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# Antitumor Activity of SCH 66336, an Orally Bioavailable Tricyclic Inhibitor of Farnesyl Protein Transferase, in Human Tumor Xenograft Models and Wap-*ras* Transgenic Mice

**Ming Liu,[1] Matthew S. Bryant, Jianping Chen, Suining Lee, Bohdan Yaremko, Phil Lipari, Michael Malkowski, Eric Ferrari, Loretta Nielsen, Nicholas Prioli, Janet Dell, Dineshwar Sinha, Jameel Syed, Walter A. Korfmacher, Amin A. Nomeir, C-C. Lin, Lynn Wang, Arthur G. Taveras, Ronald J. Doll, F. George Njoroge, Alan K. Mallams, Stacy Remiszewski, Joseph J. Catino, Viyyoor M. Girijavallabhan, Paul Kirschmeier, and W. Robert Bishop**

*Departments of Biological Research-Oncology [M. L., J. C., S. L., B. Y., P. L., M. M., E. F., L. N., N. P., J. D., L. W., J. J. C., P. K., W. R. B.], Chemical Research [A. G. T., R. J. D., F. G. N., A. K. M., S. R., V. M. G.], Drug Metabolism and Pharmacokinetics [M. S. B., W. A. K., A. A. N., C-C. L.], and Safety Evaluation Center [D. S., J. S.], Schering-Plough Research Institute, Kenilworth, New Jersey 07033*

## ABSTRACT

**We have been developing a series of nonpeptidic, small molecule farnesyl protein transferase inhibitors that share a common tricyclic nucleus and compete with peptide/protein substrates for binding to farnesyl protein transferase. Here, we report on pharmacological and *in vivo* studies with SCH 66336, a lead compound in this structural class. SCH 66336 potently inhibits Ha-Ras processing in whole cells and blocks the transformed growth properties of fibroblasts and human tumor cell lines expressing activated Ki-Ras proteins. The anchorage-independent growth of many human tumor lines that lack an activated *ras* oncogene is also blocked by treatment with SCH 66336. In mouse, rat, and monkey systems, SCH 66336 has excellent oral bioavailability and pharmacokinetic properties. In the nude mouse, SCH 66336 demonstrated potent oral activity in a wide array of human tumor xenograft models including tumors of colon, lung, pancreas, prostate, and urinary bladder origin. Enhanced *in vivo* efficacy was observed when SCH 66336 was combined with various cytotoxic agents (cyclophosphamide, 5-fluorouracil, and vincristine). In a Ha-Ras transgenic mouse model, prophylactic treatment with SCH 66336 delayed tumor onset, reduced the average number of tumors/mouse, and reduced the average tumor weight/animal. In a therapeutic mode in which gavage treatment was initiated after the transgenic mice had developed palpable tumors, significant tumor regression was induced by SCH 66336 in a dose-dependent fashion. This was associated with increased apoptosis and decreased DNA synthesis in tumors of animals treated with SCH 66336. Enhanced efficacy was also observed in this model when SCH 66336 was combined with cyclophosphamide. SCH 66336 is presently being evaluated in Phase I clinical trials.**

## INTRODUCTION

In normal cells, Ras switches between an inactive guanosine 5′-diphosphate-bound and an active GTP[2]-bound state, which can initiate several intracellular signaling pathways (1, 2). Ras signaling is terminated by hydrolysis of GTP to guanosine 5′-diphosphate 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 greatly reduced capacity to hydrolyze GTP. This leads to constitutive activation of downstream signaling pathways resulting in altered regulation of cellular proliferation (1, 3).

Four isoforms of the Ras protein exist: Ha-Ras, N-Ras, Ki-Ras 4A, and K-Ras 4B (3). They are products of three genes, with Ki-Ras4A and Ki-Ras4B being splice variants of the same gene. Oncogenic mutations of the different isoforms predominate in different tumors (4). Ha-ras mutations are found in carcinomas of the bladder, kidney, and thyroid; N-ras mutations are found in myeloid and lymphoid cancers, liver carcinoma, and melanoma; whereas Ki-ras mutations predominate in colon, lung, and pancreatic carcinoma. The functional differences of the four isoforms remain unknown.

Many lines of evidence suggest that antitumor activity can be achieved by interfering with the function of oncogenic Ras proteins (5–7). Signal transduction by Ras is dependent on its plasma membrane localization. This localization is supported by a series of post-translational modifications. The first modification is farnesylation of a cysteine residue near the COOH-terminus of Ras catalyzed by FPT. Ras prenylation is thought to be the critical modification for proper membrane localization and function (8–10). Therefore, FPT inhibition is a potential mechanism to interfere with Ras-driven tumor growth.

Recent studies suggest that prenylation of Ras proteins is complex. *In vitro*, both Ki- and N-Ras proteins are substrates for a related protein prenyl transferase, GGPT-1 (11, 12). Although this reaction occurs with a lower catalytic efficiency than the farnesylation of these proteins, geranylgeranylation of Ki- and N-Ras proteins has been observed in cells treated with FPT inhibitors (13, 14). In contrast, the Ha-Ras protein is not a substrate for GGPT-1 *in vitro* or in cells. Because geranylgeranylated forms of Ras can support cellular transformation when overexpressed (9), this reaction represents a potential resistance mechanism for Ras-transformed cells to the effects of FPT inhibitors. Despite this alternative prenylation, inhibitors of FPT demonstrate *in vitro* and *in vivo* antitumor activity in tumor models expressing a mutationally activated *Ki-ras* gene (15–19). Thus, in some cases, blocking farnesylation of proteins other than Ras may contribute to the observed antitumor properties.

We have previously reported on a series of nonpeptidic, small molecule FPT inhibitors (15, 20–24).[3] These compounds share a common tricyclic nucleus, compete with peptide/protein substrates for binding to FPT, and inhibit growth of Ras-transformed fibroblasts and human cancer cells *in vitro* and *in vivo*. Here, we report on *in vivo* studies with SCH 66336, an improved compound in this structural class. The data demonstrate that SCH 66336 possesses potent antitumor activity against a wide variety of tumors after oral administration in mice. This compound is presently undergoing Phase I human clinical trials as an anticancer chemotherapeutic agent.

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[1] To whom requests for reprints should be addressed, at Schering-Plough Research Institute, 2015 Galloping Hill Road, K-15-4-4945, Kenilworth, NJ 07033. Phone: (908) 740-7136; Fax: (908) 740-7115.

[2] The abbreviations used are: GTP, guanosine 5′-triphosphate; FPT, farnesyl protein transferase; GGPT-1, geranylgeranyl protein transferase-1; mpk, mg/kg body weight; HPβCD, hydroxypropyl-β-cyclodextrin; q.i.d., four times/day; Cytoxan, cyclophosphamide; 5-FU, 5-fluorouracil; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; b.i.d., twice/day; BrdUrd, 5-bromo-2′-deoxy-uridine; MC, methyl cellulose; Cmax, peak serum concentration.

[3] R. J. Doll, *et al.* SCH 66336 chemical synthesis, manuscript in preparation.

## MATERIALS AND METHODS

**Compound.** The structure of SCH 66336, (+) 4-{2-[4-(8-Chloro-3, 10-dibromo-6, 11-dihydro-5H-benzo (5, 6) cyclohepta [1,2-b] pyridin-11-yl)-1-piperidinyl]-2-oxoethyl}-1-piperidinecarboxamide, is shown in Fig. 1. Its synthesis will be published elsewhere.[3]

***In Vitro* Enzyme Assays.** FPT activity was determined by measuring the transfer of [$^3$H]farnesyl from [$^3$H]farnesyl $PP_i$ to trichloroacetic acid-precipitable Ha-Ras-CVLS, as described previously (20). GGPT-1 activity was similarly determined using [$^3$H]geranylgeranyl diphosphate and Ha-Ras-CVLL as substrates (20).

