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INHIBITORS OF THE RAS SIGNAL TRANSDUCTION PATHWAY AS POTENTIAL ANTITUMOUR AGENTS

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INTRODUCTION

Ras in Normal and Transformed Cells

Ras is a small (21 kD) GTP-ase protein which forms part of a cell growth-signalling pathway stretching from the plasma membrane to the nucleus. Four distinct forms of Ras protein have so far been described in mammalian cells, namely Harvey (H)-Ras, N-Ras, Kirsten (K)-Ras A and K-Ras B.

In recent years, the various elements of the signalling pathway upstream and downstream of Ras (Figure 1) have been unravelled (for reviews see References 1, 2). The constitutive activation of Ras in tumours appears to contribute to their malignant growth properties and members of this gene family are mutationally activated in a large number of malignancies. Historically, Ras was one of the first

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Abbreviations: EGF: epidermal growth factor, FPP: farnesylpyrophosphate, FPTase: farnesyl-protein transferase, GAP: GTPase-activating protein, GDI: guanine nucleotide dissociation inhibitor, GGPTase I: geranylgeranyl-protein transferase I, HFP: α-(hydroxyfarnesyl)phosphonic acid, HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A, IC_{50}: 50% inhibitory concentration, i.v.: intravenous, MAPK: mitogen activated protein kinase, MAPKK: mitogen activated protein kinase kinase, PDGF: platelet derived growth factor, PPMTase: prenylated protein methyl transferase, s.c.: subcutaneous, SSase: squalene synthase.

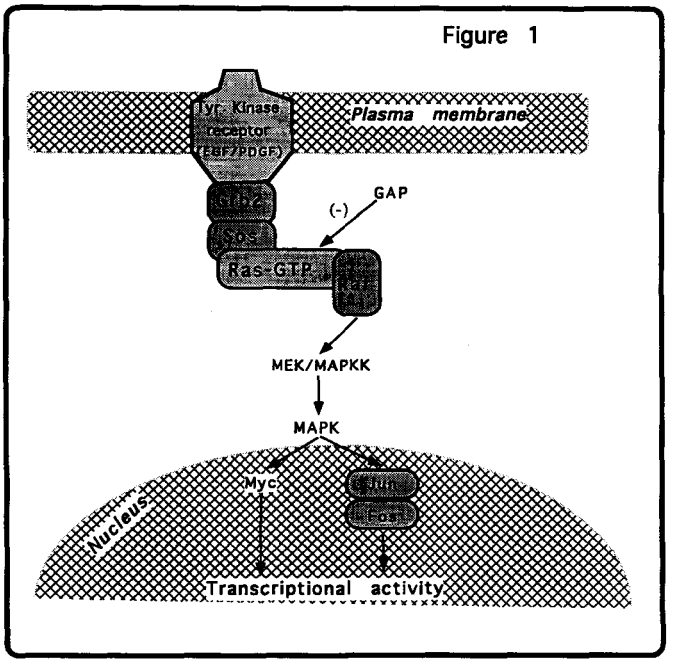

FIGURE 1 Ras signalling pathway. Upon binding of its ligand a tyrosine kinase receptor, for example, platelet derived growth factor or epidermal growth factor, autophosphorylates and recruits the cytosolic complex Grb2-Sos to the plasma membrane. In turn, Sos binds Ras localised to the plasma membrane and activates it by promoting the exchange of GDP for GTP. Activated (GTP-bound) Ras recruits Raf to the plasma membrane and activates it, which in turn induces the phosphorylation and activation of mitogen activated protein kinase kinase (MAPKK), which phosphorylates and activates the protein mitogen activated protein kinase (MAPK). Phosphorylated MAPK activates proto-oncogenic transcription factors like c-Jun, c-Myc and Elk-1.

mutated oncogenes to be identified. Ras mutations are present in around 25% of all cancers, notably 30% of lung cancer, 50% of colonic and 90% of pancreatic tumours.[3,4] A single amino acid substitution in position-12 is enough to lock Ras in its GTP bound state and constitutively to activate it (for a review see Reference 5). Activation of normal Ras proteins is recognised as an essential step in the signalling cascade of mitogenesis and normal Ras is considered necessary for the growth activity of numerous autocrine loops in tumours. Moreover, it has been suggested recently that beside its promoting effects on cell growth, oncogenic Ras could play a role in tumorigenesis by inhibition of the apoptosis phenomenon induced by the escape of epithelial cells from monolayer growth.[6] In tumour cells, constitutively active (GTP-bound) Ras could induce growth signal independent cell proliferation,

and promote escape from death via apoptosic pathways. Therefore, blocking Ras activity should result in an inhibition of the growth of tumours which depend on the activation of molecules upstream of Ras, as well as those expressing mutationally activated Ras. Moreover, inhibitors of Ras should have few or no side effects on the growth of normal cells which do not rely on a constitutively activated Ras protein for proliferation.

