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Development of Inhibitors of Protein Farnesylation as Potential Chemotherapeutic Agents

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Abstract Protein prenylation, adding either the 15-carbon isoprenoid farnesyl or the 20-carbon isoprenoid geranylgeranyl to cysteine residue(s) at or near the C-termini of proteins, is a recently identified post-translational modification that localizes some proteins to a membrane compartment. One of the most intensely studied prenylated proteins is Ras, a low molecular weight GTP-binding protein that plays an important role in the regulation of cell proliferation. Proteins encoded by *ras* genes with oncogenic mutations are capable of transforming cells in culture. Such mutated *ras* genes are frequently found in a wide variety of human tumors. Localization of the Ras oncoprotein to the cytoplasmic face of the plasma membrane via farnesylation is essential for efficient cell transforming ability. Thus, inhibition of the Ras farnesylation reaction is a possible anti-cancer strategy.

Several strategies have been employed to inhibit Ras farnesylation, including inhibition of isoprenoid biosynthesis and inhibition of the enzyme which catalyzes the farnesylation reaction, farnesyl-protein transferase (FPTase). Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate limiting enzyme in isoprenoid biosynthesis, inhibit Ras farnesylation and block the growth of *ras*-transformed cells. However, antiproliferative effects do not result from specific inhibition of Ras farnesylation; they are also observed in cells transformed by *raf*, which is independent of Ras farnesylation. A more specific approach to inhibiting Ras farnesylation is to inhibit FPTase. Using random screening of natural products and a rational design approach, a variety of compounds that specifically inhibit FPTase have been isolated. Several of these compounds were found to block the farnesylation of Ras proteins in cell culture and were able to block the anchorage-independent growth of *ras*-transformed cells and human tumor cell lines. FPTase inhibitors also blocked the morphologic alteration associated with *ras*-induced transformation of mammalian cells. In contrast, these compounds did not affect the growth or morphology of cells transformed by the *raf* or *mos* oncogenes, which do not require farnesylation to achieve biological activity. Furthermore, these compounds suppressed the growth of tumors arising from *ras*-transformed cells in nude mice in the absence of systemic toxicity. Control tumors formed by *raf*- or *mos*-transformed cells were not affected by these compounds. These studies suggest that FPTase inhibitors might be safe and effective chemotherapeutic agents.

Key words: CAAX peptidomimetic, farnesyl-protein transferase, protein prenylation, *ras* oncogene

Cellular proteins terminating in a CAAX motif can serve as substrates for one of two isoprenyl-protein transferases, farnesyl-protein transferase (FPTase) or geranylgeranyl-protein transferase type I (GGPTase I) [1,2]. FPTase catalyzes the transfer of the 15-carbon isoprenoid, farnesyl, from farnesyl diphosphate (FPP) to the C-termi-

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nal cysteine residue of proteins in which the X residue of the CAAX motif is typically serine or methionine. Substrates of FPTase include the Ras proteins, the nuclear lamins, the α and β subunits of skeletal muscle phosphorylase kinase, and several proteins involved in signal transduction in the visual system [2,3]. GGPTase I catalyzes the transfer of the 20-carbon isoprenoid, geranylgeranyl, from geranylgeranyl diphosphate to proteins in which the X residue of the CAAX motif is leucine or phenylalanine, including Rac and Rho proteins and the γ subunits of heterotrimeric G proteins [1]. Prenylation facilitates membrane localization of proteins and, for many prenylated proteins, is essential for function.

One of the most intensely studied prenylated proteins is the low molecular weight GTP binding protein Ras. Ras functions as a molecular switch in transducing growth-promoting signals from the cell surface to the nucleus, being "on" when bound to GTP and "off" when bound to GDP. Mutations which abolish the protein's intrinsic GTPase activity result in constitutively activated forms of Ras that exhibit cell transforming activity [4]. Genetic studies have demonstrated that the ability of the Ras oncoprotein to transform cells is dependent on the post-translational addition of farnesyl to the C-terminal cysteine [5–8]. Thus, inhibitors of Ras farnesylation may be useful as chemotherapeutic agents.