**Cellular Assays for Inhibition of Ha-Ras Processing and Transforming Function.** Inhibition of intracellular processing of Ha-Ras by SCH 66336 was measured in transfected Cos cells as described previously (20). To determine the inhibition of anchorage-independent growth of transformed cells, soft agar growth assays were performed using standard procedures. Briefly, $10^4$ cells were seeded on 12-well plates in 0.35% agar over a 0.7% agar layer. Drugs were included in both agar layers. Cultures were fed and treated with a drug or vehicle twice weekly, and colonies were scored manually from duplicate wells on day 14.

**Cell Lines for *in Vivo* Studies.** The construction, cloning, and selection of NIH3T3-CVLS cells transfected with activated Ha-Ras containing its native COOH-terminal sequence CVLS has been described previously (15, 20). Human tumor cell lines [A549, AsPC-1, DLD-1, DU 145, HCT 116, HPAF-II, NCI-H460 (HTB-177), Hs 700T, and MIA PaCa-2] were obtained from American Type Culture Collection (Manassas, VA).

***In Vivo* Efficacy Studies Using Tumor Xenograft Models.** All animal studies were carried out in the animal facility of Schering-Plough Research Institute in accordance with institutional guidelines. Animals were maintained in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. After a week of acclimation, female nude mice (Crl:Nu/Nu-nu Br; Charles River Laboratories, Wilmington, MA), 5–7 weeks of age, received s.c. inoculations of various tumor cell lines on day 0. Human cancer cell lines used were: lung (A549, HTB-177); pancreas (AsPC-1, HPAF-II, Hs 700T, MIA PaCa-2); colon (HCT 116, DLD-1); prostate (DU-145); urinary bladder (EJ). The number of cells inoculated were: $1.0 \times 10^6$ for NIH3T3-CVLS; $3.0 \times 10^6$ for HTB 177; $5.0 \times 10^6$ for DLD-1, DU 145, EJ, HCT 116, HPAF-II, and MIA PaCa-2; $6.0 \times 10^6$ for A549, AsPC-1, and Hs 700T. Animals were randomly assigned to control and treatment groups (10 animals/group) before the first treatment. Drug treatment at 2.5, 10, or 40 mpk (q.i.d.) was initiated on day 1. SCH 66336 was dissolved in 20% (w/v) HPβCD. Vehicle controls received 20% HPβCD. Vehicle or drug solution (0.1 ml) was administered by oral gavage every 6 h (q.i.d.) for 20 or 21 days. A no-treatment control was always included along with the vehicle control to evaluate the influence of vehicle and of the q.i.d. gavage treatment. Once palpable, tumor volume was measured in three dimensions twice weekly and calculated with the formula of $V = 1/6 \times \pi \times L \times W \times T$, where L, W, and T represent length, width, and thickness respectively (15). T/C value in percentage was calculated for each measurement at the end of each treatment, where T and C were the mean tumor volume of the treated and control groups, respectively. Average inhibition was used to compare efficacy of various treatments and was derived by subtracting the T/C values of each treatment from 100. The single-tailed Student's *t* test was used for statistical analysis. For some experiments, an every 12-h (b.i.d.) dosing schedule was used.

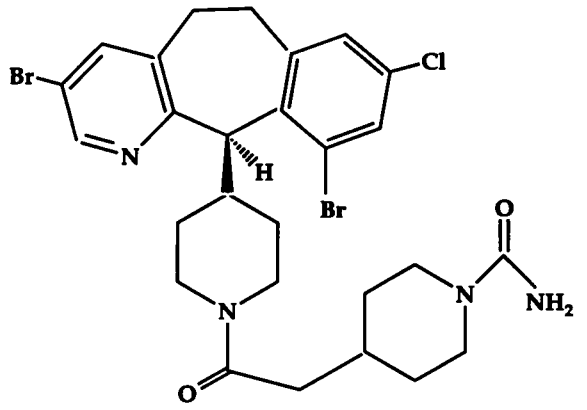


Fig. 1. Structure of SCH 66336. SCH 66336 is a lead compound in the tricyclic series of FPT inhibitors. This compound is bioavailable p.o., possesses *in vivo* antitumor activity after oral administration, and is presently being evaluated in Phase I clinical trials.

Table 1 *Biochemical and cell-based evaluation of SCH 66336*

| Assay | $IC_{50}$ (nM) |
|---|---|
| FPT enzyme (H-Ras) | 1.9 |
| FPT enzyme (K-Ras) | 5.2 |
| FPT enzyme (N-Ras) | 2.8 |
| GGPT-1 enzyme | >> 50,000 |
| H-Ras processing in Cos Cells | 10.0 |
| Soft agar growth | |
| Ha-ras-transformed NIH3T3 cells | 75.0 |
| Ki-ras-transformed NIH3T3 cells | 400.0 |
| HTB 177 lung carcinoma (K-ras mutation) | 40.0 |
| A549 lung carcinoma (K-ras mutation) | 150.0 |

Table 2 *Pharmacokinetic profile of SCH 66336 (25 mpk) in the nude mouse*

| Route | Vehicle | Cmax (μM) | AUC[a] (0–24hr; μg.hr/ml) | Bioavailability (%) | Half-life (hr) |
|---|---|---|---|---|---|
| i.v. | 20% HPβCD | – | 31.8 | – | 1.4 |
| p.o. | 20% HPβCD | 8.84 | 24.1 | 75.8 | – |
| p.o. | 0.4% MC | 16.63 | 41.45 | 100 | – |

[a] AUC, area under the curve.

Table 3 *Pharmacokinetics of SCH 66336 (10 mpk) in the cynomolgus monkey*

| Route | Vehicle | Cmax (μm) | AUC[a] (0–24hr; μg.hr/ml) | Bioavailability (%) | Half-life (hr) |
|---|---|---|---|---|---|
| i.v. | 20% HPβCD | – | 28.9 | – | 3.0 |
| p.o. | 20% HPβCD | 1.9 | 14.7 | 51.0 | – |
| p.o. | 1% PEG400 | 1.78 | 13.2 | 45.8 | – |
| p.o. | 0.4% MC | 1.92 | 14.0 | 48.5 | – |
| p.o. | 0.4% MC + 0.5% Tween 80 | 2.49 | 16.6 | 57.5 | – |

[a] AUC, area under the curve; PEG, polyethylene glycol.

HTB-177 human lung carcinoma cells were also used to evaluate the efficacy of SCH 66336 in combination with various cytotoxic agents. Cytotoxic agents, Cytoxan, 5-FU, and vincristine, were obtained from Sigma Chemical Co. HTB-177 cells were inoculated into nude mice, and efficacy was evaluated as described above. Nude mice (10 mice/group) received inoculations of HTB-177 cells on day 0 and were treated with either SCH 66336 alone (40 mpk, q.i.d., days 1–26), the cytotoxic agent alone, or the combination of SCH 66336 with the cytotoxic agent. All cytotoxics were dosed as a single i.p. injection on day 13 at the following doses: Cytoxan (200 mpk), 5-FU (50 mpk), vincristine (1 mpk).

***In Vivo* Efficacy Studies Using Wap-*ras* Transgenic Mouse Models.** To evaluate the efficacy of SCH 66336 in a model with spontaneous tumor occurrence and intact host immunity, wap-*ras* transgenic mice carrying an activated Ha-*ras* oncogene were used. Ras expression is driven by the whey acidic protein promoter in this model. Because the Ha-*ras* transgene is carried on the Y chromosome, only male mice develop tumors (mammary and salivary). The founder mice were obtained from Dr. A. C. Andres (Univ. of Berne, Switzerland; Ref. 25) and sublines (69–2 and 69–2F) were developed by Dr. L. L. Nielsen (Schering-Plough Research Institute, New Jersey; Refs. 26–28). Subline 69–2 mice spontaneously develop tumors between 3 and 6 months of age, whereas the time of tumor onset in 69–2F mice is between 6 and 9 weeks of age. These differences in the time and uniformity of tumor onset led us to use 69–2F mice for evaluating the prophylactic efficacy of SCH 66336 and 69–2 mice for evaluating the therapeutic efficacy. For prophylactic studies, 69–2F mice were enrolled when they were 35 days of age, before tumor onset. For therapeutic studies, 69–2 mice were enrolled upon development of a palpable tumor. For both studies, vehicle or SCH 66336 treatment lasted for 4 weeks on a q.i.d. schedule with 10 mice/treatment group.

