ABSTRACT

The posttranslational addition of a farnesyl moiety to the Ras oncoprotein is essential for its transforming activity. Cell-active inhibitors of the enzyme that catalyzes this reaction, protein farnesyltransferase, have been shown to selectively block ras-dependent transformation of cells in culture. Here we describe the protein farnesyltransferase inhibitor 2(S)·2(S)·2(R)-amino-3-mercapto[proplpropylamino-3(S)-methyl]pentamethylenedioxy-3-phenylpropionylmethioninemethane sulfon methyl ester (L-739,749), which suppressed the anchorage-independent growth of Ratl cells transformed with viral H-ras and the human pancreatic adenocarcinoma cell line PSN-1, which harbors altered K-ras, N-ras, and p53 genes. This compound also suppressed the growth of tumors arising from ras-transformed Ratl cells in nude mice by 66%. Under the same conditions, doxorubicin inhibited tumor growth by 33%. Control tumors formed by v-ras- or v-mos-transformed Ratl cells were unaffected by L-739,749. Furthermore, mice treated with L-739,749 exhibited no evidence of systemic toxicity. This is a demonstration of antitumor activity in vivo using a synthetic small molecule inhibitor of protein farnesyltransferase.

The protein product of the ras gene, Ras, functions in the regulation of cell proliferation (1). As for other low-molecular-weight GTP-binding proteins, Ras is active in its GTP-bound form and becomes inactive upon hydrolysis of the bound GTP to GDP by the intrinsic GTPase of the protein. Mutations at codons 12 and 61 give rise to forms of Ras that are constitutively active and can morphologically transform established cells in culture. Such oncogenically mutated forms of ras are found in a wide variety of human tumors, most notably in ~90% of human pancreatic adenocarcinomas and 50% of human colon tumors (1, 2).

Ras is synthesized as a cytosolic precursor that localizes to the plasma membrane after a series of posttranslational modifications (3, 4). The first reaction in this series involves the addition of a farnesyl group to the cysteine residue of the C-terminal CAAX sequence (where C is cysteine, A is an aliphatic residue, X represents any residue) in a reaction catalyzed by the enzyme protein farnesyltransferase (PFTase). Subsequently, the AAX residues are proteolytically cleaved, and the now C-terminal farnesylcysteine is methylated. Only the farnesylation reaction is essential for the transforming activity of the Ras oncoprotein (5–8). Thus, inhibitors of PFTase have been proposed as potential inhibitors of ras-dependent transformation that may be useful in the treatment of those cancers where Ras plays a role.

Synthetic PFTase inhibitors have been designed based on the two substrates of the reaction, farnesyl diphosphate and the Ras CAAX tetrapeptide, that portion of the Ras protein that is sufficient for interaction with the enzyme (9, 10). Recently, CAAX analogs have been synthesized that are potent inhibitors of PFTase (11–14). Several of these compounds have been shown to modulate critical aspects of Ras transformation in whole cells, including inhibition of anchorage-dependent (12) and anchorage-independent growth (13), morphological reversion (12), and inhibition of oocytic maturation induced by oncogenic Ras (11). Here we demonstrate that a potent and selective small-molecule inhibitor of PFTase will inhibit the growth of ras-dependent tumors in animals at concentrations of compound that do not cause significant toxicity to host animals.

