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Clinical report

Ras biochemistry and farnesyl transferase inhibitors: a literature survey

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Over the last decades, knowledge on the genetic defects involved in tumor formation and growth has increased rapidly. This has launched the development of novel anticancer agents, interfering with the proteins encoded by the identified mutated genes. One gene of particular interest is *ras*, which is found mutated at high frequency in a number of malignancies. The Ras protein is involved in signal transduction: it passes on stimuli from extracellular factors to the cell nucleus, thereby changing the expression of a number of growth regulating genes. Mutated Ras proteins remain longer in their active form than normal Ras proteins, resulting in an overstimulation of the proliferative pathway. In order to function, Ras proteins must undergo a series of post-translational modifications, the most important of which is farnesylation. Inhibition of Ras can be accomplished through inhibition of farnesyl transferase, the enzyme responsible for this modification. With this aim, a number of agents, designated farnesyl transferase inhibitors (FTIs), have been developed that possess antineoplastic activity. Several of them have recently entered clinical trials. Even though clinical testing is still at an early stage, antitumor activity has been observed. At the same time, knowledge on the biochemical mechanisms through which these drugs exert their activity is expanding. Apart from Ras, they also target other cellular proteins that require farnesylation to become activated, e.g. RhoB. Inhibition of the farnesylation of RhoB results in growth blockade of the exposed tumor cells as well as an increase in the rate of apoptosis. In conclusion, FTIs present a promising class of anticancer agents, acting through biochemical modulation of the tumor cells. [© 2001 Lippincott Williams & Wilkins.]

Key words: Farnesyl transferase inhibition, Ras, Rho, signal transduction,

Introduction

Cancer therapy remains a challenging issue. Despite immense advances in the field of basic and clinical research, which has resulted in higher cure rates for a number of malignancies, cancer remains one of the leading causes of death in the Western world.[1,2] Increasing knowledge on the biological complexities of cancer and the molecular genetic defects underlying tumorigenesis has provided new opportunities for rational anticancer drug discovery and development. By focusing on the primary genetic alterations in cancer cells there is a greater likelihood that the biological effects of drugs against these targets will be confined to the tumor cells rather than affecting both tumor and normal cell processes (biological specificity).[3] The therapeutic goal is to develop novel agents that will demonstrate greater efficacy and lower toxicity than can be achieved with currently available cytotoxic drugs.

The development of human cancer is thought to be the result of mutations in multiple genes that control

ATP, adenosine triphosphate; BZA, benzodiazepine, CAAX, amino acid motif where: C=cysteine, A=any aliphatic amino acid and X=serine or methionine; CDK, cyclin-dependent kinase; CVFM, amino acid motif where C=cysteine, V=valine, F=phenylalanine and M=methionine; EGF, epidermal growth factor; ERK, extracellular signal-related kinase; FAK, focal adhesion kinase, FGF, fibroblast growth factor; FPP, farnesyl pyrophosphate; FPTase, farnesyl protein transferase; FTI, farnesyl transferase inhibitor; GAP, GTPase activating protein; GDP, guanine diphosphate; GGPP geranylgeranyl pyrophosphate; GGPTase, geranylgeranyl protein transferase; GNRP, guanine nucleotide release protein; GTP, guanine triphosphate; HMG-CoA, hydroxymethylglutaryl coenzyme A; KNRK, K-*ras*-transformed normal rat kidney; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MMTV/N-, mouse mammary tumor expressing N-*ras*; NSCLC, non-small cell lung cancer; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI-3K, phosphatidyl-inositide-3-kinase; PTK, protein tyrosine kinase; Rb, retinoblastoma; RTK, receptor tyrosine kinase; SH2, Src-homology-2; SH3, Src-homology-3; VEGF, vascular endothelial growth factor.