Ras-processing is Essential for its Function

In order to be active, Ras must be associated with the plasma membrane, a feature which depends on complex post-translational processing of the Ras protein, involving notably addition of a farnesyl (a 15 carbon prenyl group) to a cysteine of the four amino acid C-terminal tetrapeptide CAAX (where A is an aliphatic amino acid and X is either methionine or serine) of all Ras proteins.[7] The farnesylation reaction is catalysed by the enzyme farnesyl-protein transferase (FPTase), while further processing involves proteolysis of the AAX terminal aminoacids[8] and methylation (Figure 2). However, these latter steps have been reported as not being essential for Ras transforming activity.[7] FPTase not only farnesylates proteins of the Ras family (H-, N- and K-Ras), but also Rho B, a protein controlling the dynamics of the actin cytoskeleton, the nuclear lamins, as well as transducin and rhodopsin kinase, two proteins involved in the visual signal-transduction pathway.[7,9] Squalene synthase is another enzyme which uses FPP as a substrate, and is an enzyme of the cholesterol biosynthesis pathway, so that some degree of specificity for inhibitors of FPTase has been considered essential to avoid perturbation of cholesterol synthesis.

Geranylgeranyl-protein transferase I (GGPTase I) catalyses transfer of a 20 carbon prenyl group to a cysteine of a somewhat different protein CAAX C-terminus (where A is an aliphatic amino acid and X is either leucine or proline), and processes important proteins such as the γ-subunit of trimeric GTP-binding proteins; Rap 1, and CDC42/G25k involved generally in growth control.[7,9] Moreover, in the cell, geranylgeranylation is 5 to 10 times more widespread than farnesylation. Furthermore, FPTase and GGPTase I are heterodimeric enzymes which share the same α-subunit.[10] Therefore, much of the chemistry has been focused on a search for potent, and specific inhibitors of FPTase, relative to GGPTase I, to prevent interference with geranylgeranylation occurring in the cell.

Another protein using geranylgeranylpyrophosphate as a substrate is geranyl-geranyl-protein transferase II, which processes proteins of the Rab family, involved in vesicle trafficking and exo-endocytosis.[11]

Therefore, the rationale behind the development of specific inhibitors of FPTase, was that these compounds would affect primarily cells relying on an activated Ras for growth. Moreover, as the number of proteins processed by FPTase appears

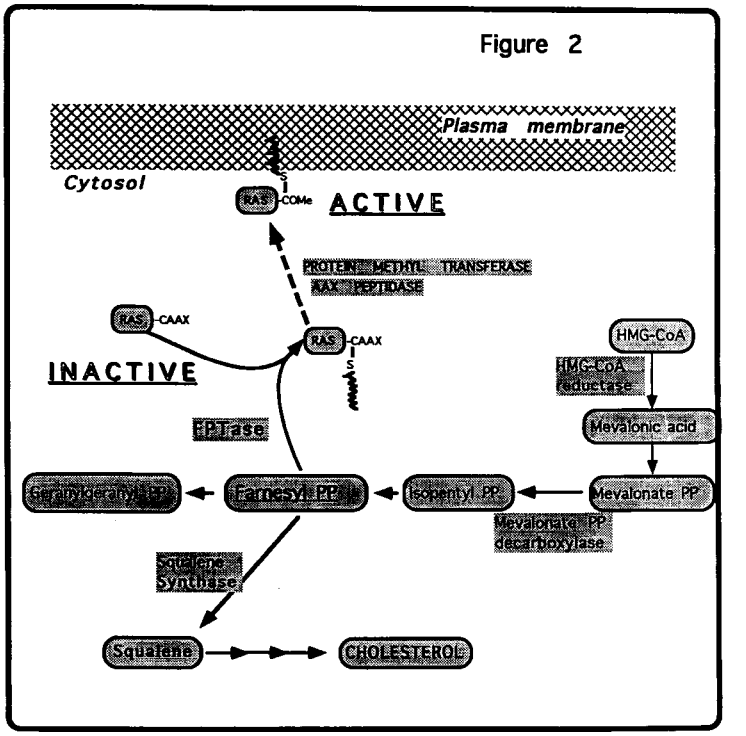

FIGURE 2 Ras-processing and cholesterol biosynthesis pathway. Ras is sequentially processed by farnesyl-protein transferase (FPTase) which farnesylates the cysteine residue of its CAAX carboxyterminus, an AAX peptidase which removes the last three C-terminal amino acids, and a protein methyl transferase which methylates the prenylated cysteine.

rather limited, fewer side effects would be expected from such compounds than from "classical" anticancer drugs targeting, for example, DNA.

The bulk of published literature in this field relates to inhibitors of FPTase, with only a few reports being published on inhibitors of prenylated protein methyl transferase, or of peptidase. Alternative targets on the Ras signalling pathway could be effectors downstream of the Ras protein, like Raf or mitogen activated protein kinase. Compounds like lovastatin or phenylacetate which depress the intracellular stores of FPP by interfering with the mevalonate biosynthesis pathway are also now being considered. Finally, some compounds, like limonene, whose mode of action is not well understood, have been reported as potential inhibitors of the Ras signalling pathway.

