Cancer Chemother Pharmacol (1999) 43: 50–58

ORIGINAL ARTICLE

Ming Liu · Matthew S. Bryant · Jianping Chen · Suining Lee · Bohdan Yaremko · Zujun Li · Janet Dell · Phil Lipari · Michael Malkowski · Nicholas Prioli · Randall R. Rossman · Walter A. Korfmacher · Amin A. Nomeir C.-C. Lin · Alan K. Mallams · Ronald J. Doll · Joseph J. Catino · Viyyoor M. Girijavallabhan · Paul Kirschmeier W. Robert Bishop

Effects of SCH 59228, an orally bioavailable farnesyl protein transferase inhibitor, on the growth of oncogene-transformed fibroblasts and a human colon carcinoma xenograft in nude mice

Received: 14 January 1998 / Accepted: 13 May 1998

Abstract The products of the Ha-, Ki-, and N-*ras* proto-oncogenes comprise a family of 21 kDa guanine nucleotide-binding proteins which play a crucial role in growth factor signal transduction and in the control of cellular proliferation and differentiation. Activating mutations in the *ras* oncogenes occur in a wide variety of human tumors. Ras proteins undergo a series of post-translational processing events. The first modification is addition of the 15-carbon isoprene, farnesyl, to a Cys residue near the carboxy-terminus of Ras. Prenylation allows the Ras oncoprotein to localize to the plasma membrane where it can initiate downstream signalling events leading to cellular transformation. Inhibitors of the enzyme which catalyzes this step, farnesyl protein transferase (FPT), are a potential class of novel anti-cancer drugs which interfere with Ras function. SCH 59228 is a tricyclic FPT inhibitor which inhibits the farnesylation of purified Ha-Ras with an IC_{50} of 95 n*M* and blocks the processing of Ha-Ras in Cos cells with an IC_{50} of 0.6 μ*M*. SCH 59228 has favorable pharmacokinetic properties upon oral dosing in nude mice. The in vivo efficacy of SCH 59228 was evaluated using a panel of tumor models grown in nude mice. These included several rodent fibroblast lines expressing mutationally-activated (val^{12}) forms of the Ha-Ras oncogene. In some cases, these proteins contain their native C-terminal sequence (CVLS) which directs farnesylation. In one model, the C-terminal sequence was altered to CVLL, making the expressed protein a substrate for a distinct prenyl transferase, geranylgeranyl protein transferase-1. When dosed orally at 10 and 50 mg/kg (four times a day, 7 days a week) SCH 59228 significantly inhibited tumor growth of cells expressing farnesylated Ha-Ras in a dose-dependent manner; over 90% growth inhibition was observed at the 50 mg/kg dose. Tumor growth of cells expressing the geranylgeranylated form of Ha-Ras was less potently inhibited. Growth of tumors derived from a rodent fibroblast line expressing activated Ki-Ras containing its native C-terminal sequence (CVIM), which preferentially directs farnesylation, was also inhibited by SCH 59228. Inhibition in the Ki-Ras model was less than that observed in the Ha-Ras model. In contrast, tumors derived from cells transformed with the *mos* oncogene were not significantly inhibited even at the highest dose level. SCH 59228 also significantly and dose-dependently inhibited the growth of human colon adenocarcinoma DLD-1 xenografts (which express activated Ki-ras). These results indicate that SCH 59228 possesses in vivo antitumor activity upon oral dosing in tumor models expressing activated *ras* oncogenes. This is the first report of oral antitumor activity with an FPT inhibitor. These results are discussed in light of recent observations on alternative prenylation of some Ras isoforms.

Key words SCH 59228 · Orally bioavailable · Tricyclic farnesyl-transferase-inhibitor · Ras · Xenograft

M. Liu (✉) · J. Chen · S. Lee · B. Yaremko · Z. Li · J. Dell
P. Lipari · M. Malkowski · N. Prioli · P. Kirschmeier
J.J. Catino · W.R. Bishop
Department of Biological Research-Oncology,
Schering-Plough Research Institute,
2015 Galloping Hill Road, Kenilworth,
NJ 07033, USA
Tel.: +1-908-298-7136; Fax: +1-908-298-7115

R.R. Rossman · A.K. Mallams · R.J. Doll · V. Girijavallabhan
Department of Chemical Research,
Schering-Plough Research Institute, Kenilworth,
NJ 07033, USA

M.S. Bryant · W.A. Korfmacher · A.A. Nomeir · C.-C. Lin
Department of Drug Metabolism and Pharmacokinetics,
Schering-Plough Research Institute, Kenilworth,
NJ 07033, USA

Introduction

Poised at the inner surface of the plasma membrane, Ras proteins normally respond to a variety of extracellular signals by undergoing exchange of bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP) [1]. In normal cells, Ras switches between these inactive GDP-bound and active GTP-bound states. GTP-Ras triggers several intracellular signalling pathways [2]. The best characterized pathway is a phosphorylation cascade involving Raf, mitogen-activated protein kinase kinase and mitogen-activated protein kinase [3]. Ras signalling is terminated by hydrolysis of GTP to GDP in a reaction that is stimulated by guanosine triphosphatase-activating proteins. Oncogenic Ras proteins have greatly reduced capacity to hydrolyze GTP as a consequence of specific mutational events in the Ras sequence. This leads to constitutive activation of downstream signalling pathways resulting in unregulated cellular proliferation [1, 4]. Oncogenic forms of the *ras* gene have been detected in around 30% of all types of human cancers, including up to 50% of colon cancers and more than 90% of pancreatic carcinomas [1].

Four isoforms of the Ras protein exist: H-ras, N-ras, K-ras 4A and K-ras 4B [4]. They are products of three genes, with K-ras4A and K-ras4B being splice variants of the same gene. Expression of the four Ras isoforms is tissue-specific in the mouse [5]. Moreover, oncogenic mutations of the different isoforms predominate in different tumors [6]. For example, H-ras mutations are found in carcinomas of the bladder, kidney and thyroid; N-ras mutations are found in myeloid and lymphoid disorders, liver carcinoma and melanoma; whereas K-ras mutations predominate in colon and pancreatic carcinoma. The functional differences of the four isoforms are unknown.