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normal cell proliferation, differentiation and apoptosis. Genes that are often found mutated in malignancies are referred to as proto-oncogenes. Activation of these proto-oncogenes to oncogenes and/or the loss of function of tumor suppressor genes may cause deranged intracellular signaling.[2,4] The most commonly altered gene products in human solid tumors include ErbB2/Her (a membrane-associated tyrosine kinase), Ras (a GTP-binding protein) and nuclear proteins that affect transcription, such as Myc, Rb or p53.[3] The *ras* (an acronym for rat sarcoma, the source of the prototypic viral gene) gene encodes 21-kDa proteins, which play an important role in signal transduction. They pass on stimuli from external growth factors to the cell nucleus, thereby initiating gene transcription.[5] Ras mutations, which are found in approximately 25% of all human malignancies, lead to an increase in cell proliferation and tumor formation.[6,7] Through blocking the mutated Ras proteins, these processes can be reversed.[8] Several strategies with this aim have been identified, the most important of which is to inhibit the post-translational modification of Ras proteins. The first and crucial step of this post-translational modification is farnesylation, by the enzyme farnesyl transferase. Without being farnesylated, *ras* proteins cannot function.[9,10] Over the past decade, several farnesyl transferase inhibitors (FTIs) have been developed, the first of which are currently undergoing clinical evaluation. Also, knowledge of the exact biochemical mechanism by which these FTIs exert their antineoplastic activity is growing. Most likely, Ras proteins are not their sole target: other cellular proteins that are farnesylated may play important roles as well. In particular, the protein RhoB, involved in regulation of cytoskeletal actin organization, adhesion and proliferation, is a likely candidate.[11] In this review, we describe the Ras signal transduction pathways, which mediate cell proliferation and differentiation, the agents inhibiting Ras and the results of (pre)clinical studies with Ras inhibitors. These drugs can be classified as promising, mechanism-based, novel agents against cancer.

Ras signal transduction

The DNA in eukaryotic cells is protected by the surrounding nuclear envelope membranes, which place multiple physical barriers between the cell's genetic material and the external cellular environment. However, this restricts the ability of nuclear genes to sense changes in the cell's environment. Extracellular signals can influence the intracellular processes via three mechanisms: (i) through internalizing a receptor, (ii) through a receptor with a second messenger system, and (iii) through migration across the plasma membrane into the cytoplasm where the agent comes into contact with and binds to target proteins or receptor molecules. The process by which these signals are passed on from the cell membrane to the cell nucleus is called signal transduction. Cells respond to extracellular mitogenic signals during the first (G_1) phase of the cell cycle.[2,12–14] An important enzymatic process in signal transduction is phosphorylation, where phosphate groups from adenosine triphosphate (ATP) are transferred to the side chain of tyrosine, serine or threonine by protein kinases. It is believed that tyrosine phosphorylation is the most important initiation of growth signal transduction in multicellular organisms. Phosphorylation activates the accepting proteins by changing their conformation, thereby creating binding sites for other target proteins. Transmembrane signaling often involves receptor tyrosine kinases (RTKs), whereas protein tyrosine kinases (PTKs) are involved in intracellular signal transduction (reviewed in Pawson[13]). Enhanced tyrosine kinase activity resulting from overexpression can lead to specific diseases (e.g. cancer and other proliferative diseases, atherosclerosis, psoriasis), as can a decrease in biochemical function (e.g. a decrease in the function of the insulin RTK can lead to diabetes mellitus).[2] Cellular functions and target proteins of PTKs can vary widely, but their chemical structure usually contains multiple related sequences of 50–100 amino acids. These sequences are named SH2, SH3 and PH. The SH2 domain recognizes short peptide motifs containing phosphotyrosine (pTyr) which are explicitly involved in tyrosine kinase signaling pathways, activation of Ras-like GTPases, phospholipid metabolism, gene expression, protein trafficking and cytoskeletal architecture. In contrast, the SH3 domain recognizes short peptide motifs containing one or more proline residues which are found in proteins involved in tyrosine kinase signaling, cytoskeletal components and subunits of the neutrophil cytochrome oxidase. SH3-binding sites are pseudo-symmetrical and can bind in two orientations: class I ligand N- to C-terminal and class II C- to N-terminal. SH3 domains have binding sites for phosphorylation by proline-directed kinases, which are involved in the control of cell morphology. Less is known about the binding properties of PH domains. They occur in serine/threonine- and tyrosine-specific protein kinases, regulators of small GTPases, cytoskeletal proteins, phospholipase C isoforms, and GTPase dynamin. PH domains bind the β/γ subunits of heterotrimeric G proteins and protein kinase C, and they may be involved in pulling signaling proteins to cell mem-

