is intact in paclitaxel-treated fibroblasts and that an intact microtubule network is required for the correct cellular localization (and appropriate activity) of K-RAS. Therefore, taxanes may interact with FTIs by further perturbing RAS prenylation and/or trafficking. In addition to stabilization of microtubules, paclitaxel can stimulate p53 and p21 protein levels in $p53^{wt}$ A549 lung tumor cells [40], suppress *bcl-xL* mRNA and protein in $p53^{wt}$ LNCaP prostate tumor cells [16], and inactive antiapoptotic BCL-2 protein [7, 31]. Docetaxel has also been shown to inactivate BCL-2 by inducing phosphorylation [7]. Thus, there are multiple steps at which FTIs and taxanes may synergize, and further work is clearly needed to biochemically define the basis for this synergy.

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