INHIBITORS OF RAS

Inhibitors of Ras-processing

Inhibitors of Farnesyl Protein Transferase

The chemical structures of most of the known FPTase inhibitors have been considered in detail in a recent and comprehensive review by Graham.[12] This report will present only a few of the essential structures (Figure 3), concentrating on the most representative and biologically-active members of the four main families of FPTase inhibitors which have been described:

- Natural products inhibitors
- Farnesyl pyrophosphate analogues
- Bisubstrate derivatives
- Peptide and peptidomimetic derivatives

Natural products inhibitors (Table I) have been discovered either by screening for activity in yeast, by monitoring the rescue of a yeast strain expressing a lethal farnesylated G protein,[13] or directly by monitoring inhibition of the isolated mammalian enzyme. Reported IC_{50} values for FPTase have varied from 0.05 to 40 μM. Where tested, with the exception of zaragozic acid A, good specificity against FPTase, as opposed to GGPTase I, has been shown. In the few cases where mechanistic studies have been carried out, FPP competition has been identified. Additionally, gliotoxin and manumycin have been shown to inhibit the *ras*-dependent multivulva phenotype in *Caenorhabditis elegans*.[14] Moreover, the manumycin derivative UCF1 appeared able to inhibit the growth of the K-*ras*-murine fibrosarcoma in BALB/c mice and of N-*ras* mutated HT1080 human fibrosarcoma in nude mice.[15] Furthermore, UCF1 blocked Ras processing and MAPK activity, while it did not decrease Rap 1 geranylgeranylation in *ras*-mutated Hep G2 human hepatoma.[16]

However, no effectiveness of the other compounds listed in Table I has yet been reported, either *in vitro* or *in vivo* in *ras*-transformed mammalian cells.

Farnesylpyrophosphate analogues (see structures in Figure 3) have shown interesting activity and selectivity against FPTase in enzyme assays, with IC_{50} values being reported of 30 nM[17] for α-(hydroxyfarnesyl)phosphonic acid (HFP), 340 nM[18] for farnesylpyrophosphonate (FPPA1) and 85 nM[19] for a phenylalanine derivative of farnesylphosphonate (Table II). However, only inhibition of Ras processing in H-*ras*-transfected Rat1 or NIH 3T3 cells has been reported with HFP[17] and FPPA1[18] respectively, with no positive effects on inhibition of cell growth being described. Farnesylamine was shown to inhibit the growth of H-*ras*-transformed

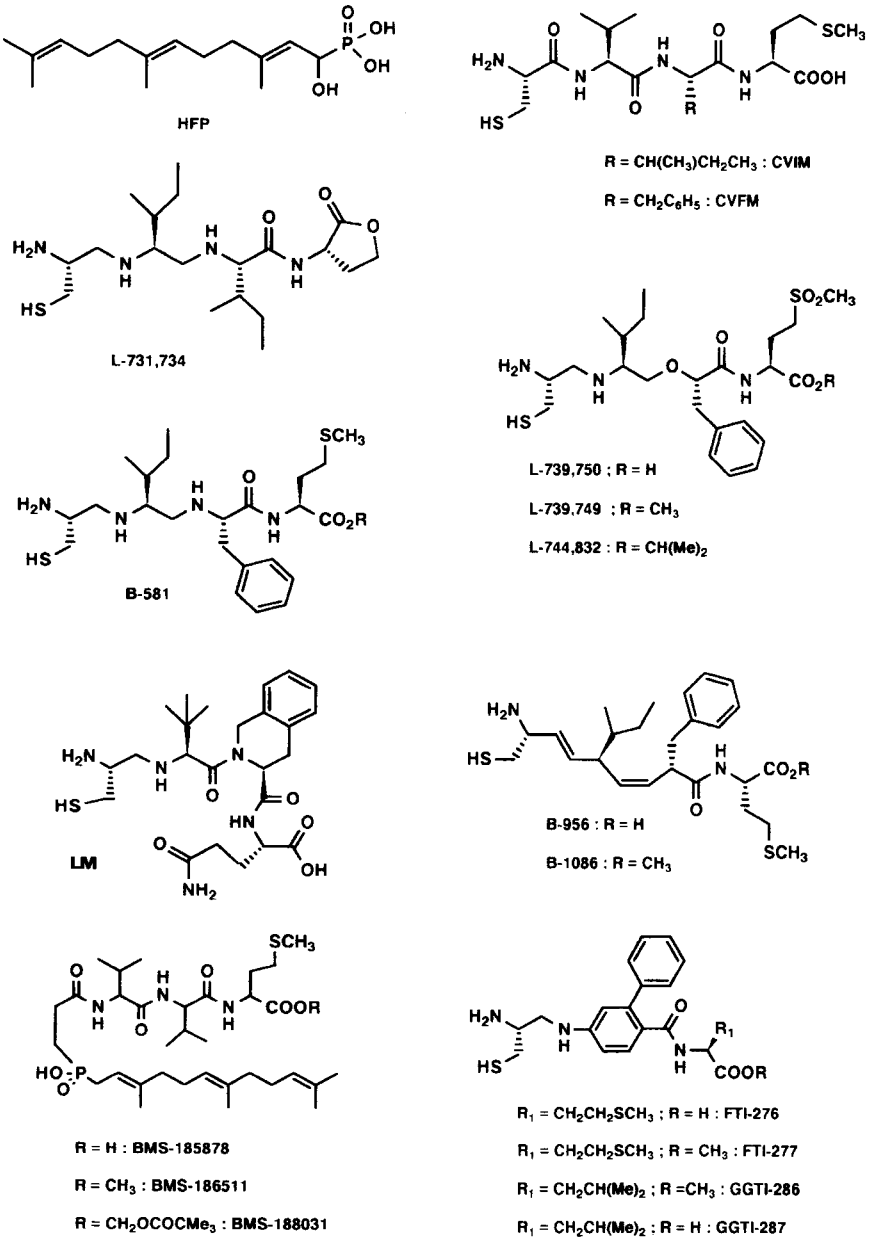

FIGURE 3 Structure of selected inhibitors of farnesyl-protein transferase and geranylgeranyl-protein transferase.

TABLE 1 Inhibitors of farnesyl protein transferase from natural sources.