Table 4 *Correlation of SCH 66336 concentration in serum and tumor xenograft with in vivo efficacy in the nude mouse*

| Dose (q.i.d. for 21 days) | Cmax SCH 66336 (serum) | Cmax SCH 66336 (tumor) | *In vivo* tumor growth inhibition |
|---|---|---|---|
| 10 mpk | 1.1 μM | 0.88 μmol/kg | 32% |
| 50 mpk | 31.5 μM | 26.7 μmol/kg | 76% |

Table 5 *Summary of in vivo efficacy of orally administered SCH 66336 in xenograft models*

| Tumor | Ras mutation status | Average growth inhibition (%) 2.5 mpk q.i.d. | 10 mpk q.i.d. | 40 mpk q.i.d. |
|---|---|---|---|---|
| A-549 (lung) | K-Ras | 42 | 61 | 70 |
| HTB-177 (lung) | K-Ras | 40 | 57 | 83 |
| AsPc-1 (pancreas) | K-Ras | 29 | 33 | 72 |
| HPAF-II (pancreas) | K-Ras | 9 | 19 | 67 |
| Hs700T (pancreas) | Not detected | 40 | 61 | 78 |
| MIA Paca (pancreas) | K-Ras | 46 | 48 | 72 |
| DU-145 (prostate) | Not detected | 29 | 59 | 86 |
| HCT-116 (colon) | K-Ras | 38 | 67 | 84 |
| EJ[a] (bladder) | H-Ras | 59 | 80 | 100 |
| NIH3T3-CVLS[a] | H-Ras | 16 | 55 | 100 |
| DLD-1[b] (colon) | K-Ras | | 32 | 76 |

[a] Result of 5, 20, or 80 mpk, b.i.d. treatment.
[b] Result of a 10 or 50 mpk, q.i.d. treatment.

**Analysis of Apoptosis in Transgenic Tumor Specimens.** Ten mammary tumor-bearing strain 69–2 wap-*ras* mice were treated with SCH 66336 at 40 mpk q.i.d. for 5 consecutive days. Every 24 h after the initiation of treatment, two mice were euthanized, and tumors were removed and preserved in 10% buffered formalin for histological study. Tumor samples were analyzed for the number of apoptotic cells using the TUNEL method, as described previously (29). The assay was performed using reagents obtained from Oncor, Inc. (Gaithersburg, MD). Cells undergoing apoptosis were evaluated by light microscopy. Positively stained cells within a 6.5 × 9-mm grid in the eyepiece were counted over 10 different ×200 magnification fields for each tumor section. The numbers were recorded, and the mean and SD were calculated. The Student's *t* test was used to compare the significance between treatments.

**Analysis of BrdUrd Labeling in Transgenic Tumor Specimens.** Inhibition of cellular proliferation *in vivo* was assessed using BrdUrd labeling. After 5 days of treatment with SCH 66336 (80 mpk, b.i.d.), two strain 69–2 wap-ras mice were given i.p. injections of 0.1 ml of BrdUrd solution (10 mpk) at 1 h after the final SCH 66336 dose. Four hours after BrdUrd injection, the mice were euthanized and mammary tumors were removed and preserved for immunohistochemical evaluation. Proliferating tumor cells were detected using antibodies directed against BrdUrd incorporated into the DNA (30). The assay was performed using reagents obtained from Boehringer Mannheim Corporation (Indianapolis, IN). Anti-BrdUrd stained cells were quantitated using light microscopy as described above.

**Pharmacokinetic Studies.** Nude mice were used to study the pharmacokinetic properties of SCH 66336. Blood samples were collected at nine time points (2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 7 h, and 24 h) after a single oral or i.v. (tail vein) dose of 25 mpk SCH 66336 in 20% HPβCD. Three mice were used for each time point, and samples were collected by cardiac puncture after euthanasia with carbon dioxide. After clotting on ice, serum was isolated by centrifugation. Quantitation of SCH 66336 serum levels was achieved using acetonitrile precipitation, followed by high performance liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry. To compare bioavailability in a different vehicle, SCH 66336 was also suspended in 0.4% MC and dosed p.o. A detailed description of the analytical methodology has been described for an earlier analogue in this series (15, 22).

In addition, both serum and tumor samples were collected from mice at various times after the final dosing in one of the efficacy studies. SCH 66336 was quantified in serum, as described above. Quantitation of SCH 66336 in tumor samples required additional processing steps including pulverizing the frozen tissue, homogenization, and protein precipitation (23).

The pharmacokinetics of SCH 66336 were also studied in male cynomolgus monkeys. SCH 66336 in 20% HPβCD was given by oral or i.v. administration at a dose of 10 mpk. To compare bioavailability between various vehicles, SCH 66336 was also suspended at 10 mpk in 1% Polyethylene Glycol 400, 0.4% MC, or 0.4% MC + 0.5% Tween 80 and administered to the monkeys. Blood samples were collected up to 48 h after dosing. Plasma was isolated and SCH 66336 was quantified using methods described previously (22).

## RESULTS

**SCH 66336 Is a Potent and Selective FPT Inhibitor.** SCH 66336 demonstrates potent and selective inhibition of FPT in various biochemical and cell-based assays (Table 1). SCH 66336 blocks farnesylation of Ha-Ras *in vitro* by purified human FPT with an $IC_{50}$ value of 1.9 nM. SCH 66336 also inhibits farnesylation of N-Ras (2.8 nM) and K-Ras-4B *in vitro* (5.2 nM), although it does not inhibit GGPT-1 at concentrations up to 50 μM. Kinetic studies of compounds in this series demonstrate that they are competitive with the protein substrate (20). The ability of SCH 66336 to inhibit farnesylation of Ha-Ras in cells was assessed using immunoblot analysis of cell lysates to distinguish between the posttranslationally processed and unprocessed forms of this protein (20). SCH 66336 inhibits farnesylation of Ha-Ras in Cos cells with an $IC_{50}$ value of 10 nM, indicating that it penetrates the cell membrane readily. SCH 66336 also blocks many of the features of the transformed phenotype in rodent fibroblasts expressing a mutationally-activated form of Ha-Ras, including anchorage-independent, soft agar growth ($IC_{50}$ = 75.0 nM). In contrast, SCH 66336 and related compounds do not revert the transformed phenotype of rodent fibroblasts expressing a form of activated H-Ras engineered to be a substrate for GGPT-1 (20).