MATERIALS AND METHODS

Cell Culture. The Ratl/Ras, Ratl/Mos, and Ratl/Raf cell lines were derived by transformation of Ratl cells by v-ras, v-mos, or v-raf, respectively, as described (13). The Ratl/huH-Ras cells were derived from Ratl cells by transformation with plasmid pZIP-rasH61L (5), which contains a 1.2-kb BamHI fragment encoding human H-Ras with a Leu-61 substitution ligated into the BamHI site of the pZIP-NeoSV(x)1 retrovirus vector. Ratl/huN-Ras cells were derived from Ratl cells by transformation with plasmid pZIP-rasN12D (16), which contains a 1.0-kb BamHI fragment encoding human N-Ras with an Asp-12 substitution ligated into the BamHI site of the pZIP-NeoSV(x)1 retrovirus vector. Ratl/huK-Ras cells were derived from Ratl cells by transformation with plasmid pZIP-rasK4B(12V) (17), which contains a 1.0-kb BamHI fragment encoding human K-Ras4B with a Val-12 substitution and a vector-derived 10-amino acid leader sequence ligated into the BamHI site of the pZIP-NeoSV(x)1 retrovirus vector. All of the Ratl derivatives were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/10% fetal bovine serum. The human pancreatic adenocarcinoma cell line PSN-1 (18) was maintained in RPMI 1640/10% fetal bovine serum.

Protein Prenyltransferase Assays. Protein prenyltransferase assays were done essentially as described (19) and contained the following: for PFTase, 100 nM [3H]farnesyl diphosphate, 100 nM Escherichia coli-produced Ras-CVIM (where C is cysteine, V is valine, I is isoleucine, and M is methionine), and 1 nM PFTase; for protein geranylgeranyltransferase (PGGTase-I), 100 nM [3H]geranylgeranyl diphosphate, 1000 nM E. coli-produced Ras-CAIL (where A is alanine and L is leucine), and 6.8 nM PGTase-I. Both enzymes were human recombinant enzymes partially purified from E. coli lysates (20, 21).

Soft Agar Assays. Soft agar assays were done essentially as described (13). Briefly, Ratl/Ras or Ratl/Raf cells were defrayed in part by page charge. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
seeded at a density of $1 \times 10^6$ cells per plate (35 mm in diameter) in a 0.3% top agarose layer in medium A (DMEM/10% fetal bovine serum) over a bottom 0.6% agarose layer. PSN-1 cells were seeded as above, but RPMI 1640 was used in place of the DMEM (medium B). Both layers contained 0.1% MeOH or the indicated concentration of L-739,749. The cells were fed twice weekly with 0.5 ml of medium A or medium B containing 0.1% MeOH or the indicated concentration of L-739,749. Photomicrographs were taken 15 days after seeding cultures.

Nude Mouse Assays. All animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and experimental protocols were reviewed by the Merck Animal Care and Use Committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee. Female and Use of Care committee.

RESULTS

L-739,750 is a Potent and Selective Inhibitor of PFTase in Vitro. We have previously described a CAAX-based inhibitor of PFTase, L-731,735, and its produg derivative L-731,734 (13). L-731,734 inhibited PFTase in whole cells [50% inhibition (IC50) of Ras processing at 100 μM] and selectively blocked the anchorage-independent growth of ras-transformed cells (at 1 μM). The modest potency of this compound in cell culture is due, in part, to chemical instability: at neutral pH, rapid cyclization of the lactone carbonyl group to the proximal amino group of the backbone of L-731,734 occurs to form a diketopiperazine, which is >100-fold less potent than an inhibitor of PFTase (S.D. M., S.J.d., J.B.G., and S.L.G., unpublished data). The current compounds were designed to eliminate this mode of reactivity by replacing the nucleophilic nitrogen with an ether oxygen. Structural modification in this series of compounds led to 2(S)-[2(S)-[2(R)-amino-3-mercaptopropylamino-3(S)-methyl]penteltyloxy-3-phelypropionylmethionine-sulfone (L-739,750) and 2(S)-[2(S)-[2(R)-amino-3-mercaptopropylamino-3(S)-methyl]penteltyloxy-3-phelypropionylmethionine-sulfone methyl ester (L-739,749) (Fig. 1). The stability of the esters in this series of compounds was enhanced >10-fold relative to their nitrogen-containing analogs (S.J.d., S.L.G., and R.L.S., unpublished data).