Compound	*IC_{50} for FPTase (μM)*	*IC_{50} for GGPTase I (μM)*	*Inhibition mechanism*	*Reference*
Manumycin	5	180	FPP competition	15
Gliotoxin	1.1	nr	nr	94
Pepticinnamin	0.1	nr	nr	95
Chaetomellic acid A	0.06	92	FPP competition	96
Chaetomellic acid B	0.19	54	nr	96
Zaragozic acid A	0.22	0.62	FPP competition	17
Actinoplanic acid A	0.23	nr	FPP competition	97
Actinoplanic acid B	0.05	nr	nr	97
Cylindrol A	2.2	nr	nr	98
Fusidienol	0.3	nr	nr	99
Preussomerins G/D	1.2	20	nr	100
Barceloneic acid A	40	nr	nr	101
RPR 113228	2.1	59	nr	102
SCH 58450	29	740	nr	103

nr: not reported.

NIH 3T3 cells and blocked the processing of Ras,[20] while (*E,E*)-2-((dihydroxyphosphonyl)methyl)-3-oxo-3-((3-, 7, 11-trimethyl-2, 6, 10-dodecatrienyl)-amino) propanoic acid[21] not only inhibited FPTase *in vitro* with an IC_{50} value of 83 nM, but also inhibited the growth of H-*ras*-transformed NIH 3T3 cells in soft agar, and blocked Ras prenylation.

Bisubstrate derivatives developed by Bristol Myers Squibb (Table II), notably BMS-186511, seem to be more promising, since the latter blocked Ras processing and cell growth in H-*ras*-transformed NIH 3T3 cells, but was less effective in K-*ras*-transformed NIH 3T3 cells, and ineffective against untransformed cells.[22,23] Moreover, BMS-185611 inhibited FPTase activity, Ras processing and the growth of ST88-14, a Schwannoma cell line of a neurofibromatosis type I (NF1) established from a biopsy from a patient.[24] The product of the normal NF1 gene is neurofibromin, a Ras GTPase-activating protein, and ST88-14 cells express a normal but constitutively activated (GTP-bound) Ras protein. Therefore, an inhibitor of Ras processing could be useful in correcting genetic defects upstream of Ras in the Ras signal transduction pathway.

TABLE II Selected synthetic inhibitors of farnesyl protein transferase.

Compound	*IC_{50} for FPTase (μM)*	*IC_{50} for GGPTase I (μM)*	*Inhibition mechanism*	*Effects in vivo/in vitro*	*Reference*
Farnesylpyrophosphate derivatives					
HFP	0.03	36	FPP comp.	Blocks Ras processing	17
FPPA1	0.34	nr	FPP comp.	Blocks Ras processing	18
PheFPP	0.085	nr	FPP comp.	nr	19
FA	0.083	26	FPP comp.	Blocks Ras processing, inhibits cell growth	21
Bisubstrate derivatives					
BMS-186878	0.006	21	FPP & Ras comp.	nr	22
BMS-186511	nr	nr	FPP & Ras comp.	Blocks Ras processing, inhibits cell growth	23
Peptide derivatives					
CVIM	0.15	35	Ras comp.	nr	25
CVFM	0.06	11.0	Ras comp.	nr	26
BZA-5B	0.04	nr	Ras comp.	Inhibition of cell growth, blocks Ras processing	27
L-731,735	0.02	>100	Ras comp.	nr	28
L-731,734	0.3	>100	Ras comp.	Blocks Ras processing, inhibits cell growth	28
L-739,750	0.002	3.0	Ras comp.	nr	32
L-739,749	0.2	>100	Ras comp.	Inhibits Ras processing, cell growth, and tumour growth in nude mice	32
L-739,832	nr	nr	Ras comp.	Inhibits Ras processing, cell growth, and tumour growth in *ras*-transgenic mice	33
B 581	0.02	0.38	Ras comp.	Blocks Ras processing	29
B 956	0.01	nr	Ras comp.	Inhibits Ras processing, cell growth, and tumour growth in nude mice	36
FTI-277			Ras comp.	Inhibits Ras processing, cell growth, and tumour growth in nude mice	31, 34
LM	0.037	8.1	Ras comp.	Inhibits Ras processing, cell growth, and tumour growth in nude mice	35

L-731,734 is the lactone form of L-731,735; L739,749 and L-739,832 are respectively the methyl ester and isopropyl ester forms of L-739,750; BMS-186511 is the methyl ester form of BMS-186878; FTI-277 is the methyl ester derivative of FTI-276 (Cys-4-aminobenzoic acid-Met); HFP: α-(hydroxyfarnesyl)phosphonic acid; FPPA1: farnesylphosphonate; PheFPP: (2*S*) 3-phenyl-2-((4*E*,8*E*,12*E*)-2-(dihydroxy-phosphoryl)-5,9,13-trimethyl-tetradeca-4,8,12-trienoylamino)-propionic acid; FA: (*E*,*E*)-2-((Dihydroxyphosphonyl)methyl)-3-oxo-3-((3-,7,11-trimethyl-2,6,10-dodecatrienyl)-amino)propanoic acid; FPP: farnesylpyrophosphate; LM: (*S**,*R**)-*N*-((2-(*N*-(2-amino-3-mercapto-propyl)-L-tert-leucyl)-1,2,3,4-tetrahydro-3-isoquinolinyl)carbonyl)-L-methionine; Ras comp.: Ras competition; FPP comp.: FPP competition.