Several lines of evidence suggest that antitumor activity may be achieved by interfering with the function of oncogenic Ras proteins [7, 8]. Among these proof-of-principle studies, the most compelling study to validate Ras as a therapeutic target is that of Shirasawa et al. [9]. Using homologous recombination, they knocked out the activated *ras* allele in human colorectal carcinoma DLD-1 cells. The resulting cell line was no longer able to grow in soft agar and was not tumorigenic in nude mice. Despite the fact that these cells harbor multiple genetic changes (including amplification of the *myc* gene and mutational loss of the p53 tumor suppressor gene) deletion of the activated *ras* allele alone suppressed the malignant phenotype. A second similar study, found a significant decrease in the tumorigenic properties of these cells, although some residual tumor growth was observed [10].

Signal transduction by Ras is dependent on its plasma membrane localization. This localization is supported by a series of posttranslational modifications. The first step in this pathway is farnesylation of a Cys residue near the C-terminus of Ras proteins catalyzed by farnesyl protein transferase (FPT). Prenylation is thought to be the critical modification for membrane location and function of Ras [11–13]. Therefore, FPT inhibition is a potential mechanism for interfering with Ras-driven tumor growth.

Recent studies by us and others suggest that prenylation of Ras proteins is complex. In vitro, both Ki- and N-Ras proteins can serve as substrates for a related protein prenyl transferase, geranylgeranyl protein transferase-1 (GGPT-1) [14, 15]. 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 [16, 17]. In contrast, the Ha-Ras protein is not a substrate for GGPT-1 in vitro or in cells. Since geranylgeranylated forms of Ras can support cellular transformation when overexpressed (for example, see reference 12), this reaction represents a potential resistance mechanism for Ras-transformed cells to the effects of FPT inhibitors.

We have been working on the development of a series of non-peptidic, small molecule FPT inhibitors [18]. These compounds share a common tricyclic nucleus and compete with peptide/protein substrates for binding to FPT. Here, we report on in vivo studies with SCH 59228, an improved compound in this structural class. The results demonstrate that, despite the complexity of Ras prenylation, these compounds possess antitumor activity following oral administration in nude mice. This is the first detailed report of compounds possessing oral antitumor activity with this mechanism of action.

Materials and methods

Compound

The structure of SCH 59228 [1-(3-bromo-8-chloro-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2b]pyridin-11-yl)-4-(4-pyridinylacetyl)-piperazine N4-oxide] is shown in Fig. 1. Its synthesis is reported elsewhere [19].

In vitro enzyme assays

FPT activity was determined by measuring transfer of [^{3}H]farnesyl from [^{3}H]farnesyl pyrophosphate to trichloroacetic acid-precipitable

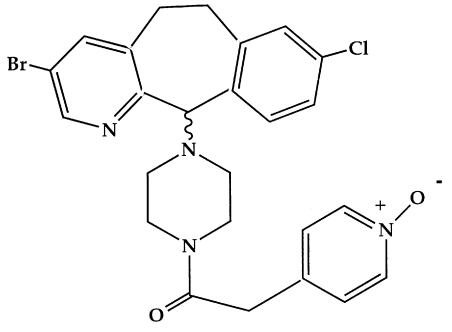


Fig. 1 Structure of SCH 59228. SCH 59228 is a representative compound in the tricyclic series of FPT inhibitors. This compound is orally bioavailable and possesses in vivo antitumor efficacy following oral administration

Ha-Ras-CVLS as previously described [18]. GGPT-1 activity was similarly determined using [^{3}H]geranylgeranyl diphosphate and Ha-Ras-CVLL as substrates [18].

Cellular assays for inhibition of Ha-Ras processing and transforming function

Inhibition of intracellular processing of H-Ras by SCH 59228 was measured in transfected Cos cells as described previously [18]. To determine the inhibition of anchorage-independent growth of transformed cells, soft agar growth assays were employed (Kirschmeier et al., in preparation). In brief, logarithmically growing oncogene-transformed fibroblasts or human tumor cells were trypsinized and plated in top LMP agarose (0.35%) at 10^4 cells/well of a six-well cluster dish in DMEM containing 10% FBS and various amounts of SCH 59228. The bottom layer (0.6% LMP agarose) contained the same concentration of SCH 59228. After 14 days, colonies were stained with MTT and quantitated.

Cell Lines for in vivo studies

The PT24 cell line is derived from BALB c/3T3 cells transfected with activated Ha-Ras-CVLS. NIH3T3 cells transfected with activated Ha-Ras containing its native C-terminal sequence (CVLS) or an altered C-terminal sequence (CVLL) were constructed by removing H-ras coding sequences from the pSV Sport expression vectors by restriction digestion [18]. These fragments were incubated with the Klenow fragment of DNA polymerase I and subcloned into pOPRSVI (Stratagene) by standard methods [20]. The resulting pOPRSVI-H-ras CVLS and pOPRSVI-H-ras CVLL plasmids were transfected into NIH-3T3 cells using Lipofectamine (GIBCO-BRL) under the conditions suggested by the manufacturer. G-418-resistent clones were isolated and screened for morphological transformation and their ability to grow in soft agar. NIH3T3 cells transfected with activated Ki-Ras containing its native C-terminal sequence of CVIM were similarly constructed. MSV-3T3 cells are NIH3T3 cells transfected with the *mos* oncogene. The human colon carcinoma DLD-1 cell line was obtained from the American Type Culture Collection (Rockville, Md.).

In vivo efficacy studies

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 National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experimental protocols were reviewed by and the experimental progress was supervised by the Schering Plough Animal Care and Use Committee. After a week of acclimation, 5–7-week-old female nude mice (Crl:Nu/Nu-nu Br, Charles River Laboratories, Wilmington, Mass.), were subcutaneously inoculated with various cell lines on day 0. The number of cells inoculated were: 3.0×10^5 for NIH3T3 Ki-Ras-CVIM; 2×10^6 for NIH3T3 Ha-Ras-CVLL and CVLS; 3×10^6 for MSV-3T3; 5×10^6 for DLD-1 and PT-24. All the models have 100% take rate and grew consistently in every animal. Animals were randomly assigned to control and treatment groups (ten animals per group) before the first treatment.