Anchorage-independent growth of fibroblasts expressing muta-

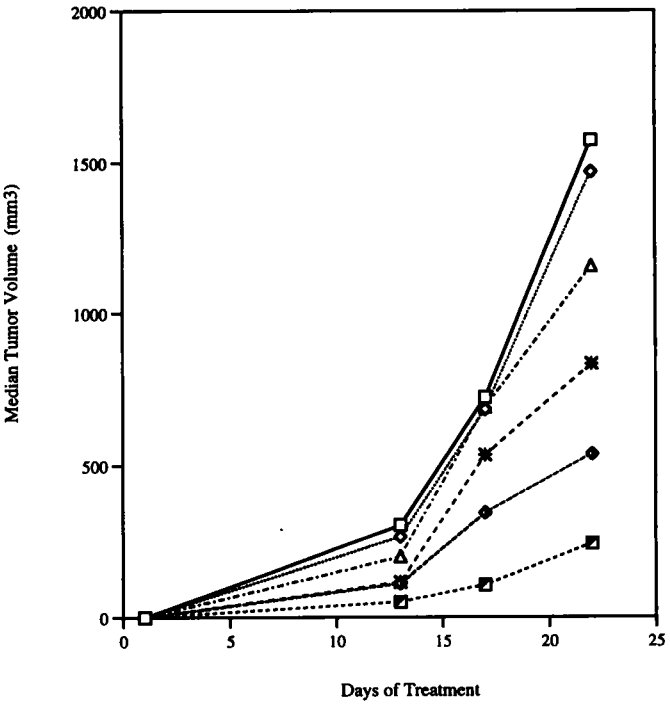


Fig. 2. SCH 66336 inhibits HTB177 human lung carcinoma xenograft growth in nude mice in a dose-dependent fashion. Nude mice bearing HTB177 xenografts were treated with various doses of SCH 66336, as described in "Materials and Methods." Tumor cells were inoculated on day 0, and q.i.d. oral treatment with SCH 66336 was started on day 1. Each treatment group included 10 mice. Median tumor volume for each group is plotted *versus* days after tumor cell inoculation. □, no treatment; ◇, 20% HPβCD; ◩, 66336/40 mpk; ◆, 66336/10 mpk; ✱, 66336/2.5 mpk; △, 66336/0.625 mpk.

tionally-activated Ki-Ras is also inhibited by SCH 66336 with an $IC_{50}$ value of 400 nM. In addition, soft agar growth of a variety of human tumor cell lines containing activated Ha-Ras and Ki-Ras is inhibited by SCH 66336.[4] Representative data for two human lung carcinoma cell lines, HTB177 and A549, both of which contain an activating mutation in Ki-Ras, are shown in Table 1. As observed with other farnesyl transferase inhibitors, some tumor cell lines lacking Ras mutations are also growth inhibited by SCH 66336 in soft agar (data not shown). Human tumor cell lines derived from a variety of tissues of origin are susceptible to anchorage-independent growth inhibition by SCH 66336. Overall, the concentration of SCH 66336 needed to achieve 50% reduction of soft agar growth ($IC_{50}$) was ≤500 nM for approximately 60% of the human tumor cell lines tested.

**Oral Pharmacokinetics and Tumor Levels of SCH 66336.** The pharmacokinetics of SCH 66336 were evaluated in mice, rats, and cynomolgus monkeys. In athymic nude mice (Table 2), a Cmax of 8.8 μM was achieved after an oral dose of 25 mpk. At this dose, drug levels persisted at a concentration above 700 nM for 9 h. The oral bioavailability of SCH 66336 in the mouse was 76%, and the half-life after i.v. administration was 1.4 h.

A dose-response study was conducted in the rat where plasma Cmax values of 3, 10, and 30 μM were achieved after a single oral dose of 10, 30, and 100 mpk, respectively. SCH 66336 was also administered p.o. in four different vehicles or by i.v. administration to cynomolgus monkeys at 10 mpk (Table 3). Plasma levels were similar for all vehicles and Cmax values of 1.8–2.5 μM were reached after oral dosing. The half-life was 3.0 h., and oral bioavailability was approximately 50% in this species.

In a human colon carcinoma DLD1 xenograft study in the nude mouse, the concentration of SCH 66336 in the tumor tissue after the final dosing was very similar to the steady-state concentration achieved in the serum (Table 4). Although this cell line is relatively resistant to the growth inhibitory effects of SCH 66336 in soft agar ($IC_{50}$ = 2.5 μM), these high tumor levels supported antitumor efficacy. At a tumor level of 0.88 μmol/kg, 32% tumor growth inhibition was achieved, whereas a tumor level of 26.7 μmol/kg resulted in 76% inhibition. In a second tumor model using HTB177 human lung carcinoma cells, tumor levels of SCH 66336 ranged from 14–33% of that in the serum (data not shown). However, because this cell line displays much greater sensitivity to soft agar growth inhibition by SCH 66336, these lower tumor drug levels also supported antitumor efficacy. The reason for differences in tumor drug levels between the two xenograft models is unknown but may reflect, in part, differences in the extent of tumor vascularization.

The observed pharmacokinetic properties described above support the use of SCH 66336 as an oral agent and indicate that SCH 66336 can readily reach the target tumor tissue.

**SCH 66336 Inhibits Tumor Growth by Rodent Fibroblasts Transformed with Activated Ha-Ras.** NIH 3T3 cells transformed with activated Ha-Ras-CVLS were used to evaluate the antitumor effect of SCH 66336. Oral gavage treatment with SCH 66336 (every 12 h at 5, 20, or 80 mpk) was initiated the day after tumor inoculation and continued for 15 days. SCH 66336 dose-dependently inhibited the growth of tumors derived from these cells (Table 5), with 16, 55, and 100% tumor growth inhibition observed at the 5, 20, and 80 mpk dose levels, respectively. This inhibition was statistically significant at $P < 0.005$ for the 20 and 80 mpk treatment groups. The efficacy of SCH 66336 observed in this model is consistent with the results observed with an earlier tricyclic analogue (*e.g.*, SCH 59228), which selectively inhibited growth of tumors from cells transformed with a farnesylated *versus* geranylgeranylated form of Ha-Ras (15).

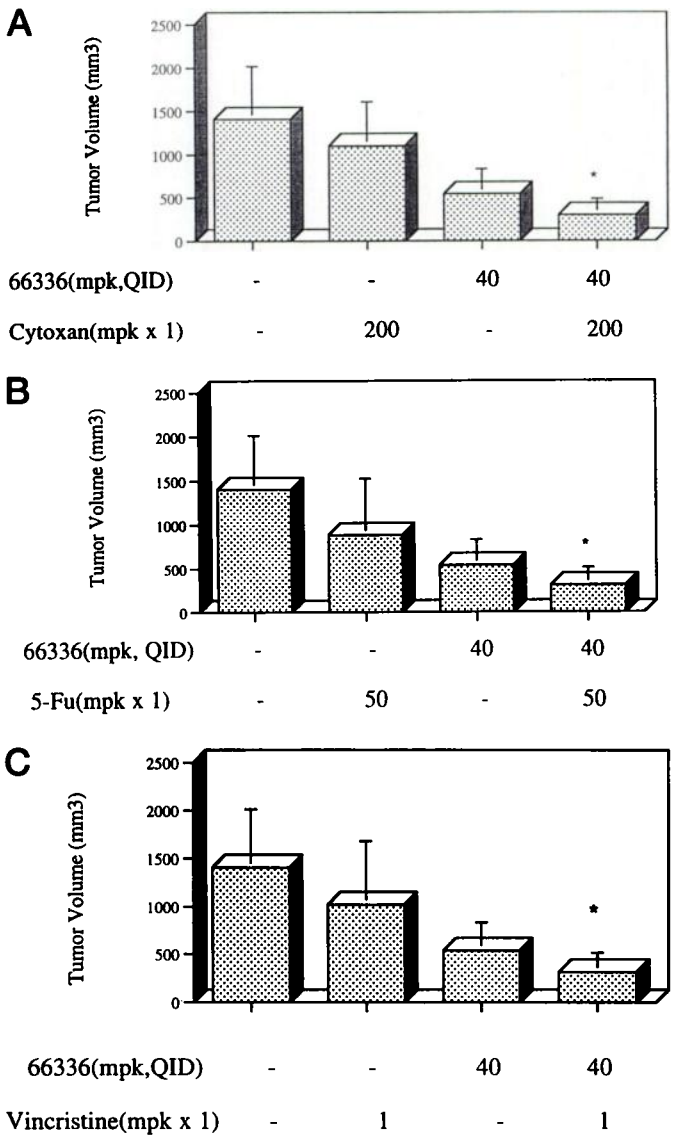


Fig. 3. Combination of SCH 66336 with cytotoxic agents in the HTB177 human lung carcinoma xenograft. Female athymic nude mice received s.c. inoculations of $3 \times 10^6$ HTB 177 lung carcinoma cells on day 0. Animals were treated with SCH 66336 (40 mpk, p.o., q.i.d.), the indicated cytotoxic agent (i.p., day 13), or a combination of treatments as described in "Materials and Methods." Results are shown for Cytoxan (*A*), 5-FU (*B*), and vincristine (*C*). Mean tumor volumes for each group at the end of the experiment are compared. SDs are indicated by error bars. *, $P < 0.05$ compared with each single agent treatment.