Peptide derivatives (See Table II) are currently receiving most interest, since peptide analogues or peptidomimetics designed on the basis of the last four amino acids of a Ras protein can inhibit the FPTase-catalysed farnesylation of Ras, at a concentration at which it does not interfere with GGPTase I (see Table II). These products constitute the most numerous family of FPTase inhibitors studied, with several major pharmaceutical companies investing in this area of research. Since peptides and peptidomimetics contain a free carboxylic acid group and permeate cells poorly, a universally-followed strategy has been the synthesis of prodrugs of active peptides in the ester form (see structures displayed in Figure 3).

The grand ancestors of this family of products were the tetrapeptides CVIM[25] and CVFM,[26] shown in Figure 3, with IC_{50} values against FPTase of 150 and 60 nM respectively, but these proved inactive against intact cells. A major breakthrough, however, occurred with the publication in the journal *Nature* of two papers back to back reporting benzodiazepine-based BZA-5B[27] and peptidomimetic L-731,734[28] (see structures in Figure 3) as inhibitors not only of Ras processing but also of *ras*-dependent cell growth in H-*ras*-transformed Rat1 cells, while being inactive against untransformed and *src*-transformed cells[27] or *raf*- and *mos*-transformed cells[28] respectively.

Subsequently, other peptide derivatives, like B581 from Eisai,[29] (imidazol-4-yl-ethyl)-Val-Tic-Met[30] from Bristol-Myers Squibb and FTI 277 from the group of Sebti,[31] have been described as displaying similar characteristics involving interference with Ras processing in H-*ras*-transformed NIH 3T3 cells, while displaying no effect on the processing of the geranylgeranylated protein Rap 1. Moreover, FTI 277 blocked the constitutive activation of mitogen activated protein kinase (MAPK) in H-*ras*-transformed NIH 3T3 cells at concentrations between 300 nM and 1 mM. Interestingly, concentrations 100 times higher were required to obtain the same degree of inhibition of MAPK in K-*ras4B*-transformed NIH 3T3 cells.[31]

Another milestone was the report of *in vivo* growth inhibition activity by L-739,749 (Figure 3), of *ras*-transfected Rat1 cells subcutaneously (s.c.) xenografted onto nude mice.[32] This compound, when injected intravenously (i.v.) at 20 mg/kg daily, from day 2 to 7 post-tumour implant, induced a 66% decrease in tumour weight 5 days after the last treatment. Interestingly, this compound had no effect on the growth of either *raf*- or *mos*- transfected Rat1 cells. Furthermore, while the "classical" anticancer agent doxorubicin, used at its maximal tolerated dose of 2 mg/kg induced only a 33% reduction in tumour weight, at the cost of systemic toxicity, including weight loss, anorexia and inactivity, L-739,749 did not induce any visible side-effects, and microscopic post-mortem examination of tissues like retina, bone marrow and gastrointestinal tract did not reveal any evidence of abnormalities.

More significantly, its isopropyl ester analogue L-739,832[33] showed impressive activity against spontaneous mammary and salivary carcinoma in H-*ras* transgenic mice. In this model, treatment was started later, when spontaneously occurring tumours reached a volume of 50 to 350 mm^3. In 7 out of 7 mice treated daily at a dose of 40 mg/kg i.v., no detectable tumour could be found within 2 weeks of treatment. Following cessation of treatment, tumour regrowth or growth at another site occurred in 5 out of 5 mice. However, retreatment at a dose of 40 mg/kg daily starting when the tumour reached a volume of 1200 mm^3 induced further regression of tumour size in 2 out of 3 mice within 2 to 6 weeks. In untreated animals, when tumours were treated at a late stage (having reached a volume of 1200 mm^3), 3 out of 5 mice responded within 10 days with a 50% decrease in tumour volume. In this study, doxorubicin, used at its maximal tolerated dose (2 mg/kg) actually only slowed tumour growth, inducing no regressions. No visible toxicity was detected after 11 weeks of treatment with L-739,832 at the dose of 40 mg/kg/day. Moreover histological examination of 28 organs did not reveal any clinical manifestation of toxicity. Systemic toxicity and some animal deaths were observed only at doses in excess of 100 mg/kg/day.

When administered at a dose of 50 mg/kg daily, another peptide, FTI-277 (Figure 3) from the laboratory of S. Sebti, selectively inhibited the growth of H-*ras*-NIH 3T3 cells by 50% over a 10 day period, while showing no inhibitory effect on *raf*-transformed NIH 3T3 cells. Similarly, the effect of FTI-277 on the growth of two human lung carcinoma, one presenting a K-*ras* mutation (Calu-1) and the other none (NCI-H810), xenografted onto nude mice, was studied.[34] Treatment involved a dose of 50 mg/kg daily and was started 36 days after s.c. implantation. FTI-277 after up to 14 days of treatment had no effect on the growth of NCI-H810, while the inhibitory effect on Calu-1 was spectacular, with growth inhibition being almost complete during the 32 days of treatment. In this study too, the authors stressed the absence of visible toxicity following 36 days of continuous treatment.[34]