L-739,750 was a potent inhibitor of PFTase in vitro. In assays containing [3H]farnesyl diposphate, E. coli-produced Ras-CVIM and 1 nM partially purified E. coli-produced human PFTase (20), 50% inhibition was observed at a concentration of 1.8 nM (Table 1). This assay may underestimate the potency of compounds such as L-739,750, for which the IC50 is similar to the concentration of PFTase used in the assay. Thus, L-739,750 afforded a significant improvement in intrinsic potency relative to our previously reported CAAX-based PFTase inhibitor, L-731,735 (IC50 = 18 nM) (13). L-739,750 was highly selective for inhibition of PFTase and exhibited $>1000$-fold lower potency for inhibition of the related enzyme, PGGTase type I. PGGTase type I catalyzes the transfer of a geranylgeranylated isoprene to proteins having a C-terminal CAAX sequence in which X is a leucine. A methyl ester derivative of L-739,750 (L-739,749) was >100-fold less potent than L-739,750 at inhibiting PFTase and retained selectivity for PFTase versus PGGTase-I (Table 1).

L-739,749 Inhibits PFTase in Whole Cells. Despite its moderate potency against the enzyme in vitro, L-739,749 was a potent inhibitor of PFTase in whole cells. Cell activity was assessed by monitoring the extent of farnesylation of the PFTase substrate Ras. As reported elsewhere (22), 50% inhibition of Ras processing in cells was observed between 0.1 and 1 μM L-739,749. In contrast, an >10-fold higher concentration of L-739,750 was required to produce equivalent inhibition. The superior inhibitory activity of L-739,749 in cells relative to L-739,750 is consistent with the previous observation that masking the C-terminal carboxylate facilitated entry of the drug into mammalian cells (12, 13).

L-739,749 was a potent and selective inhibitor of the anchorage-independent growth of ras-transformed cells. In the presence of diluent alone, the Rat1/Ras cells formed multiple, large colonies (Fig. 2). Complete inhibition of the growth of the Rat1/Ras cells was observed at 10 μM L-739,749, and partial inhibition was achieved with concentrations of L-739,749 as low as 2.5 μM (Fig. 2). L-739,749 similarly inhibited the anchorage-independent growth of Rat1 cells transformed by oncogenically mutated human H-, K-, and N-ras genes (data not shown). To evaluate the specificity of L-739,749 for Ras-induced cell transformation, Rat1 cells transformed with either the v-raf or v-mos oncogenes were included in the analysis. Neither Raf nor Mos requires farnesylation to achieve biological activity, and both proteins appear to transform cells independently of Ras (23-26). No effect was observed on the anchorage-independent growth of the Rat1/Raf (Fig. 2) or Rat1/Mos cells at a concentration (10 μM) of L-739,749 that completely inhibited the anchorage-independent growth of the Rat1/Ras cells. Treatment of the Rat1/Raf or Rat1/Mos cells with L-739,749 up to 100 μM also did not affect their ability to grow in soft agar. L-739,750 showed selective inhibition of the growth of ras-transformed cells in soft agar but required 2- to 4-fold higher doses than L-739,749 to produce the same effect.

L-739,749 also inhibited the anchorage-independent growth of the human pancreatic adenocarcinoma cell line.

Table 1. Selective inhibition of PFTase in vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50, nM</th>
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<tbody>
<tr>
<td>L-739,749</td>
<td>240 ± 23</td>
</tr>
<tr>
<td>L-739,750</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>L-739,749</td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>L-739,750</td>
<td>3,000</td>
</tr>
</tbody>
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Protein prenyltransferase assays were done as described (see ref. 19 and Materials and Methods). PFTase values are the average of three (L-739,749) or seven (L-739,750) separate determinations ± SEM.
PSN-1 (Fig. 2; ref. 18). Typical of human tumor cells, the PSN-1 cells have multiple genetic alterations, including amplified activated c-K-ras and c-myc and mutated p53 genes. Inhibition of the growth of the PSN-1 cells was observed with concentrations of L-739,749 as low as 2.5 μM. Complete inhibition of the anchorage-independent growth of the PSN-1 cells required >100 μM L-739,749. However, complete inhibition of PSN-1 growth was achieved with another compound related to L-739,749 at 10 μM, which was the same drug concentration that completely inhibited the growth of Rat1/Ras cells (F.R.W., N.E.K., and J.B.G., unpublished data).