Drug treatment at either 10 mg/kg body weight or 50 mg/kg was initiated on day 1. SCH 59228 was dissolved in 20% (w/v) hydroxypropyl-β-cyclodextrin (HPβCD). Vehicle controls received 20% HPβCD. Vehicle or drug solution (0.1 ml) was administered by oral gavage every 6 h (four times daily) 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 gavage treatment. In some experiments, vehicle-treated animals experienced 5–10% weight loss, while no additional weight loss was observed in SCH 59228 treated animals. Once palpable, tumors were measured in three dimensions twice weekly and volumes calculated with the formula $V = 1/6 \times \pi \times L \times W \times T$, where L, W, and T represent length, width, and thickness respectively [18]. The T/C value in percent was calculated where T and C were the mean tumor volume of the treated and control groups, respectively, at the end of each experiment. 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.

Pharmacokinetic studies

Nude mice were also used to study the pharmacokinetic properties of SCH 59228. 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 intravenous dose of 25 mg/kg SCH 59228 in 20% HPβCD. Oral dosing was administered by gavage and intravenous doses were administered by a single bolus injection into the tail vein. Two 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 59228 serum levels was achieved using acetonitrile precipitation, followed by high-performance liquid chromatography-atmospheric pressure chemical ionization (APCI) tandem mass spectrometry. Our pharmacokinetic calculations utilized a simple noncompartmental model. Half-lives were estimated graphically using a plot of log (plasma concentration) versus time. AUC calculations utilized the linear trapezoidal rule. Mean serum concentrations were utilized to calculate a single AUC or half-life value. A detailed description of the analytical methodology has been presented for an earlier analog in this series [21].

In addition, both serum and tumor samples were collected from two mice at various times (1, 3, 6 h) following the final dosing in one of the efficacy studies. SCH 59228 was quantified in serum as described above. Quantitation of SCH 59228 in tumor samples required additional processing steps including pulverizing the frozen tissue, homogenization and then protein precipitation [22].

The pharmacokinetics of SCH 59228 were also studied in male cynomologus monkeys. SCH 59228 in 20% HPβCD was given by oral or intravenous administration at doses of 5 or 10 mg/kg. Different monkeys (two per dose and route) were utilized for each study and given the compound in restraining chairs according to institutional guidelines. Intravenous administration was by a single bolus injection into a cephalic vein and the sampling was from a saphenous vein. Blood samples were collected up to 48 h after dosing. Plasma was isolated and SCH 59228 was quantified using previously described methods [21].

Results

SCH 59228 is a potent and selective FPT inhibitor

We have previously reported on a series of novel farnesyl transferase inhibitors which possess a substituted tricyclic ring system [18, 23]. SCH 44342, an early compound in this series, possessed modest FPT inhibitory potency (IC_{50} 250 n*M*; Table 1).

This compound was found to be orally bioavailable, but rapidly metabolized in vivo (Table 1). Identification of the principal metabolites of SCH 44342 led to the synthesis of a variety of new compounds possessing 3-bromo- and 4-pyridylacetyl-N-oxide modifications [19]. Among them, SCH 59228 was found to possess improved efficacy, bioavailability and pharmacokinetic stability (Table 1). SCH 59228 was approximately five-fold more potent than SCH 44342, inhibiting human

Table 1 Biochemical and pharmacologic evaluations of SCH 59228 and SCH 44342. IC50 values are shown for SCH 59228 and SCH 44342 for inhibition of the enzymatic activity of farnesyl protein transferase (FPT) and geranylgeranyl protein transferase-1 (GGPT-1). IC50 values are also shown for inhibition of Ha-Ras processing following transient expression in Cos cells and for the anchorage-independent growth of Ha-Ras-CVLS transformed fibroblasts and DLD-1 colon carcinoma cells. Farnesyl transferase inhibitors were dosed either orally (PO) or intravenously (IV) into nude mice using hydroxypropyl-β-cyclodextran as vehicle. All assays were carried out as described in Materials and methods

Parameter	SCH 59228	SCH 44342
FPT enzyme IC_{50} (μM)	0.059	0.25
GGPT-1 enzyme IC_{50} (μM)	$\gg 38$	$\gg 46$
Ha-Ras-CVLS processing in Cos cells IC_{50} (μM)	0.6	3.0
Ha-Ras-CVLS soft agar growth IC_{50} (μM)	1.2	10.0
DLD-1 soft agar growth IC_{50} (μM)	2.0	n.d.
Serum AUC, oral dosing (μg h/ml)	19.6	0.37
Serum AUC, intravenous dosing (μg h/ml)	23.4	1.75
Serum half-life, intravenous dosing	1.2 h	< 10 min
Oral bioavailability (%)	84	20

FPT with an IC_{50} of 59 nM, while retaining selectivity against GGPT-1. To examine the effect of SCH 59228 on Ras processing in whole cells, Cos-7 monkey kidney cells transiently expressing Ha-Ras-[Val^{12}]CVLS were treated with various concentrations of drug. Processed and unprocessed Ras proteins were resolved on SDS-polyacrylamide gels and detected by immunoblotting [18]. SCH 59228 resulted in a dose-dependent inhibition of Ha-Ras processing. At 2 μM, greater than 95% of the overexpressed Ha-Ras was present as unprenylated precusor. The IC_{50} for SCH 59228 in this assay was 0.6 μM, compared to 3.0 μM for SCH 44342. SCH 59228 also suppressed the soft agar growth and transformed morphology of cells overexpressing activated Ha-Ras-CVLS.

SCH 59228 is bioavailable upon oral dosing

When tested in nude mice, SCH 44342 was found to be rapidly metabolized (Table 1). Following intravenous administration at 25 mg/kg, SCH 44342 demonstrated a half-life of less than 10 min and an AUC of 1.75 μg · h/ml. When orally administered, SCH 44342 was 20% bioavailable and did not achieve serum levels necessary for 50% inhibition of Ha-Ras processing in cell culture [23].

Mass spectral identification of the major metabolites of SCH 44342 and extensive structure-activity efforts led to the identification of analogs in which the susceptible metabolic sites were blocked. One compound which resulted from these efforts, SCH 59228, displayed a significantly better pharmacokinetic profile than SCH 44342 in the nude mouse. After intravenous administration at 25 mg/kg, SCH 59228 had a serum half-life of 1.2 h and AUC of 23.4 μg · h/ml. Bioavailability of SCH 59228 was 84% and serum levels after oral administration were nearly 20-fold higher than those achieved with SCH 44342. Serum concentrations of SCH 59228 were maintained above the IC_{50} for inhibition of Ha-Ras processing for more than 6 h after a single oral dose of 25 mg/kg (Fig. 2A).