Several strategies have been employed to inhibit the Ras farnesylation reaction, including inhibition of isoprenoid biosynthesis and inhibition of FPTase (Fig. 1). Inhibitors of the rate-limiting enzyme in isoprenoid biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase, such as lovastatin [9], block the post-translational modification of Ras and other farnesylated proteins by blocking the synthesis of FPP. Moreover, lovastatin inhibits cell growth at concentrations that parallel those required to inhibit protein prenylation. However, the antiproliferative effect of lovastatin is likely not due to inhibition of Ras farnesylation since cells transformed by viral Raf, an oncoprotein whose function is not dependent on farnesylation, are similarly growth-inhibited [10]. Rather, the antiproliferative effects are probably due to inhibition of synthesis of other mevalonate derivatives essential for cell growth.

A more direct approach to inhibiting Ras farnesylation is to inhibit FPTase. Inhibitors of the

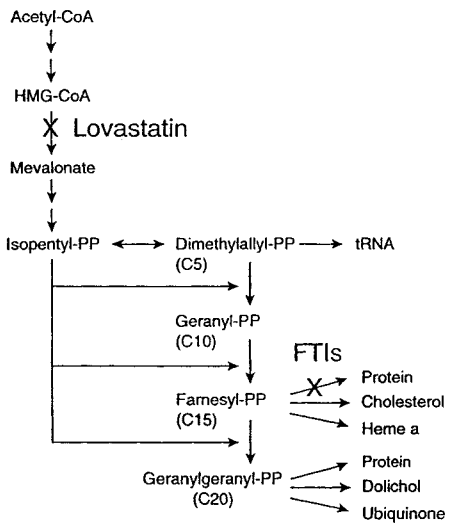


Fig. 1. Isoprenoid biosynthetic pathway. The Xs indicate steps in the pathway which are targets for inhibition of protein prenylation. FTI, farnesyl-protein transferase inhibitor.

enzyme have been identified both through targeted screens [11] and rational design based on the two substrates of the reaction, FPP and the Ras CAAX tetrapeptide [12–16]. The C-terminal CAAX tetrapeptide of Ras contains all the determinants required for interaction of the protein with FPTase [17,18]. While analogs of both protein and isoprenoid substrates have proven to be potent inhibitors of the enzyme, the most significant advances have been made with CAAX peptidomimetics.

One of the more thoroughly characterized CAAX peptidomimetics is L-739,749, the methyl ester derivative of L-739,750 (Fig. 2). L-739,749 contains several structural modifications relative to the CAAX tetrapeptide CIFM, a potent, non-substrate FPTase inhibitor [19], which are critical for *in vitro* potency and cell activity. Deletion of the carbonyl between the first and second and second and third residues in L-739,749 confers stability against cleavage by aminopeptidases present in cells [20]. Substitution of an oxygen for the nitrogen atom between the second and third residues increases the chemical stability of L-739,749 relative to less potent compounds in this series. Finally, replacement of the carboxylate on the C-terminal methionine sulfone (as is found in L-739,750) with a methyl ester confers

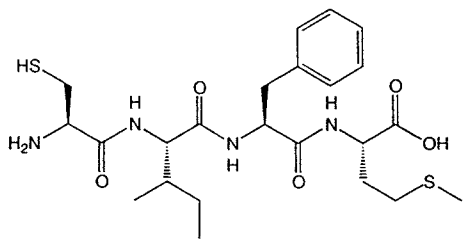
increased cell activity (see below), presumably by increasing membrane permeability [20]. Since the C-terminal carboxylate is an important determinant of intrinsic FPTase inhibitory potency [15, 21], L-739,749 functions as a prodrug, cleaved by intracellular esterases to form L-739,750, the active form of the compound.

L-739,750 is a potent and specific inhibitor of FPTase. In assays containing [^{3}H]FPP, *E. coli*-produced Ras protein and 1 nM partially purified *E. coli*-produced human FPTase [22], 50% inhibition (IC_{50}) was observed at 1.8 nM [23]. In contrast, L-739,750 inhibited the related GGPTase I with an IC_{50} of 3,000 nM. As expected, the prodrug, L-739,749, was a less potent inhibitor of FPTase (IC_{50} = 240 nM) but maintained its specificity for FPTase relative to GGPTase I [23].