**SCH 66336 Inhibits the Growth of Human Tumor Xenografts Representing a Wide Variety of Histologies.** SCH 66336 has demonstrated significant *in vivo* antitumor activity in a variety of human tumor xenograft models when dosed p.o. in nude mice (Table 5). In most of these studies, tumor cells were implanted s.c. on day 0 and SCH 66336 was administered q.i.d. beginning on day 1 and continuing throughout the study. Typically, q.i.d. doses of 2.5, 10, and 40 mpk were administered by oral gavage using 20% HPβCD as a vehicle. Using this paradigm, antitumor activity was observed in xenograft models of lung carcinoma (A549 and HTB-177), pancreatic carcinoma (AsPC-1, HPAF-II, HS 700T, and MIA PaCa-2), colon carcinoma (HCT 116 and DLD-1), and prostate

[4] P. Kirschmeier, *et al.*, manuscript in preparation.

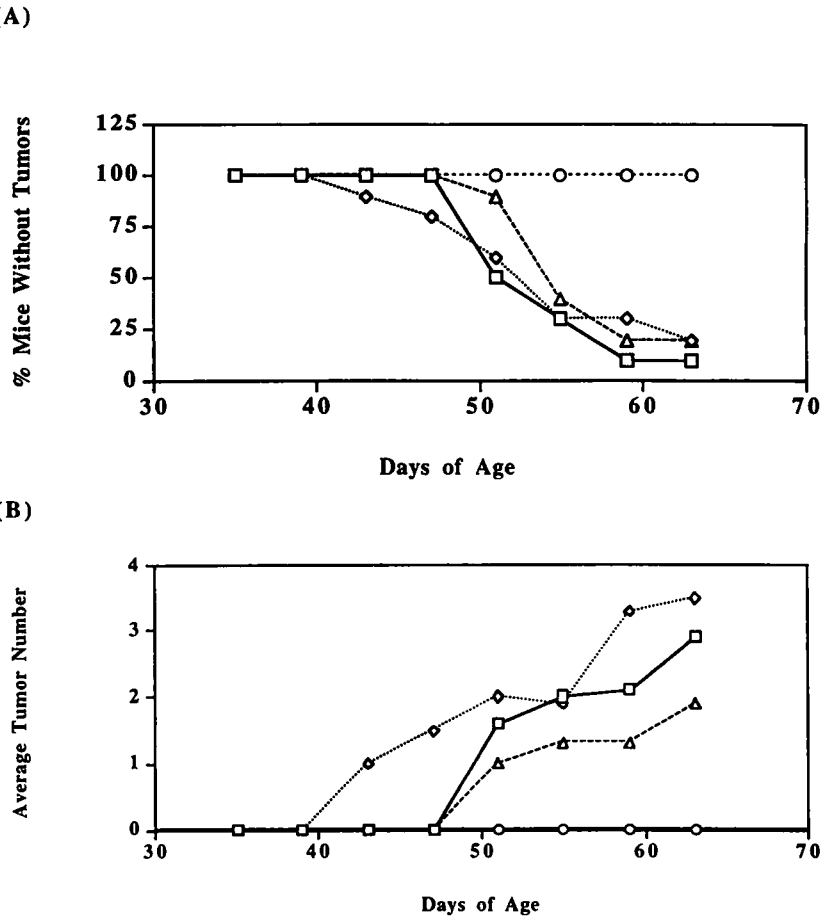


Fig. 4. SCH 66336 delays tumor onset when dosed prophylactically to wap-*ras* transgenic mice. Wap-*ras* transgenic mice (strain 69–2F) were used to evaluate SCH 66336 in a prophylactic fashion. SCH 66336 dosing was initiated at 35 days of age, before tumor onset, and continued for 4 weeks (to 63 days of age) at either 10 or 40 mpk q.i.d. Results are shown as the percentage of tumor-free mice (*A*) and as the average number of tumors/mouse (*B*). □, no treatment; ◇, 20% HPβCD; ○, SCH66336/40 mpk; △, SCH66336/10 mpk.

carcinoma (DU 145). Average inhibition of tumor volume in drug-treated *versus* vehicle control groups at the end of the experiment ranged from 67–86% at the 40 mpk q.i.d. level ($P < 0.01$ in each model). Significant antitumor activity was also observed at lower dose levels (10 and 2.5 mpk q.i.d.). Growth curves from a representative experiment using the HTB-177 lung carcinoma model are shown in Fig. 2.

Antitumor activity is also observed when SCH 66336 is administered with a b.i.d. schedule in the HTB-177 lung carcinoma model. Doses of 80 and 20 mpk b.i.d. produced equivalent tumor growth inhibition (87 and 59%, respectively) compared with q.i.d. doses of 40 and 10 mpk. This result was anticipated based on the known pharmacokinetic properties of SCH 66336 in mice. Using the b.i.d. dosing schedule in a human bladder carcinoma (EJ) model, tumor growth inhibition of 59% and 80% was observed with doses of 5 and 20 mpk, respectively. Complete inhibition was achieved with a dose of 80 mpk. These data, along with the promising pharmacokinetic properties of SCH 66336 in primates, indicate that a less frequent dosing schedule is achievable clinically. Present Phase I clinical trials with SCH 66336 are being carried out with b.i.d. oral dosing.

**Enhanced Antitumor Efficacy Using Combination Therapy of SCH 66336 with Cytotoxic Agents.** The HTb 177 xenograft model was used in preliminary experiments designed to examine the combination of SCH 66336 with cytotoxic agents (Fig. 3, *A-C*). In one study, SCH 66336 (40 mpk, q.i.d., days 1–26) resulted in a 60% tumor growth inhibition when dosed alone. Various cytotoxic agents were administered i.p. on day 13 as single agents and yielded 9, 28, and 7% tumor growth inhibition for Cytoxan (200 mpk), 5-FU (50 mpk), and vincristine (1 mpk), respectively. When SCH 66336 was used in combination with these agents, tumor growth inhibition of 81, 80, and 80% was observed for the combination with Cytoxan, 5-FU, and vincristine, respectively. These results are statistically significant compared with the SCH 66336 alone result with $P < 0.05$. The greater efficacy observed with the combinations indicates that there is no antagonism upon combining SCH 66336 with the cytotoxics tested.

**SCH 66336 Delays Tumor Onset and Induces Tumor Regression in Wap-ras Transgenic Mice.** SCH 66336 was also evaluated in a transgenic model in which an activated ($Val^{12}$) Ha-Ras oncogene is expressed from the whey acidic protein promoter (25, 27). Because the transgene is carried on the Y chromosome, male transgenic mice reproducibly develop tumors of the mammary and salivary gland. Two substrains of these mice (69–2 and 69–2F) were used for these studies. SCH 66336 demonstrated antitumor activity when dosing at either 10 or 40 mpk q.i.d. was initiated before tumor onset (at 35 days of age) and continued for 4 weeks (to 63 days of age) in the 69–2F strain. SCH 66336 treatment delayed tumor onset, reduced the average number of tumors/mouse, and reduced the average tumor weight/animal. More significant antitumor effects were seen at the 40 mpk dose level, where animals remained tumor-free throughout the dosing period (Fig. 4). These animals remained tumor-free for a minimum of another 20 days after treatment was terminated, at which time tumors did develop in some mice. This recurrence of tumors is anticipated because these transgenic animals still harbor the activated Ha-ras transgene.