Less impressive *in vivo* results have been reported for (*S**,*R**)-*N*-((2-(*N*-(2-amino-3-mercapopropyl)-L-tert-leucyl)-1,2,3,4-tetrahydro-3-isoquinolinyl)carbonyl)-L-methionine, another peptide derivative from the group at Bristol-Myers Squibb, using as a model H-*ras*-Rat1 cells injected intraperitoneally in athymic BALB/c mice. After 11 consecutive days of twice daily treatment at 45 mg/kg starting on day one post-tumour implant, a prolonged survival time of 54% (i.e. 18.5 instead of 12 days) over the controls was observed.[35]

Recently, it has been reported that the peptidomimetic B956 (Figure 3) from Eisai Company inhibited human tumours xenografts in nude mice, presenting *ras* mutations.[36] Interestingly, in the anchorage-independent *in vitro* cell growth assay, an IC_{50} value for B956 of between 0.2 and 1 μM was noted in cell lines with H-Ras mutations, while in cell lines harbouring an N-Ras mutation and in 50% of those

presenting K-Ras mutations, IC_{50} values ranged between 2 and 10 μM. With the other 50% of the K-Ras mutated cells together with those cells characterised by wild type Ras, the IC_{50} values for B956 were within the range of 10–100 μM. Similarly, *in vivo*, B956 induced complete inhibition of the growth of EJ-1 bladder tumours (mutated in H-Ras) at a dose of 100 mg/kg daily, with treatment starting on day 1 after s.c. tumour cell implantation, while at the same concentration it only slowed the growth of HT-1080 tumours (mutated in N-Ras) and had merely a marginal effect on the growth of HCT-116 tumours (mutated in K-Ras).

As stressed in a recent paper,[37] in the clinic K-Ras is the most frequently mutated member of the Ras family encountered. K-Ras is a substrate[38] for GGTase I and compounds designed selectively as inhibitors of the farnesylation of H-Ras, were generally less potent inhibitors, both *in vivo* and *in vitro*, of the growth of various cell lines displaying K-Ras mutations as compared to H-Ras-mutated cells.[36,37,39] Interestingly, it has been shown[37] that a CAAX peptidomimetic inhibitor of GGTase I, GGTI-287 (Figure 3) displayed selectivity for GGTase I (IC_{50}: 5 nM) as opposed to FPTase (IC_{50}: 25 nM) *in vitro*. Moreover, GGTI-286, the methyl ester of GGTI-287, was a more potent inhibitor of the processing of Rap1 and K-Ras than of H-Ras in cells, and markedly inhibited the activation of MAPK by K-Ras. Furthermore, FTI-277 (Figure 3), a potent and selective inhibitor of FPTase did not display these characteristics[37] but, unfortunately, no data on cell growth inhibition were reported.

Inhibitors of other Ras-processing Enzymes

Inhibitors of prenylated protein methyl transferase or Ras processing peptidase. The natural product dorrigocin has been shown to inhibit carboxyl methylation of Ras *in vitro*, and caused reversion of the morphology of *ras*-transformed NIH 3T3 cells.[40] Acetyl-farnesyl-cysteine[41] and its derivatives[42] inhibitors of prenylated protein methyl transferase (PPMTase), were shown to block the carboxyl methylation of Ras *in vitro*, but they failed to control the growth of *ras*-transformed cells. Inhibition of the growth of H-*ras* transformed Rat1 cells[43] was, however, reported with farnesyl thiosalicylic acid, an inhibitor of PPMTase,[44] with a K_i of 2.6 μM.

In addition, inhibitors of isoprenylated protein endoprotease,[45] and more recently, a dual peptidic inhibitor of both FPTase and peptidase,[46] have been described.

Inhibitors acting downstream of Ras. The plant flavonoid apigenin, an inhibitor of MAPK activity in cells, caused reversion of the morphology of *ras*-transformed NIH 3T3 cells *in vitro*.[47] Similarly, the natural product radicicol (UCS1006) inhibited MAPK activity in cells, induced reversion of their morphology and inhibited the growth of *ras*-transformed cells.[48]

The synthetic inhibitor PD 098059 (2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one) developed by Parke Davis inhibited MAPK-kinase with an IC_{50} of approximatively 10 μM, caused reversion of the phenotype and inhibited the growth of *ras*-transformed BALB 3T3 cells.[49]

Since it inhibited the growth of *ras-/raf-/mos*-transformed, or MAPKK mutant NIH 3T3 cells, compound SCH 51344 (6-methoxy-4(2-((2-hydroxyethoxyl)-ethyl)amino)-3-methyl-1M-pyrazolo(3,4-b)quinoline) appeared to target a yet unknown downstream element of the Ras signalling pathway.[50] The antibiotic azatyrosine, was also found to result in reversion of *ras-/raf-/erbB-2*-transformed, NIH 3T3 cells, and to inhibit the growth *in vitro* and *in vivo* of human pancreatic carcinoma PSN-1 cells, which express an activated K-*ras*.[51]

Proteins regulating Ras. Ras is active in its GTP-bound state, the GTPase-activating protein (GAP) activates the endogenous rate of GTP hydrolysis of Ras. In turn, GAP activity is inhibited by guanine-nucleotide-dissociation-inhibitors (GDIs).[52] Inhibitors of GDIs or compounds activating or mimicking GAP, like a peptidic analogue of the Src homology region 3 of GAP, could play a role in regulating Ras activity.[53] Interestingly, a recent paper[54] reported inhibitory effects on Ras-mediated activation of MAPK in cell extracts, by a peptide containing a consensus Ras binding sequence from Raf-1 and the GAP protein NF1.