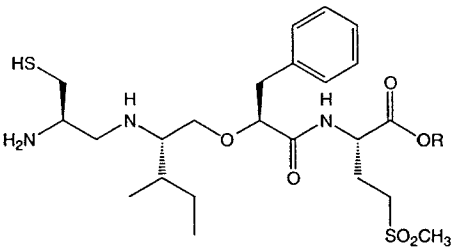
L-739,749 was a potent inhibitor of FPTase in cells. In assays which measure the fraction of processed (farnesylated) and unprocessed Ras in immunoprecipitates from cells treated with L-739,749, 50% inhibition was observed between 0.1 and 1 μM [24]. In contrast, approximately 10-fold higher concentrations of L-739,750 were required to produce the same effect. This observation is consistent with the role of L-739,749 as a prodrug.

Inhibition of FPTase in *ras*-transformed Rat1 cells by L-739,749 inhibited anchorage-independent growth. Complete inhibition of growth in soft agar was achieved with 10 μM L-739,749, and partial inhibition with concentrations as low as 2.5 μM [23]. In contrast, L-739,749 (at concentrations up to 100 μM) had no effect on the anchorage-independent growth of Rat1 cells transformed by v-*raf* and v-*mos*. The Raf and Mos oncoproteins do not require farnesylation for biological activity and appear to transform cells independently of Ras [25–28]. This observation suggested that the effect of L-739,749 on the *ras*-transformed cells was not due to general cytotoxicity. In addition, L-739,749 also inhibited the anchorage-independent growth of the human pancreatic adenocarcinoma cell line, PSN-1 [23]. Like human tumors, the PSN-1 cells have multiple genetic alterations, including amplified activated c-K-*ras* and c-*myc* and mutated p53 genes [29].

Treatment of *ras*-transformed cells with a single dose of L-739,749 (as low as 1 μM) caused the cells to acquire a flat phenotype similar to that of the untransformed parental cells [24]. The



CIFM



L-739,749, R=CH_3
L-739,750, R=H

Fig. 2. Structure of the CAAX tetrapeptide CIFM and the farnesyl-protein transferase inhibitors, L-739,749 and L-739,750.

reverted phenotype was detectable within 18 hours after addition of L-739,749 and persisted for 8–10 days, at which time the transformed phenotype began to reappear. In contrast, treatment of *raf*-transformed cells with L-739,749 at concentrations up to 50 μM had no effect on their morphology [24], consistent with the compound's lack of effect on the anchorage-independent growth of these cells.

Most recently, the *in vivo* efficacy of L-739,749 has been demonstrated using a nude mouse explant model [23]. Mice injected subcutaneously with *ras*- (oncogenically mutated human H-, K- or N-*ras*), v-*raf*-, or v-*mos*-transformed cells were treated with phosphate-buffered saline (PBS) or 20 mg L-739,749/kg intraperitoneally once daily for 5 days beginning 2 days after injection of the cells. The tumors were excised and weighed 5–9 days following the last treatment. There was a significant decrease in the average weight of all the *ras*-dependent tumors from mice treated with L-739,749 relative to those treated with PBS; the percentage decrease ranged from 66% for H-*ras*-dependent tumors to 51% for N-*ras*-dependent tumors. In contrast, L-739,749 had no effect on the weights of the *raf*- or *mos*-dependent tumors.