This model was also used in a therapeutic mode in which treatment was initiated after mice had developed palpable tumors. Strain 69–2 mice were treated with SCH 66336 at 2.5, 10, 20, or

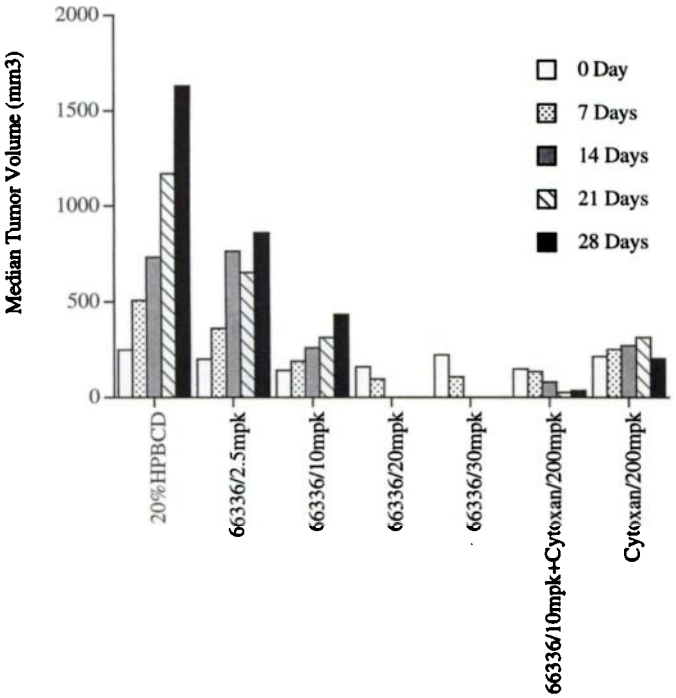


Fig. 5. SCH 66336 induces tumor growth inhibition and tumor regression when dosed therapeutically to wap-*ras* transgenic mice. Wap-*ras* transgenic mice (strain 69–2) were used to evaluate SCH 66336 in a therapeutic mode in which treatment was initiated after mice had developed palpable tumors (>100 $mm^3$). Mice were treated q.i.d. with escalating doses (2.5, 10, 20, or 30 mpk) of SCH 66336 for 4 weeks. One group of mice was treated with weekly i.p. injections of Cytoxan at 200 mpk alone, and another group was treated with a combination of Cytoxan at 200 mpk and SCH 66336 10 mpk, q.i.d. Growth curves for the various treatment groups are shown. The median tumor size measured on days 0, 7, 14, 21, and 28 is plotted for each group.

30 mpk (q.i.d.) for 4 weeks. Some groups of mice were also treated with weekly i.p. injections of cyclophosphamide at 200 mpk. Growth curves for the various treatment groups are shown in Fig. 5. The mean tumor size at the start of dosing (day 0) was 200 $mm^3$. In the vehicle-treated group, tumors grew throughout the course of the experiment to a volume of >1500 $mm^3$ by the end of the study. SCH 66336 at 2.5 or 10 mpk significantly slowed the rate of tumor growth. SCH 66336 at the 20- or 30-mpk dose level resulted in significant tumor regression. Regression was measurable by day 7 and essentially complete by day 14. Similar results were obtained in strain 69–2F mice (data not shown). Significant and rapid tumor regressions were also observed when SCH 66336 treatment was initiated in 69–2 mice after tumors had achieved sizes in the 2000 $mm^3$ range (data not shown).

Interestingly, although 200 mpk Cytoxan or 10 mpk SCH 66336 as single agents did not result in tumor regression, the combination of these treatments resulted in significant regression. This result corresponds well with the enhanced efficacy observed with the combination of SCH 66336 and Cytoxan in the HTB177 xenograft model and supports the possibility that SCH 66336 may act in an additive or greater than additive manner when combined with standard cytotoxic cancer therapy.

In cross-over studies, mice initially in the vehicle control or low-dose SCH 66336 groups (2.5 or 10 mpk) were subsequently treated with 30 mpk SCH 66336 for 3 more weeks. As soon as the high-dose treatments were initiated, all tumors regressed. Such cross-over efficacy was demonstrated in both the 69–2 and 69–2F wap-ras strains.

**SCH 66336 Induces Apoptosis and Reduces Cell Proliferation *in Vivo*.** When H&E-stained tissue sections of tumors from these transgenic animals were examined microscopically, a marked increase in apoptosis and a decrease in mitotic figures was observed upon SCH 66336 treatment (data not shown). The TUNEL assay was used to evaluate the degree of apoptosis, and BrdUrd incorporation was used to evaluate the degree of cell proliferation in both wap-ras transgenic tumors and EJ human bladder xenografts. A marked increase in apoptosis was observed as early as 24 h after the initiation of SCH 66336 treatment in the wap-*ras* tumors (Fig. 6 and Table 6), as well as the EJ xenograft (data not shown). In the wap-*ras* model, maximal apoptosis was observed at 24 h, however, the apoptotic index remained elevated for at least 5 days. A significant dose-dependent decrease of BrdUrd labeling was also detected at day 5 after dosing was initiated in both wap-ras transgenic tumors (Fig. 7 and Table 6) and EJ xenograft tumors (data not shown), indicating a significant reduction in cell proliferation.

## DISCUSSION

The studies reported here demonstrate that the tricyclic FPT inhibitor, SCH 66336, has potent *in vivo* antitumor activity when administered p.o. to nude mice and to wap-*ras* transgenic mice. Tumor growth inhibition is dose-dependent, and tumor regression is achieved in some models. Significantly, in models where regression was seen, increased apoptosis and decreased cell proliferation were observed and are likely to contribute to the observed efficacy. In addition, this is the first report of enhanced *in vivo* efficacy when a FPT inhibitor is used in combination with cytotoxic agents.

SCH 66336 and related tricyclic FPT inhibitors are structurally distinct from other reported FPT inhibitors, many of which were derived from peptidomimetic approaches (31–40). These differences, including the absence of a sulfhydryl function, are likely to contribute to the favorable oral pharmacokinetics of SCH 66336. Initial compounds in this series, such as SCH 44342, displayed less than optimal pharmacokinetic properties in the mouse, including a very rapid oxidative metabolism (15, 22). Blocking the susceptible metabolic sites on SCH 44342 greatly improved the pharmacokinetic properties of compounds in this series. SCH 66336, an 11-piperidinyl trihalogenated analogue, was one of the lead compounds that emerged from these efforts. This compound possesses not only excellent bioavailability in both the mouse and monkey, but it also displays improved metabolic stability (t1/2 of 1.4 h in the mouse and 3 h in the monkey). Due to these improvements, SCH 66336 persists in mouse serum at a concentration $\geq 1$ $\mu$M for over 7 h after a single oral dose of 25 mpk. Significant steady-state drug levels are also achieved in tumor xenograft tissues after continuous dosing for 3 weeks. In addition to the improved pharmacokinetics of SCH 66336, the intrinsic potency ($IC_{50}$ = 1.9 nM) is also substantially improved compared with earlier compounds in this series. The enhanced potency and pharmacokinetic properties of SCH 66336 are critical for its antitumor efficacy when administered p.o. on either a q.i.d. or a b.i.d. schedule.