Other inhibitors of Ras function. Farnesyl pyrophosphate, the source of the farnesyl residue required as a cosubstrate in the prenylation of Ras proteins catalysed by FPTase, is also a key intermediate in the biosynthesis of cholesterol, ubiquinone and dolichol. The cellular origin of FPP is under the control of the "mevalonate pathway": mevalonate is synthesized from HMG-CoA by the enzyme HMG-CoA reductase; a pyrophosphate group is then added to mevalonic acid which is then cleaved by mevalonate-pyrophosphate decarboxylase to isopentenyl pyrophosphate. Successive polymerization of these molecules leads to several isoprenoids including the 15-carbon FPP but also the 20-carbon geranyl-geranyl pyrophosphate (the substrate for GGPTase). Control of the mevalonate pathway has been widely and successfully exploited in the design of cholesterol-lowering drugs. A variation of this approach has been suggested for cancer chemotherapy.[55,56] This proposal was based initially on the observation that cells treated by compactin, a potent inhibitor of HMG-CoA reductase, would not grow in the absence of lipoproteins unless large amounts of mevalonate are supplied.[57,58]

In addition, lovastatin (another HMG-CoA reductase inhibitor) has been shown to inhibit isoprenoid biosynthesis, Ras farnesylation and membrane association[59–61] and to inhibit the growth of H-*ras*-BALB/c 3T3 cells in nude mice.[62] Moreover, lovastatin can also reverse the radio resistance of *ras*-transformed human osteosarcoma cells[63] and reverse tumour resistance to oxidative stress in the same model.[64]

The control of protein prenylation by HMG-CoA reductase inhibition has also been proposed[65,66] as the putative mechanism of action of lovastatin, simvastatin and fluvastatin as inhibitors of human vascular smooth muscle cell proliferation. In a phase I clinical study[67] of lovastatin involving 88 patients with solid tumours, one minor response (45% reduction in size) was observed in one patient with recurrent glioma out of 24 patients with brain tumours. The dose-limiting toxicity encountered was myopathy. Prophylaxis with ubiquinone reduced the severity, but not the incidence, of the musculoskeletal toxicity.

It is also noteworthy that phenylacetate, which blocks mevalonate-pyrophosphate decarboxylase, (the next step in the mevalonate pathway) has demonstrated anti-tumour activity in tissue culture[68] and in animal models.[69] Recent data indicate that phenylacetate can suppress the growth of various *ras*-transformed cells through interference with Ras isoprenylation,[70] and may function by down-regulating the apoptosis inhibitor Bcl-2.[71] In the clinic, limited efficacy of phenylacetate against refractory malignant glioma (1/7 patients) and a 50% decrease in prostate specific antigen level (1/9 patients) has been described. Dose limiting toxicity was a reversible central nervous system depression.[72]

More intriguing is the case of limonene, a major constituent of citrus oil, which blocks the isoprenylation of Ras proteins at a point in the mevalonic acid pathway distal to HMG-CoA-reductase, but without affecting cholesterol biosynthesis.[73]

Limonene and its metabolite perillyl alcohol have been reported to inhibit the growth of a chemically-induced breast carcinoma in mice.[74–76] At concentrations up to 10 mM, limonene is not an inhibitor of FPTase, but on the other hand, limonene metabolites such as perillyl alcohol and perillic acid methyl ester are weak inhibitors of FPTase with IC_{50} values of 1 mM and 50 μM, respectively.[77] However, as pointed out recently, the antitumour effects of limonene and its derivatives,[78] although probably related to Ras prenylation, remain controversial, since perillyl alcohol while reducing the total amount of Ras proteins expressed in certain cultured tumour cells, does not prevent prenylation.[79] These data reveal that the mechanisms underlying limonene activity are, unlike lovastatin, probably not related either to FPP depletion nor due to direct inhibition of *ras* farnesylation.[80] Limonene is now in phase I clinical trials, and seems at least to be devoid of toxicity at a dose of 100 mg/kg.[81]

In conclusion, lovastatin, phenylacetate and limonene have demonstrated anti-proliferative activity, especially in *ras*-transformed cells. Although more mechanistic studies remain to be carried out so as to elucidate their respective modes of action, their involvement in the *ras* transduction pathway is certainly of primary importance. It is noteworthy that these three compounds are currently evaluated as anti-cancer agents in clinical trials and that both phenylacetate and lovastatin show minor activity against glioma.

A number of miscellaneous type compound have been reported on in the literature recently. Tiazofurin, an inhibitor of IMP dehydrogenase, an enzyme of the GTP biosynthesis, has been shown to decrease the concentration of Ras-GTP in human leukemia K562 cells, probably via depletion of the intracellular GTP pool.[82] Fumagillin[83] inhibited the growth of *ras*-transformed HT1080 cells, while trichostatin A[84] caused the reversion of *ras*-transformed NIH 3T3 cells to normal morphology. In addition, Umezawa[85] reported that a new Vinca alkaloid, II-121C inhibited the growth of K-*ras*-NRK cells. However, it needs to be remembered that there is no evidence that the biological activity of any of these miscellaneous compounds is actually mediated through a direct inhibition of FPTase.