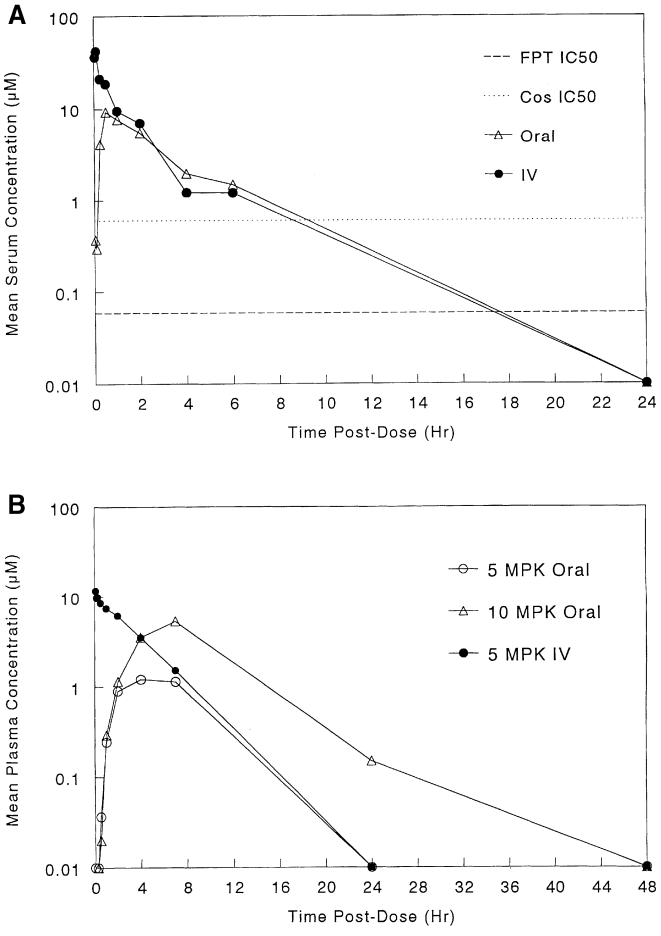


Fig. 2A,B Pharmacokinetic profiles of SCH 59228 in nude mice (**A**) and cynomologus monkeys (**B**) after single-dose administration. **A** Serum concentration of SCH 59228 after oral and intravenous administration to nude mice at a dose of 25 mg/kg. **B** Plasma concentration of SCH 59228 after oral administration to cynomologus monkeys at doses of 5 or 10 mg/kg and intravenous administration at a dose of 5 mg/kg

Blood and tumor samples were also collected at 1, 3 and 6 h following the final dosing of SCH 59228 in a nude mouse efficacy study (employing xenografts derived from the DLD-1 colon carcinoma line). Steady-state levels of SCH 59228 were quantified in both compartments. Concentrations of SCH 59228 achieved in the tumor tissue were similar to those observed in serum. After 21 days of dosing with SCH 59228 at 10 mg/kg and 50 mg/kg (four times daily), serum AUCs were 4.63

Table 2 Pharmacokinetic profile of SCH 59228 in the cynomolgus monkey. Farnesyl transferase inhibitors were dosed either orally (PO) or intravenously (IV) into cynomolgus monkeys using hydroxypropyl-β-cyclodextran as vehicle. Serum samples were analyzed for the presence of the parent compound as described in Materials and methods.

SCH 59228 dose	Plasma AUC (μg · h/ml)
5 mg/kg, oral	9.4
10 mg/kg, oral	35.6
5 mg/kg, intravenous	24.4

and 70.2 μg · h/ml, respectively, and tumor AUCs were 5.24 and 70.0 μg · h/ml, respectively. This indicates that SCH 59228 readily reaches the target tissue after oral administration.

The pharmacokinetics of SCH 59228 were also evaluated in the cynomologus monkey. SCH 59228 displayed an excellent pharmacokinetic profile in this species (Fig. 2B and Table 2). The half-life after intravenous administration approached 2.6 h and the oral bioavailability was 39%.

SCH 59228 preferentially inhibits tumor formation by rodent fibroblasts transfected with farnesylated vs geranylgeranylated H-Ras

Three transformed cell lines were initially used to examine the antitumor selectivity of SCH 59228: NIH 3T3 cells transformed with activated Ha-Ras containing its native C-terminal sequence of CVLS (farnesylated); NIH 3T3 cells transformed with activated Ha-Ras in which its C-terminal sequence was modified to CVLL (geranylgeranylated); and PT-24 cells, BALB c/3T3 fibroblasts transfected with activated Ha-Ras-CVLS (farnesylated). Cells were inoculated subcutaneously into nude mice. Interestingly, NIH 3T3 cells transformed with Ha-Ras-CVLS were the most aggressive model, forming tumors of greater than 1000 mm^3 in volume by the end of the study. In contrast, tumors formed by NIH 3T3 cells expressing Ha-Ras-CVLL only grew to about 250 mm^3 in volume. Whether this reflects clonal variation or a diminished tumorigenic potential of cells transformed by geranylgeranylated Ha-Ras is not known.

Oral gavage treatment with SCH 59228 (every 6 h at 10 or 50 mg/kg) was initiated the day after tumor inoculation and continued for 20 days. SCH 59228 dose-dependently inhibited the growth of tumors derived from cells expressing farnesylated forms of Ha-Ras. In the NIH 3T3 model, 58 and 95% tumor growth inhibition was observed at the end of the study at the 10 and 50 mg/kg dose levels, respectively ($P \ll 0.0005$ for both doses). In the PT-24 model, 48% and 91% inhibition was observed at 10 and 50 mg/kg, respectively ($P \ll 0.0005$ for both doses; Fig. 3). In contrast, SCH 59228 had a reduced effect on the growth of tumors derived from cells expressing geranylgeranylated forms of Ha-Ras (20% and 45% tumor growth inhibition at 10 and 50 mg/kg; $0.1 < P < 0.25$ and $P < 0.005$, respectively). The greater efficacy observed in models expressing farnesylated Ha-Ras reflects the in vitro specificity of SCH 59228 for inhibition of FPT over GGPT-1. However, antitumor activity was also observed against cells transformed by the geranylgeranylated form of this protein (see below).