branes.[13] Various combinations of SH2, SH3 and PH domains are frequently found in the same polypeptide, and they can all collaborate in forming signaling complexes downstream of PTKs. For example, the proteins Grb2 and Crk (so-called adapter molecules, transferring a signal from an activated receptor to a target protein, e.g. Ras) are composed almost entirely of SH2 and SH3 domains, which enables them to participate in complex protein–protein interactions. Proteins containing SH2, SH3 and/or PH domains can regulate many facets of the signaling process, such as signaling from the cell surface to the nucleus, protein trafficking and subcellular localization, control of cell architecture, cell–cell interactions, and cellular responses to infection.[2–4,13,15] Proteins involved in signal transduction are usually capable of activating a variety of target proteins and in turn can be activated themselves by multiple signals. For example, to transmit signals to the nucleus, the insulin-like growth factor receptor (IGFR-1) can utilize both the Ras protein or phospholipase C and protein kinase C as intermediates.[16]

Receptors

Several types of receptors are involved in Ras signal transduction, e.g. the integrins, which are localized at the cell surface. They are composed of membrane-spanning glycoproteins, which bind extracellular matrix proteins, complement components and cell-surface adhesive molecules. When extracellular ligands bind to integrin receptors, a signal is passed on through the cell membrane that ultimately leads to linkage and organization of cytoskeletal proteins at the cytoplasmatic surface.[17] An early event during integrin signaling is tyrosine phosphorylation of the enzyme focal adhesion kinase (FAK), in response to cell adhesion. FAK lacks SH2 and SH3 domains, and the mechanism by which integrins activate FAK is incompletely understood. Upon FAK phosphorylation, a cascade of other phosphorylation reactions is initiated resulting in protein interactions required for adhesion-dependent signaling complexes. Autophosphorylation of FAK results in the binding of Src, which creates binding sites for, for example, the Grb2–mSos complex and phosphatidylinositol-3-OH kinase (PI-3K).[17,18] Among others, the Ras/Mitogen-activated protein kinase (MAPK) pathway is stimulated through integrin signaling. Another class of receptors are the RTKs, that are characterized by an extracellular ligand-binding domain, a transmembrane spanning region and a cytoplasmatic tyrosine kinase domain. These domains can be parts of a single polypeptide. Ligands for these receptors include growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF). RTKs signal cells through tyrosine phosphorylation reactions. Some of the receptors, like EGFR, ERbB2/Her and PDGFR, are overexpressed in many tumors and/or are persistently activated by autocrine loops.[2,19–21] Binding of a growth factor to its receptor leads to intracellular dimerization and autophosphorylation, thereby creating specific phosphorylated tyrosine residues that serve as binding sites for downstream signal transducers. The autophosphorylation site recognizes the SH2 domain of specific target enzymes, which stimulates their activities. Some receptors have multiple autophosphorylation sites, each of which is specific for a particular target protein. Some of the affected targets may be redundant, especially at the level of Ras activation.[2,3,12,13,21] Stimulation of the Ras pathway has also been observed with several agents that bind to so called serpentine receptors, that have seven ('serpentine') transmembrane spanning regions. Agents binding to these receptors include thrombin, lysophosphatidic acid, bradykinin, and agents that act on the α_2-adrenergic and M_2-muscarine receptors.[22] Finally, cytokines such as the interleukins are capable of stimulating Ras via transmembrane receptors.[23–25]