L-739,749 Inhibits the Growth of ras-Dependent Tumors in Nude Mice. A nude mouse explant assay was used to evaluate the effects of L-739,749 on the formation of ras-dependent tumors in animals. Rat1 cells transformed with either onco-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Days after injection of cells</th>
<th>PBS</th>
<th>L-739,749</th>
<th>Difference, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat1/huH-Ras</td>
<td>11</td>
<td>0.71 ± 0.12</td>
<td>0.24 ± 0.08</td>
<td>−66*</td>
</tr>
<tr>
<td>Rat1/huN-Ras</td>
<td>11</td>
<td>1.58 ± 0.37</td>
<td>0.78 ± 0.07</td>
<td>−51*</td>
</tr>
<tr>
<td>Rat1/huK-Ras</td>
<td>11</td>
<td>1.48 ± 0.27</td>
<td>0.62 ± 0.17</td>
<td>−58*</td>
</tr>
<tr>
<td>Rat1/Raf</td>
<td>15</td>
<td>0.51 ± 0.11</td>
<td>0.47 ± 0.07</td>
<td>−8</td>
</tr>
<tr>
<td>Rat1/Mos</td>
<td>11</td>
<td>0.42 ± 0.05</td>
<td>0.47 ± 0.06</td>
<td>+12</td>
</tr>
</tbody>
</table>

Rat1 cells transformed with oncogenically mutated human H-ras (Rat1/huH-Ras), K-ras (Rat1/huK-Ras), or with N-ras (Rat1/huN-Ras) or with v-mos (Rat1/Mos) or v-raf (Rat1/Raf) were injected s.c. into nude mice on day 0. Sixteen mice were used for each cell line. On day 2, eight animals from each group were assigned to the control (PBS) group and eight to the L-739,749 (20 mg/kg) treatment group. Animals were dosed i.p. once daily for 5 days beginning on day 2. Tumors were excised and weighed on day 11 (or day 15 for the Raf tumors). Data are presented as the average weight of the eight tumors in each treatment group for each cell line. Statistical significance of the difference between the average tumor weight in the PBS and L-739,749 treatment groups for each cell line was evaluated by using Student's one-sided t test (α = 0.05). *Statistically significant difference (P < 0.05).
from 51% for the N-ras-dependent tumors to 66% for the H-ras-dependent tumors. In contrast, there was no significant difference in the average weights between the PBS- and L-739,749-treated ras- and mos-dependent tumors. The inhibition of tumor growth by L-739,749 was dose-dependent. A 10-fold dilution of the compound did not significantly decrease the weights of the H-ras-dependent tumors relative to controls.

Having found that L-739,749 would inhibit ras-dependent tumor growth by 51–66% in nude mice, it was important to determine the extent of tumor inhibition that a standard chemotherapeutic agent would produce in the same model system. In parallel studies, Rat1/huH-Ras cells were again injected into nude mice on day 0, and the animals were treated with doxorubicin at 2 mg/kg per day for 5 days beginning on day 2. Doxorubicin produced a 33% reduction in the weight of the Rat1/huH-Ras tumors relative to controls treated with PBS. It should be emphasized that doxorubicin was used at its maximally tolerated dose in these studies. All of the doxorubicin-treated animals exhibited signs of systemic toxicity—including weight loss, anorexia, and inactivity during the week after their doxorubicin therapy. In contrast, mice receiving the PFTase inhibitor appeared normal and did not suffer any weight loss. The pathology of animals treated with L-739,749 was also analyzed. Gross and microscopic examinations of rapidly dividing tissues (bone marrow and gastrointestinal tract) and tissues where farnesylated proteins are important for normal function (retina and skeletal muscle) revealed no treatment-related abnormalities.