SCH 59228 preferentially inhibits tumor growth of rodent fibroblasts transformed with Ki-Ras vs the *mos* oncogene

We also evaluated the effect of SCH 59228 on tumors derived from fibroblastic cell lines transformed by activated Ki-Ras-CVIM or by the *mos* oncogene (MSV-3T3). Both of these cell lines were highly tumorigenic in nude mice, resulting in tumors of >1000 mm^3 for the *mos*-transformed cells and >2500 mm^3 for the Ki-Ras-transformed cells.

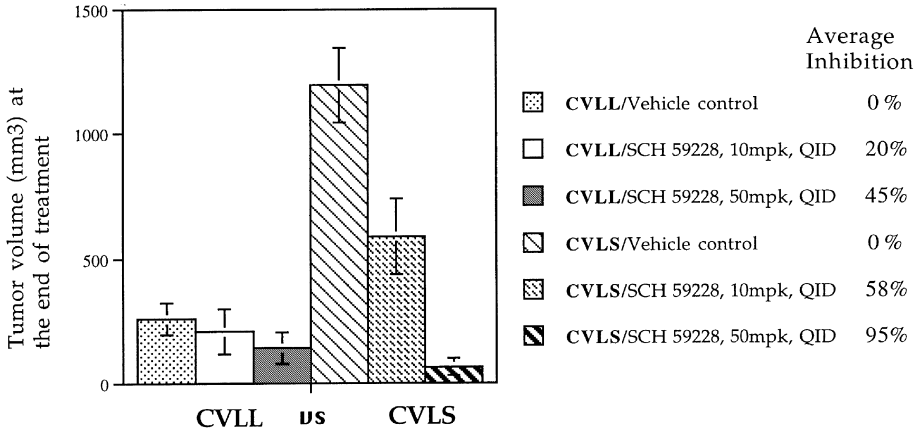
Beginning on the day following tumor cell inoculation, mice were treated with vehicle or increasing doses (5, 20 or 50 mg/kg) of SCH 59228 every 6 h for 20 days. SCH 59228 treatment did not affect the growth of tumors derived from *mos* oncogene-transformed cells (Fig. 4) except for minimal inhibition at the highest dosage group (19% tumor growth inhibition at 50 mg/kg, $0.05 < P < 0.1$). In contrast, SCH 59228 dose-dependently inhibited the growth of tumors derived from Ki-Ras-CVIM-transformed cells (21%, $0.025 < P < 0.05$; 47%, $P \ll 0.0005$; and 63%, $P \ll 0.0005$; at 5, 20 and 50 mg/kg).

SCH 59228 inhibits the growth of a human tumor xenograft

To evaluate the efficacy of SCH 59228 against a human tumor model, nude mice bearing xenografts of DLD-1 cells were used. This cell line is a clinically isolated, well-characterized human colonic adenocarcinoma [24]. The day after inoculation of 5×10^6 DLD-1 cells per mouse, oral treatment with SCH 59228 (four times a day) was initiated and continued for 22 days. In vehicle-treated animals, tumors reached a volume of 300 mm^3. Growth curves for various treatment groups are shown in Fig. 5. Average tumor inhibition of 15% ($0.05 < P < 0.1$) and 43% ($P < 0.005$) was observed at 10 and 50 mg/kg, respectively.

Discussion

The studies reported here demonstrate that the tricyclic FPT inhibitor, SCH 59228, has in vivo antitumor activity when administered orally to nude mice. The tricyclic class of FPT inhibitors are structurally distinct from other classes of FPT inhibitors reported to date,



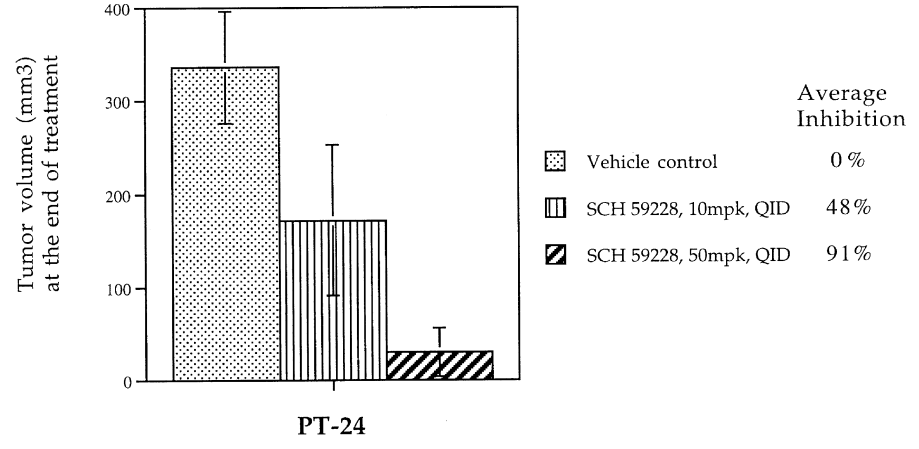


Fig. 3A,B SCH 59228 selectively inhibits the growth of tumors expressing farnesylated (Ha-Ras-CVLS) vs geranylgeranylated (Ha-Ras-CVLL) forms of Ras. **A** Ha-Ras-CVLS or Ha-Ras-CVLL tumors were initiated in nude mice and animals were dosed orally with SCH 59228 as described in Materials and methods. Tumor volumes measured at the end of the treatment period (day 20) are shown. Average inhibitions, calculated as described in Materials and methods, section, are shown at the right side of the graph. **B** PT-24 tumors were initiated in nude mice and animals were dosed orally with SCH 59228 as described in Materials and methods. Tumor volumes measured at the end of the treatment period (day 20) are shown. Average inhibitions, calculated as described in Materials and methods section, are presented at the right side of the graph