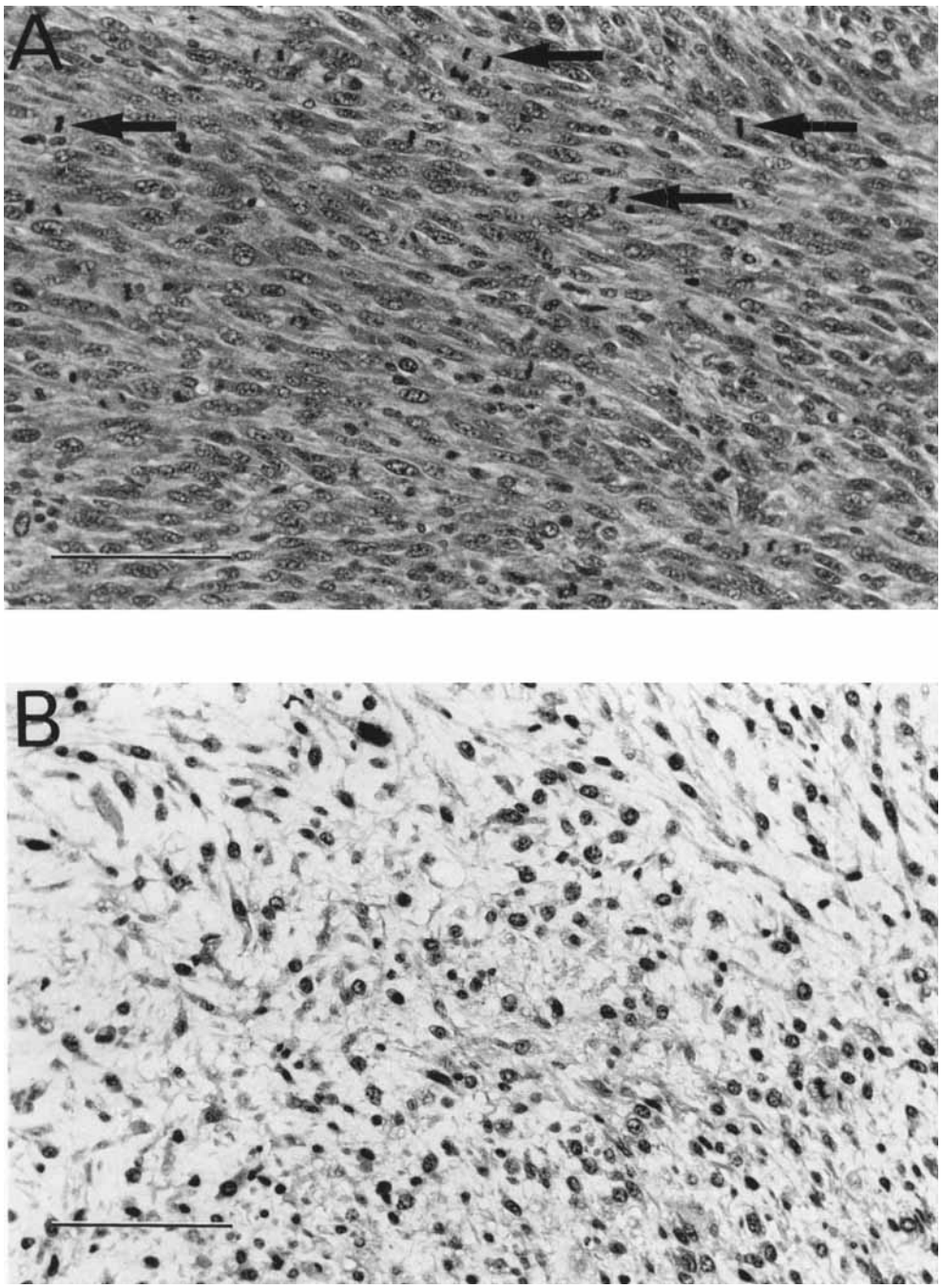


Fig. 3. Microscopic appearance of H-*ras*-dependent tumors from control (A) and CAAX peptidomimetic-treated (B) animals. The arrows indicate mitotic figures. Hematoxylin and eosin. Bar = 100 μm.

A 10-fold dilution of L-739,749 had no effect on the growth of the *ras*-dependent tumors, indicating that the effect of the compound was dose dependent.

Histological examination of tumors from treated (with a CAAX peptidomimetic similar in potency to L-739,749) and untreated animals revealed dramatic differences (Fig. 3). Tumors from control animals exhibited a high degree of cellularity and numerous mitotic figures indicative of rapid growth. In contrast, tumors from treated animals exhibited reduced cellularity and fewer mitotic figures.

In comparison, doxorubicin administered at 2 mg/kg (the maximally tolerated dose) resulted in a 33% decrease in the weight of H-*ras*-dependent tumors. While the doxorubicin-treated animals showed signs of toxicity, such as weight loss, the L-739,749-treated animals appeared normal. Gross and microscopic examination of rapidly dividing tissues (bone marrow and gastrointestinal tract) and tissues where farnesylated proteins are important for normal function (retina and skeletal muscle) from L-739,749-treated animals revealed no treatment-related abnormalities.

The FPTase inhibitors, exemplified here by L-739,749, appear to be effective and safe chemotherapeutics in animal models. While much remains to be learned about the mechanism of action of these compounds, the current data suggests their efficacy against human cancers.

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