We observed a broad spectrum of *in vivo* antitumor activity with SCH 66336. This compound dose-dependently inhibits the *in vivo* growth of a panel of human tumor xenografts of lung, colon, pancreas, bladder, and prostate origin. In some experiments, 5–10% weight loss was observed in SCH 66336-treated animals. However, weight loss was also seen in some vehicle-treated groups and attributed to frequent oral gavage. No other overt toxicity was observed. Similar to the results with other FPT inhibitors (41, 42), the presence of an activated *ras* oncogene is not required for a tumor cell line to be sensitive to SCH 66336-mediated growth inhibition either in soft agar[4] or as xenografts in mice. For example, significant tumor growth inhibition is observed in models that are wild-type for *ras* alleles, such as the pancreatic tumor line Hs700T and the prostate carcinoma line

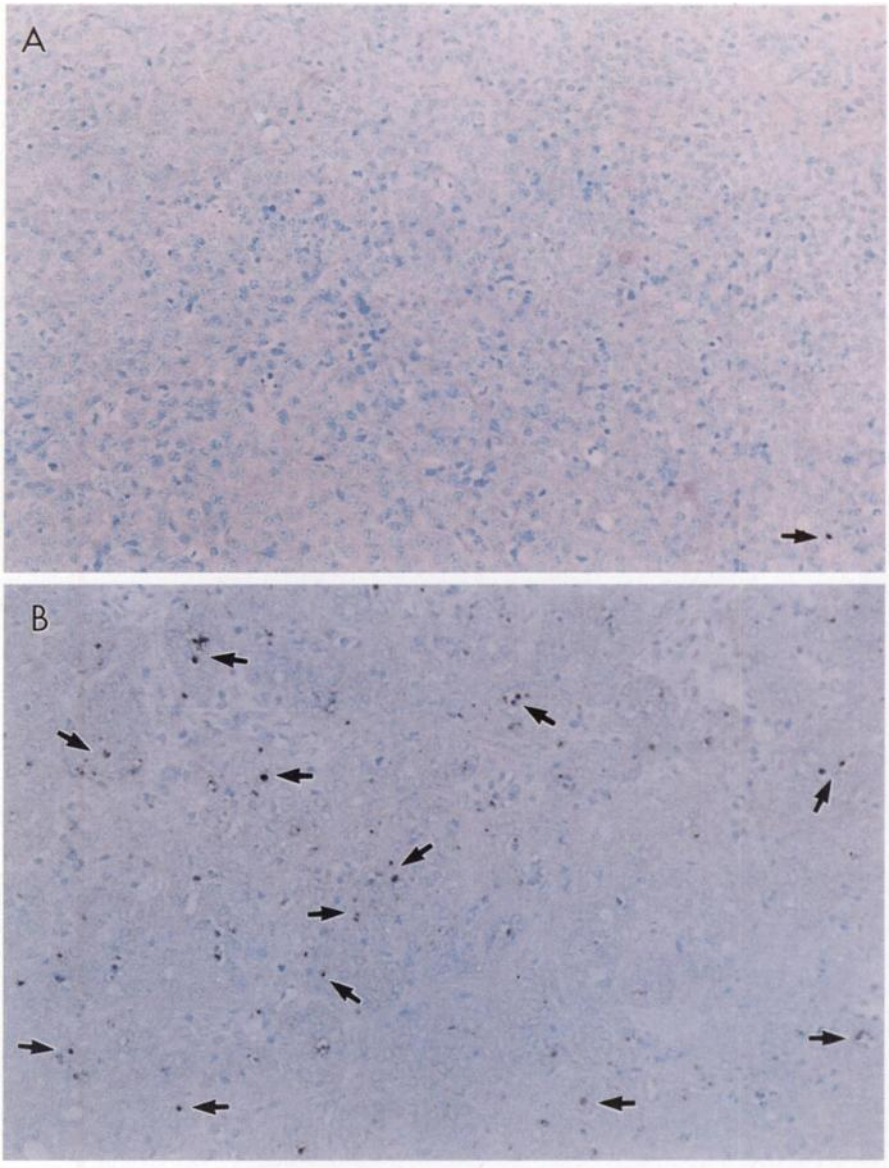


Fig. 6. SCH 66336 induces apoptotic cell death in tumors from wap-*ras* transgenic mice. The photomicrographs show mammary adenocarcinomas from wap-*ras* transgenic mice (strain 69–2) either treated with vehicle control (*A*) or SCH 66336 40 mpk (*B*; p.o., q.i.d.) for 24 h (four doses). The tissue sections were stained using the TUNEL assay to label apoptotic cells, as described in "Materials and Methods." *Arrows*, the only positively stained cell in *A* and some of the positively stained cells in *B*.

DU-145. Sensitive cells with a wild-type *ras* genotype may, nevertheless, be dependent on Ras-mediated signaling for maintenance of the transformed phenotype (due to mutational activation of oncogenes or reliance on autocrine growth factors, either of which may activate the Ras signal transduction pathway).

Tumor growth inhibition was also observed using xenografts expressing either a mutant allele of Ha-*ras* (*e.g.*, EJ bladder carcinoma) or a mutant allele of Ki-*ras* (*e.g.*, A549 lung carcinoma, etc.). Most of these models were run in an unstaged manner, where dosing was initiated on day 1 after tumor cell implantation. In models containing an activated Ki-Ras, tumor growth inhibition of 67–84% was observed at the high dose of SCH 66336. In contrast, 100% tumor growth inhibition was seen in the Ha-Ras models examined including EJ bladder, Ha-Ras-transformed NIH3T3 fibroblasts, and the wap-*ras* transgenic mice. In the transgenic model, significant tumor regressions were observed. Tumors of 200 $mm^3$ regressed to an unpalpable state within 14 days after initiating treatment with the high dose of SCH 66336. Tumors as large as 2000 $mm^3$ also underwent significant regression upon treatment. Both a decrease in proliferation (as measured by BrdUrd labeling) and an increase in apoptosis (as measured by TUNEL staining) contribute to the regressions in this model. Induction of apoptosis and tumor regression has also been observed in the EJ bladder model (data not shown). The role of the Ha-Ras mutation in these responses remains to be established. Efforts to demonstrate an apoptotic response and/or tumor regression in a Ki-Ras-driven model are on-going.

There have been several other reports of *in vivo* activity with various FPT inhibitors (dosed i.p.) in mice bearing human tumor xenografts. L-739,749 inhibited the growth of tumors derived from Rat fibroblasts transformed with activated Ha-, N- or Ki-*ras* in nude mice (16). A distinct class of FPT inhibitor, FTI-276, was reported to be active against xenografts of Calu-1 lung carcinoma cells (containing a Ki-*ras* mutation), but not against NCI-H810 (lacking a *ras* mutation; Ref. 18). Similarly, the peptidomimetic B956 was active against activated Ha-*ras*-expressing EJ bladder carcinoma xenografts, although significantly poorer activity was reported in xenografts expressing activated N-*ras* (HT-1080 fibrosarcoma) or Ki-*ras* (HCT 116 colon carcinoma; Ref. 19).

Reduced response of Ki-*ras*-driven models may be due, in part, to the alternative prenylation of the Ki-Ras protein, which occurs in the presence of FPT inhibitors (13, 14). The fact that tumor cells (and

Table 6 *SCH 66336 increases apoptosis and decreases cell proliferation in in vivo tumors*

$P < 0.01$ when each SCH 66336 treatment was compared with its vehicle control, respectively. Wap-*ras* transgenic mice (strain 69-2) were orally-dosed with SCH 66336 either 40 mpk q.i.d. for the TUNEL assay or 80 mpk b.i.d. for the BrdUrd labeling assay. Positively stained cells within a 6.5 × 9-mm grid in the eyepiece were counted over 10 different magnification fields (× 200) for each tumor section. The mean and the SD were calculated and are shown here.

| | Cells with positive staining (mean ± SD) | | | | | |
|---|---|---|---|---|---|---|
| | | SCH 66336-treated | | | | |
| Assay | Vehicle-treated | 24 h | 48 h | 72 h | 96 h | 120 h |
| TUNEL | 0.9 ± 1.2 | 28.9 ± 11.4 | 17.4 ± 9.1 | 15.1 ± 4.8 | 10.2 ± 4.1 | 12.2 ± 4.8 |
| BrdUrd labeling | 73.8 ± 25.1 | | | | | 12.4 ± 9.1 |

fibroblasts) expressing an activated Ki-*ras* gene respond at all to FPT inhibitors may be due to: (*a*) geranylgeranylated forms Ki-Ras proteins being less efficient in driving transformation; or (*b*) the possibility that there is a distinct farnesylated protein lying downstream of Ras in the transformation pathway, the function of which is blocked by the FPT inhibitor. Clearly, critical mechanistic questions regarding FPT inhibitors remain to be addressed. These were recently reviewed by Der and Cox (43).