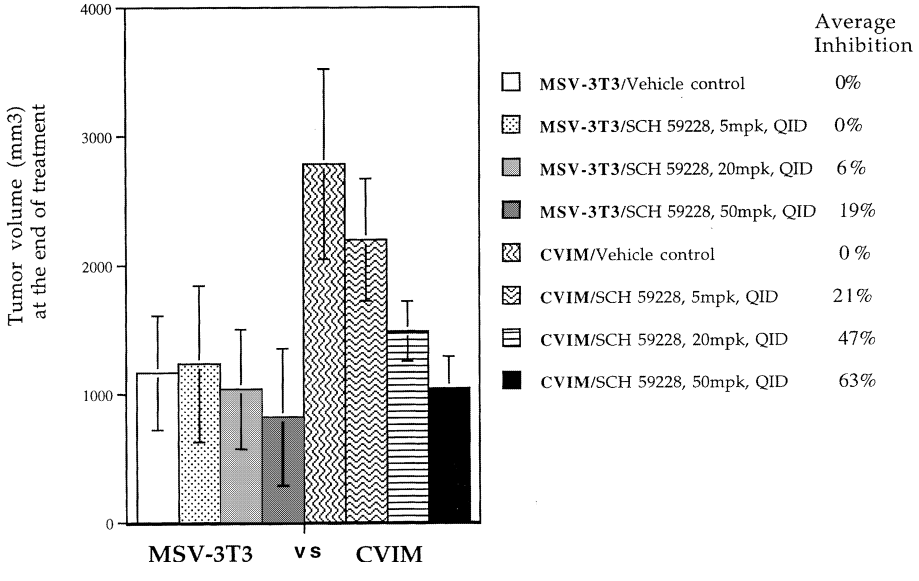


Fig. 4 SCH 59228 selectively inhibits growth of tumor cells transformed with Ki-Ras-CVIM vs the *mos* oncogene. Ki-Ras-CVIM or *mos*-transformed tumors were initiated in nude mice and animals were dosed orally with SCH 59228 as described in Materials and methods. The in vivo efficacy of SCH 59228 against tumor transformed by activating K-Ras was determined in comparison with a *mos*-transformed tumor. Tumor volumes measured at the end of the treatment period (day 20) are shown. Average inhibitions, calculated as described in Materials and methods, are presented at the right side of the graph

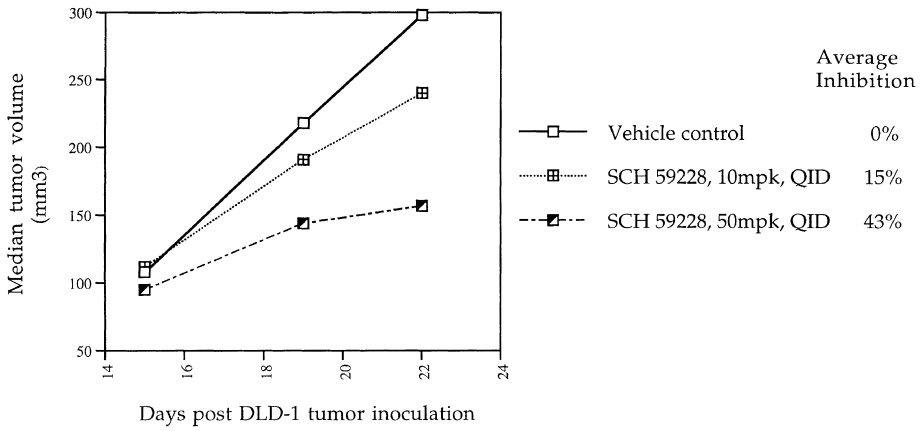


Fig. 5 SCH 59228 inhibits the growth of human colon DLD-1 tumor xenografts. Xenograft studies were performed as described in Materials and methods. The growth curves of the DLD-1 tumors following various treatments are shown. The data are plotted according to the median tumor volume and average inhibitions are presented at the right side of the graph

many of which were derived from peptidomimetic approaches (for example, see references 25–33). These differences, including the absence of a sulfhydryl function, are likely to contribute to the potential for more favorable oral pharmacokinetic properties of the tricyclic class of inhibitors.

Earlier compounds in this series, such as SCH 44342, display less than optimum pharmacokinetic properties in the mouse, including a very rapid oxidative metabolism. Blocking the susceptible metabolic sites on SCH 44342 greatly improves the pharmacokinetic properties of compounds in this series. SCH 59228, an 11-piperazinyl analog, was one of the lead compounds that emerged from these efforts. In addition to improved metabolic stability, its intrinsic potency (IC_{50} 59 n*M*) was also greater than that of earlier compounds. As a result of these improvements, SCH 59228 persisted in mouse serum at a concentration greater than its IC_{50} for Ha-Ras processing in Cos cells for over 6 h following a single oral dose of 25 mg/kg. In addition, significant steady-state drug levels were also achieved in the tumor xenografts following 3 weeks of dosing.

The data in the Ha-Ras models suggest that SCH 59228 exerts its antitumor efficacy in these models by blocking farnesylation of the Ha-Ras protein. This hypothesis is supported by the greater antitumor efficacy observed against cells expressing the farnesylated vs the geranylgeranylated form of this protein. However, the inhibition of tumors expressing geranylgeranyled Ha-Ras was statistically significant at the 50 mg/kg dose level. The finding that efficacy was also seen in this model suggests that inhibition of farnesylation of proteins in addition to Ras may contribute to the antitumor effect (see below).

SCH 59228 also displayed antitumor activity against cells transformed with activated Ki-Ras-CVIM. The inhibition observed in the Ki-Ras model is less than that seen in the Ha-Ras-CVLS models and more similar to that observed using the geranylgeranylated forms of Ha-Ras. In contrast, little antitumor activity was observed against cells transformed by the *mos* oncogene, which is thought to transform cells in a Ras-independent manner. The results with the *mos*-transformed cells suggest that SCH 59228 does, in fact, target the Ras signal transduction pathway to exert its antitumor activity.

Recent studies have clearly demonstrated that the Ki- and N- isoforms of the Ras protein are geranylgeranylated in cells treated with FPT inhibitors [16, 17]. This is presumably also the case in the in vivo, animal setting, although this has not been directly demonstrated. There are two possible (although not mutually exclusive) explanations for the sensitivity of the transformed phenotype of Ki-Ras-transformed cells to the effects of FPT inhibitors despite alternative prenylation. First, geranylgeranylated forms of activated Ras proteins may not transform cells as efficiently as the farnesylated species. Clearly, the geranylgeranylated proteins are transforming when overexpressed in fibroblasts (for example, see references 12 and 34). However, in our hands, cells transformed with the geranylgeranylated form of Ha-Ras are less tumorigenic in nude mice and have a poorer plating efficiency on plastic (unpublished data). Similarly, Cox et al. [35] have shown that expression of a geranylgeranylated form of Ha-Ras is growth suppressive in normal fibroblasts.