PERSPECTIVES

Until recently the main focus of research into inhibitors of the Ras signal transduction pathway has been on inhibitors of FPTase and, more specifically, inhibitors of the farnesylation of H-Ras, with great care being taken to show significant specificity for inhibition of FPTase relative to that of GGPTase I (Tables I and II). This emphasis was probably related to the fact that GGPTase I prenylates important GTP-binding proteins in the cell. Therefore, the most promising candidate as an inhibitor of the Ras-signalling pathway seemed to be the peptide derivative L-744,832, which showed impressive *in vivo* activity in H-*ras*-transformed transgenic mice.[33] Moreover, this latter compound also showed an inhibitory effect on the growth of some tumour lines which did not present a mutation in *ras* but were characterised by activated protein tyrosine kinase receptors, like epidermal growth factor receptor.[39] Therefore it was considered that such inhibitors of FPTase might be useful not only against tumours presenting an activated Ras, but also in correcting the effects of activated effectors upstream of Ras in the signal transduction pathway.

However, as stressed in a recent paper,[37] K-Ras is the most frequently mutated member of the Ras family encountered in the clinic, and K-Ras is a substrate for GGPTase I.[38] More worryingly, inhibitors designed selectively to inhibit the farnesylation of H-Ras, had littie effect on cell lines displaying K-Ras mutations.[36,37,39] Therefore, selecting for FPTase inhibitors without GGTase I activity could be considered detrimental in this respect. On the other hand, it is possible that targeting effectors downstream of Ras, like Raf or MAPK, could circumvent these problems and provide good inhibitory activity.

Therefore, in citing the importance of the specificity of inhibitors for FPTase versus GGPTase, the question of the models used to select for the inhibitors is also raised. The first step has consisted generally of an enzymatic assay based on the

farnesylation of H-Ras by a mammalian FPTase. The battle horse of screening for cellular activity *in vitro* has been the H-*ras*-transformed NIH 3T3 or Rat1 cell lines. Similarly, the first models used for determining *in vivo* antitumour activity consisted of nude mouse xenografts of H-*ras*-transformed NIH 3T3 cells. These models were invaluable for establishing proof of the principle that an anti-Ras strategy might be relevant in chemotherapy, however it is likely that a bias was introduced by selecting for highly specific and highly potent inhibitors of H-Ras prenylation and function. Since K-*ras* mutations seems to be more relevant in the clinic, cellular models based on K-*ras*-transformed cells or human cell lines harbouring *ras* mutation, and their xenografts in nude mice might be more predictive of efficacy in humans. A very attractive *in vivo* model actually is the transgenic mouse expressing human H-*ras* in spontaneously arising tumours. A transgenic mouse expressing K-*ras* mutations might even be more interesting, and closer to the problematic human pathology.

Another unanswered question is the identity of protein(s) whose processing, and therefore activity, is hindered by FPTase inhibitors. Beside Ras itself, Rho B is an interesting candidate. Rho and its counterpart Rac are related to Ras, and have been shown to control the shape of the actin cytoskeleton[86] and therefore cell morphology. One of the hallmarks of transformation is a change of morphology. Moreover, Rho[87,88] and Rac[89] activity is necessary for Ras transformation, and while Rho proteins are geranylgeranylated, Rho B is both geranylgeranylated and farnesylated.[90] The FPTase inhibitor L731,734 has been shown preferentially to inhibit the processing of Rho B over Ras.[91] Moreover, it has been reported that the effects of L-731,734 on cell morphology preceded those on cell growth.[92] It will also be informative to know whether all FPTase inhibitors inhibit the processing of the same subset of target proteins or whether indeed this is an area where some selectivity/specificity might be achieved or achievable.

An exciting feature of potential inhibitors of Ras function is their apparent selectivity and lack of reported cytotoxic effects *in vitro* and toxic side effects *in vivo*. Ras is part of a normal signalling pathway in all cells, and yet all the reports dealing with Ras inhibitors *in vitro* have shown a clear selectivity for inhibiting Ras processing and cellular growth in *ras*-transformed cells over *raf*- or *mos*-transformed and untransformed cells. This aspect might be explained[93] on the one hand, in transformed cells, by a dominant negative effect exerted by cytosolic oncogenic Ras accumulated upon treatment with FPTase inhibitors, over oncogenic Ras still associated to the plasma membrane. On the other hand, wild type cytosolic Ras would not exert such a dominant negative effect in untransformed cells. However, some of the substrates of FPTase such as rhodopsin kinase and transducin are involved in the visual signalling pathway, and the lamins A and B are involved in the control of nuclear membrane assembly. Still, there have not yet been any reports of major side effects or gross toxicities in mice treated with

any of these potential inhibitors, for periods up to and including 11 weeks of daily treatment.[33] However, the inhibitory effects of the various reported Ras inhibitors on tumour growth appeared dependent on continuous treatment. Therefore, successful treatment in the clinic might depend on continuous or at least a lengthy period of therapy, and the question of potentially adverse and long-term effects of treatment with inhibitors of the Ras signalling pathway obviously needs to be addressed.

Finally, Ras inhibitors are unconventional anticancer agents inasmuch as they are likely to exert cytostatic rather than cytotoxic effects on Ras-dependent tumours. Accordingly, the question of the end point to be used in assessing clinical studies is raised: should clinicians look for tumour regression, tumour stabilisation, increase in median survival time or quality of life?

The search for potent FPTase inhibitors has been built directly upon the results of fundamental research on Ras function and over recent years has been taken up by an increasing number of major pharmaceutical companies. Clearly, inhibitors of FPTase, and more broadly those of the Ras signal transduction pathway, are exciting molecules, both as research tools for dissecting the physiological role of the elements of this pathway, and as potential candidates for consideration as novel anticancer agents.

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