**DISCUSSION**

We have demonstrated that a synthetic, cell-active inhibitor of PFTase, L-739,749, can selectively inhibit the growth of ras-dependent tumors in a nude mouse explant model. Treatment with L-739,749 at 20 mg/kg i.p. once daily for 5 days resulted in a 51–66% decrease in the average tumor weight (relative to the vehicle control) depending on the ras allele used. It is important to note that 5–9 days elapsed after the last treatment dose before tumor excision. It is possible that altering the dosing protocol such that compound is administered for the duration of the experiment would result in a further decrease in tumor weights. In preliminary studies, an analog of L-739,749 produced a 93% inhibition of tumor growth after a more chronic treatment regimen.

Two other inhibitors of cellular prenylation, lovastatin (27) and limonene (28), have been shown to block tumor growth. Lovastatin, an inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (29), blocks the synthesis of isoprenyl pyrophosphates, as well as cholesterol. The mechanism by which limonene, the major monoterpene in orange peel oil, inhibits cellular isoprenylation is unclear. Manumycin is capable of inhibiting PFTase in vitro and has been reported to inhibit the growth of a ras-dependent tumor in nude mice (30). However, the mechanism by which this antibiotic produces its inhibitory effect in vivo is unclear because PFTase inhibition was not evaluated in cultured cells.

It is likely that the antitumor effects of L-739,749 are due to its inhibition of PFTase activity. L-739,750, the acid of L-739,749, is a potent and specific inhibitor of PFTase in vitro (Table 1). L-739,749 also exhibits selectivity in cell culture where processing of Ras proteins is blocked, but proteins engineered to be substrates for PGGTase-I are unaffected (ref. 13; F.R.W., N.E.K., A.O., and J.B.G., unpublished data). Similar specificity of CAAX peptidomimetics for PFTase versus PGGTase-I in mammalian cells and Xenopus oocytes has been observed (11). These results suggest that the antitumor effects of L-739,749 are due to its inhibition of PFTase activity. The ability of L-739,749 to block the anchorage-independent growth of ras-transformed cell lines in soft agar is consistent with previous observations showing that genetic disruptions of a mutant K-ras gene in two human colon cancer cell lines also resulted in their loss of anchorage-independent growth (31).

The current studies suggest that PFTase inhibitors such as L-739,749 will not only be effective antitumor drugs but may also prove to be remarkably safe chemotherapy agents. No evidence of gross toxicity or microscopic histological abnormalities was noted at autopsy in any of the L-739,749-treated mice. The ability of PFTase inhibitors to selectively target the fundamental mechanism responsible for oncogenesis in ras-transformed cells (i.e., ras-mediated signal-transduction events) rather than to interfere with the secondary biological properties common to all transformed cells (e.g., increased DNA and protein synthesis) may account for the remarkable therapeutic ratio (efficacy versus toxicity) exhibited by L-739,749 in animals.

The therapeutic ratio achieved with L-739,749 is somewhat surprising, given that farnesylation is necessary for the biological activity of a number of essential cellular proteins, such as the nuclear lamins (32). While the reasons for the lack of systemic cytotoxicity of L-739,749 are not known, several points deserve consideration (see also ref. 33). First, farnesyalted proteins may display differential sensitivity to the inhibitor. In this regard, it has been shown that higher concentrations of CAAX-based PFTase inhibitors are required to inhibit farnesylation of the nuclear lamins than are required to inhibit Ras processing (11, 12). Additionally, among the farnesylated proteins, there may be variability in the minimum amount of functional (farnesylated) protein required for cellular proliferation. Finally, the lack of absolute specificity of the prenyltransferases has been demonstrated in vitro with mammalian enzymes (19) and in the yeast Saccharomyces cerevisiae (34, 35). Thus, the possibility exists that, in mammalian cells lacking PFTase activity, PGGTase-I may prenylate some proteins that would otherwise be farnesylated, thus restoring function.

We thank E. Sendor for performing the statistical analyses and C. Der for the human H-, K-, and N-ras plasmids.

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