The second possible explanation for the antitumor activity in the Ki-Ras model is that the geranylgeranylated form of this protein can still drive transformation but that there is a distinct farnesylated protein lying downstream of Ras in the transformation pathway. In this scenario, the observed antitumor activity would be a result of blocking the farnesylation of this alternative protein. The small GTPase RhoB has been suggested to be a candidate for such a protein (for example, see reference 36), although the prenylation of RhoB is complex (Bishop et al. unpublished data; [37]). A role for another farnesylated protein in the observed antitumor activity may also explain the efficacy of SCH 59228 in the Ha-Ras-CVLL model. Clearly, critical mechanistic questions regarding FPT inhibitors remain to be addressed. A recent review by Der and Cox [38] further addresses these critical questions.

Our results also show that SCH 59228 dose-dependently inhibits the in vivo growth of human colon carcinoma DLD-1 cells, a tumor cell line which expresses an activated form of Ki-Ras [9]. Recently, using an

analog of SCH 59228 with enhanced potency, we screened a panel of in vitro and in vivo models including human tumor xenografts of lung, colon, pancreas, and prostate origin, and observed a broad spectrum of antitumor activity (unpublished data). Similar to the results of others [39, 40], there is no apparent correlation between the sensitivity of the transformed phenotype of a cell line to FPT inhibitors and its Ras mutational status. Many of the sensitive cell lines which lack activating ras mutations are, nevertheless, likely to be dependent on enhanced Ras signalling for transformation (owing to mutational activation of oncogenes or reliance on autocrine growth factors, either of which may activate the Ras signal transduction pathways). It is possible that alternative geranylgeranylation of Ki- and N-Ras proteins may not support full activation of the Ras signalling pathways, particularly when the misprenylated Ras protein is not overexpressed. As described above, it is also possible that a farnesylated protein downstream of Ras serves as a target for FPT inhibitors in these cells.

There have been several other reports of in vivo activity with various classes of FPT inhibitors. Kohl et al. [41] have reported that L-739,749, when dosed via an intraperitoneal route of administration, inhibits the growth of tumors derived from Rat fibroblasts transformed with activated Ha-, N- or Ki-Ras in nude mice. Unlike our results, they report essentially equivalent reduction in tumor volume using cells transformed with any of the Ras isoforms. Similar to the data reported here, they observed that the growth of tumors derived from *mos*-transformed cells is unaffected. Another compound in this series, when dosed subcutaneously, has been reported to induce regressions of mammary tumors in MMTV-v-Ha-ras transgenic mice [42].

A distinct class of peptidomimetic FPT inhibitor, represented by FTI-276, has also been reported to inhibit growth of NIH 3T3 cells transformed by Ha-Ras-CVLS in nude mice [43]. Growth of Raf-transformed tumors was not inhibited. This compound has also been 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). Similarly, the peptidomimetic B956 suppresses the growth of Ha-Ras-CVLS transformed NIH 3T3 cell-derived tumors in nude mice [40]. This compound is also active against activated Ha-Ras-expressing EJ bladder carcinoma xenografts, although significantly poorer activity is seen in xenografts expressing activated N-ras (HT-1080 fibrosarcoma) or Ki-Ras (HCT 116 colon carcinoma). These studies all employed intraperitoneal dosing [40, 43].

This is the first detailed report of oral antitumor activity using an inhibitor of farnesyl protein transferase. Compounds related to SCH 59228 have also demonstrated oral activity in a wide battery of human tumor xenograft models and in a Ha-Ras transgenic model where significant tumor regressions have been observed (unpublished data). Compounds in this series are being progressed into early phase clinical trials to establish proof-of-principle in humans. The recent demonstration of alternative processing of N- and Ki-Ras proteins in the presence of these inhibitors indicates that their mechanism of action is likely to be more complex than originally anticipated. Further efforts to delineate the proteins whose prenylation status is either blocked or altered by FPT inhibitors and the downstream consequences of these changes are a major focus of current research. Such studies should help shed light on the consequences of long-term inhibition of FPT and the mechanism(s) of sensitivity and resistance of human cancers to these inhibitors.

References

1. Barbacid M (1987) Ras genes. Annu Rev Biochem 56: 779
2. Joneson T, White MA, Wigler MH, Bar-Sagi D (1996) Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. Science 271: 810
3. Moodie SA, Willumsen BM, Weber MJ, Wolfman A (1993) Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. Science 260: 1658
4. Lowy DR, Willumsen BM (1993) Function and regulation of Ras. Annu Rev Biochem 62: 851
5. Leon J, Guerrero I, Pellicer A (1987) Differential expression of the ras gene family in mice. Mol Cell Biol 4: 1493
6. Bos JL (1989) ras oncogenes in human cancer: a review. Cancer Res 49: 4682
7. Gibbs JB, Oliff A, Kohl NE (1994) Farnesyltransferase inhibitors: ras research yields a potential cancer therapeutic. Cell 77: 175
8. Gibbs JB, Kohl NE, Koblan KS, Omer CA, et al (1996) Farnesyltransferase inhibitors and anti-Ras therapy. Breast Cancer Res Treat 38: 75
9. Shirasawa S, Furuse M, Yokoyama N, Sasazuki T (1993) Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science 260: 85
10. Plattner R, Anderson MJ, Sato KY, Fasching CL, et al (1996) Loss of oncogenic ras expression does not correlate with loss of tumorigenecity in human cells. Proc Natl Acad Sci USA 93: 6665
11. Jackson JH, Cochrane CG, Bourne JR, Solski PA, et al (1990) Farnesol modification of Kirsten-ras exon 4B protein is essential for transformation. Proc Natl Acad Sci USA 87: 3042
12. Kato K, Cox AD, Hisaka MM, Graham SM, et al (1992) Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc Natl Acad Sci USA 89: 6403
13. Schaefer WR, Kim R, Sterne R, Thorner J, et al (1989) Genetic and pharmacological suppression of oncogenic mutations in RAS genes of yeast and humans. Science 245: 379
14. James GL, Goldstein JL, Brown MS (1995) Polylysine and CVIM sequences of K-RasB dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic in vitro. J Biol Chem 270: 6221
15. Zhang F, Bond R, Wang L, Windsor W, et al (1997) Characterization of H, K and N-Ras as in vitro substrates for isoprenyl protein transferases. J Biol Chem 272: 10232
16. Whyte DB, Kirschmeir P, Hockenberry TN, Nunez-Oliva I, et al (1997) K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem 272: 14459
17. Rowell CA, Kowalczyk JJ, Lewis MD, Garcia AM (1997) Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo. J Biol Chem 272: 14093
18. Bishop WR, Bond R, Petrin J, Wang L, et al (1995) Novel tricyclic inhibitors of farnesyl transferase. J Biol Chem 270: 30611