FPT inhibitors have previously been reported to induce an apoptotic response *in vitro* in Ha-Ras-transformed Rat1 fibroblasts denied substratum attachment by growth on a nonadhesive substrate (44). Kohl *et al.* (17) using mouse mammary tumor virus-v-Ha-*ras* transgenic mice demonstrated that the FPT inhibitor L-744,832 also induced regressions of mammary tumors *in vivo* when dosed s.c. In these studies, transgenic animals that failed to respond or showed only a partial response to a low dose of L-744,832, did not demonstrate tumor regression when subsequently treated at a higher dose level (17). This is in contrast to our observations with SCH 66336. When animals that were initially treated with a low dose of SCH 66336 (sufficient to yield only a partial response) were crossed over to high-dose treatment, complete tumor regressions occurred.

L-744,832 was also recently reported to induce apoptosis and result

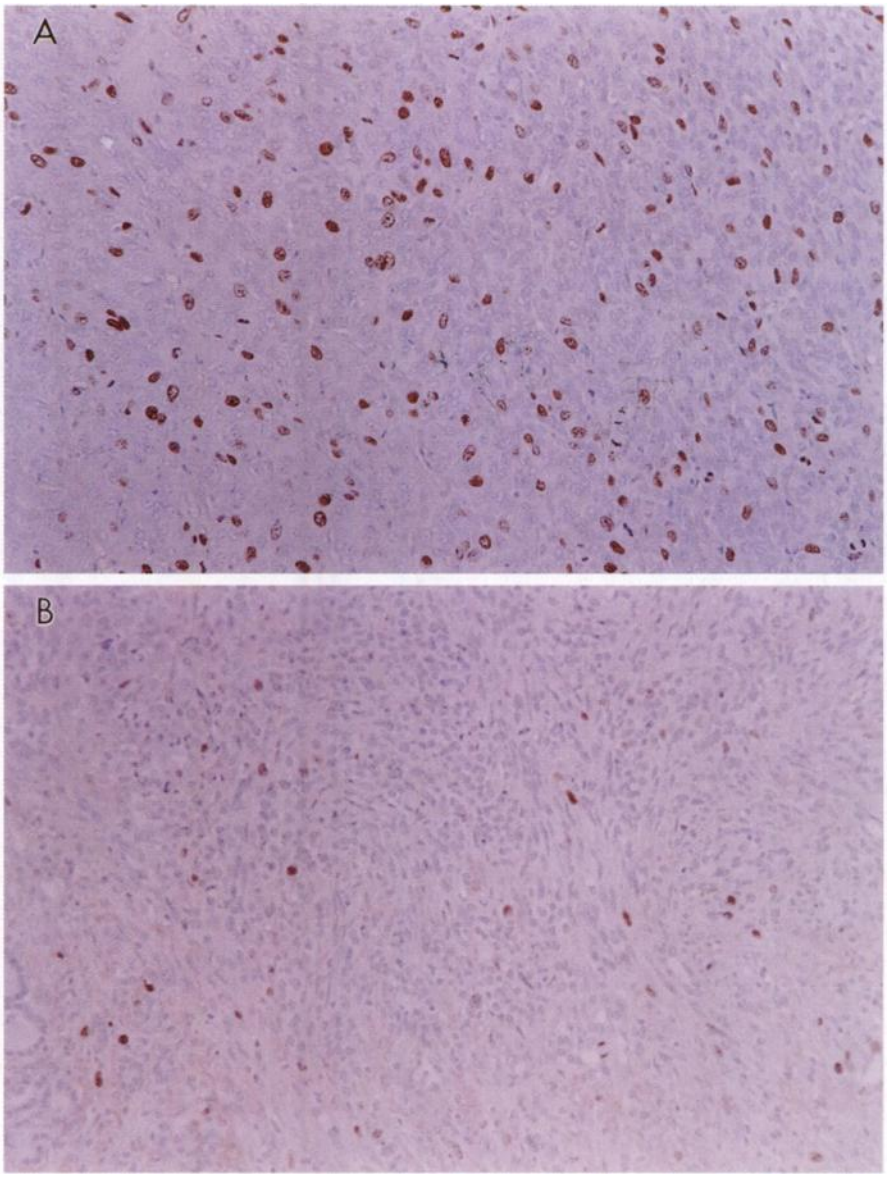


Fig. 7. SCH 66336 reduces cellular proliferation in tumors from wap-*ras* transgenic mice. The photomicrographs show mammary adenocarcinomas from wap-*ras* transgenic mice (strain 69–2) either treated with vehicle control (*A*) or SCH 66336 at 80 mpk (*B*; p.o., b.i.d.) for 5 consecutive days. The mice received i.p. injections of BrdUrd solution before sampling of tumors, and the tumor tissue sections were stained with antibodies against BrdUrd to label proliferating cells, as described in "Materials and Methods."

in tumor regression in the mouse mammary tumor virus-Ha-*ras* mice when this mutation was introduced into a $p53^{-/-}$ background (30). Tumor regression was equally efficient in the $p53^{-/-}$ or the $p53^{wt}$ background. Similarly, tumor regressions were reported with this compound in double *ras/myc* transgenic animals (29) as well as in N-*ras* transgenic mice (45). In the latter model, efficacy was reduced relative to the Ha-*ras* transgenics. Unlike the studies reported here with SCH 66336 that used oral dosing, all studies with L-744,832 used daily s.c. injection of the inhibitor.

The present work is the first report of *in vivo* combination studies using an FPT inhibitor with various cytotoxic agents. Each of the cytotoxic agents used in the present work has a distinct mechanism of action: Cytoxan is an alkylating agent, 5-FU is an antimetabolite that interferes with nucleic acid metabolism, and vincristine is a *Vinca* alkaloid that binds tubulin and interferes with spindle formation, leading to mitotic arrest. The cytotoxic action of 5-FU is primarily exerted during the S phase of the cell cycle, whereas vincristine acts in M phase and Cytoxan is not considered to be cycle-specific. There is concern that signal transduction inhibitors may be cytostatic agents, which would lead to $G_1$ cell cycle arrest, potentially antagonizing the action of classical cytotoxic agents. Our data indicate that is not the case. When SCH 66336 was used in combination with cytotoxics in the HTB177 lung carcinoma xenograft model, enhanced efficacy was observed compared with the single agent treatments. Similarly, when a low dose of SCH 66336 was combined with Cytoxan in the wap-*ras* transgenic model, tumor regression was achieved at doses where the single agents resulted in tumor growth inhibition, but not regression. These data suggest that SCH 66336 does not antagonize the action of cytotoxic chemotherapy and may have a greater than additive effect in some models. Recently, Moasser *et al.* (46) examined *in vitro* effects of combining a FPT inhibitor with various cytotoxic agents, including doxorubicin, cisplatin, 5-FU, and vinblastine. They also reported no antagonism in the *in vitro* setting and, in fact, a synergistic interaction was noted when the FPT inhibitor was combined with microtubule stabilizing agents such as taxol.

SCH 66336 is being evaluated in early phase clinical trials to establish proof-of-principle for farnesyl transferase inhibition in man. The preclinical studies described here suggest that SCH 66336 may have use against a wide array of human cancers and may, at least in some cases, lead to tumor regression. In addition, the results to date in combination with cytotoxic chemotherapeutic agents in animal models indicates that these combinations may enhance the clinical efficacy of FPT inhibitors. Additional preclinical studies should help to guide the clinical development of this class of novel antitumor agents.

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