19. Mallams AK, Rossman RR, Doll RJ, Girijavallabhan V, et al (1998) Inhibitors of farnesyl protein transferase. 4-amido, 4-carbamoyl and 4-carboxamido derivatives of 1-(8-chloro-6, 11-dihydro-5H-benzo[5,6]cyclohepta[1,2-b]pyridin-11-yl)piperazine and 1-(3-bromo-8-chloro-6,11-dihydro-5H-benzo[5,6] cyclohepta[1,2-b]pyridin-11-yl)piperazine. J Med Chem (in press)
20. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Press, Cold Spring Harbor
21. Bryant MS, Korfmacher WA, Wang S, Nardo C, et al (1997) Pharmacokinetic screening for selection of new drug discovery candidates is greatly enhanced through the use of liquid chromatography-atmospheric pressure ionization tandem mass spectrometry. J Chromatogr 777: 61
22. Bryant MS, Liu M, Korfmacher WA, Nardo C, et al (1997) LC-APCI/MS/MS analysis of serum and tumor samples from mice treated with a potent antitumor compound (SCH 59228). Proceedings the 45th ASMS Conference on Mass Spectrometry and Allied Topics, Palm Springs, CA
23. Mallams AK, Njoroge FG, Doll RJ, Snow ME, et al (1997) Antitumor 8-chlorobenzocycloheptapyridines: a new class of selective, nonpeptidic, nonsulfhydryl inhibitors of Ras farnesylation. Bioorg Med Chem 5: 93
24. Dexter DL, Barbosa JA, Calabresi PN (1979) N-dimethylformamide-induced alteration of cell culture characteristics and loss of tumorigenicity in cultured human colon carcinoma cells. Cancer Res 39: 1020
25. Kohl NE, Mosser SD, DeSolms J, Giuliani EA, et al (1993) Selective inhibition of ras-dependent transformation by a farnesyltransferase inhibitor. Science 260: 1934
26. James GL, Goldstein JL, Brown MS, Rawson TE, et al (1993) Benzodiazepine peptomimetics: potent inhibitors of Ras farnesylation in animal cells. Science 260: 1937
27. Garcia AM, Rowell C, Ackermann K, Kowalczyk JJ, et al (1993) Peptidomimetic inhibitors of Ras farnesylation and function in whole cells. J Biol Chem 268: 18415
28. Vogt A, Qian Y, Blaskovich MA, Fossum RD, et al (1995) A non-peptide mimetic of Ras-CAAX: selective inhibition of farnesyltransferase and Ras processing. J Biol Chem 270: 660
29. Hunt JT, Lee VG, Leftheris K, Seizinger B, et al (1996) Potent cell active non-thiol tetrapeptide inhibitors of farnesyl-transferase. J Med Chem 39: 353
30. Leftheris K, Kline T, Vite GD, Cho YH, et al (1996) Development of highly potent inhibitors of Ras farnesyltransferase possessing cellular and in vivo activity. J Med Chem 39: 224
31. Graham SL, deSolms SJ, Giuliani EA, Kohl NE, et al (1994) Pseudopeptide inhibitors of Ras farnesyl-protein transferase. J Med Chem 37: 725
32. deSolms SJ, Deana AA, Giuliani EA, Graham SL, et al (1995) Pseudodipeptide inhibitor of protein farnesyltransferase. J Med Chem 38: 3967
33. Singh SB, Lingham RB (1996) Farnesyl-protein transferase inhibitors in early development. Exp Opin Invest Drugs 5: 1589
34. Cox AD, Garcia AM, Westwick JK, Kowalczyk JJ, et al (1994) The CAAX peptidomimetic compound B581 specifically blocks farnesylated, but not geranylgeranylated, or myristylated, oncogenic ras signalling and transformation. J Biol Chem 269: 19203
35. Cox AD, Hisaka MM, Buss JE, Der CJ (1992) Specific isoprenoid modification is required for function of normal, but not oncogenic, ras protein. Mol Cell Biol 12: 2606
36. Lebowitz PF, Sakamuro D, Prendergast GC (1997a) Farnesyl transferase inhibitors induce apoptosis of ras-transformed cells denied substratum attachment. Cancer Res 57: 708
37. Lebowitz PF, Casey PJ, Prendergast GC, Thissen JA (1997b) Farnesyltransferase inhibitors alter the prenylation and growth-stimulating function of RhoB. J Biol Chem 272: 15591
38. Der CJ, Cox AD (1997) Farnesyltransferase inhibitors and cancer treatment: targeting simply ras? Biochim Biophys Acta Rev Cancer 1333: F51
39. Sepp-Lorenzino L, Ma Z, Rands E, Kohl NE, et al (1995) Peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and -independent growth of human tumor cell lines. Cancer Res 55: 5302
40. Nagasu T, Yoshimatsu K, Rowell C, Lewis MD, et al (1995) Inhibition of human tumor xenograft growth by treatment with the farnesyl transferase inhibitor B956. Cancer Res 55: 5310
41. Kohl NE, Wilson FR, Mosser SD, Giuliani E (1994) Protein farnesyltransferase inhibitors block the growth of ras-dependent tumors in nude mice. Proc Natl Acad Sci USA 91: 9141
42. Kohl NE, Omer CA, Conner MW, Anthony NJ, et al (1995) Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. Nat Med 1: 792
43. Sun J, Qian Y, Hamilton AD, Sebti SM (1995) Ras CAAX peptidomimetic FTI 276 selectively blocks tumour growth in nude mice of a human lung carcinoma with K-Ras mutation and p53 deletion. Cancer Res 55: 4243