Pathways upstream of Ras

Proteins with SH2 and SH3 domains mediate intracellular signaling by PTKs. The Ras pathway can be activated by a complex network (reviewed in Pawson;[13] Figure 1). First, the activated receptors, PDGFR and EGFR, bind Grb2 directly. These receptors, however, can also bind and phosphorylate SH2-containing proteins, such as Shc and Syp, which then bind and activate Grb2. Through this mechanism, PTKs without intrinsic Grb2-binding sites can activate Ras as well. Another function of this network might be to potentiate Ras activation. Second, the SH3 domains of Grb2 interact with a target protein, e.g. mSos1 and 2, or other guanine nucleotide exchange factors (GNRPs, which can activate Ras by stimulating Ras from its GDP state into its active GTP state). Among others, the proteins C3G and dynamin (a GTPase that may link Ras signaling to clathrin-mediated endocytosis) belong to the class of GNRPs. C3G binds the SH3 domain of the adapter protein Crk and this complex couples tyrosine phosphorylation to Ras activation. Crk can also bind mSos1, which increases the number of pathways activating Ras by tyrosine phosphorylation.[12,13,15] Binding of Grb2 to a phosphotyrosine of an activated receptor pulls along the Sos protein, which is then positioned in the membrane next to Ras. Sos

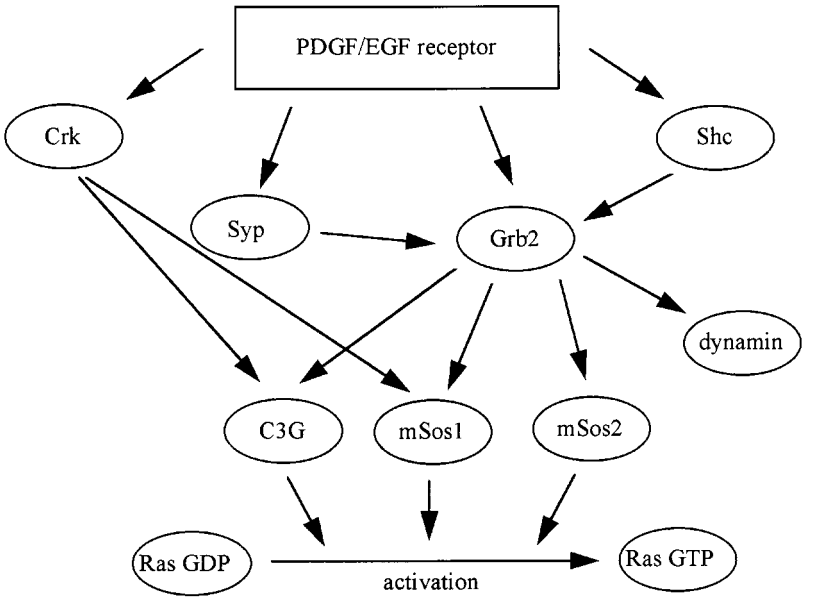


Figure 1. Signaling through the Ras pathway in mammalian cells. The activated receptors bind several SH2-containing proteins, e.g. Crk, Syp, Grb2 and Shc. Syp and Shc can activate Grb2 as well. Grb2 in turn activates mSos1 and 2, which stimulate Ras to exchange GDP for GTP. Crk can stimulate Ras activation independently of Grb2, through the protein C3G.

forms a complex with the Ras protein and helps it to exchange GDP for GTP. Ras becomes activated when it binds GTP and inactivated when it hydrolyzes GTP to GDP. Ras has intrinsic GTPase activity, which can be stimulated by RasGAPs. Hence, RasGAPs can limit the lifetime of activated Ras, thus functioning as a regulator of Ras activity.[2,3,13] Apart from the network just described, there may also be one or more Grb2-independent pathways to Sos.

Pathways downstream of Ras

Until now, the Ras/Raf pathway has been the best understood signal transduction pathway (reviewed in Khosravi and Der[5]). This pathway starts with the activation of Ras, when it exchanges GDP for GTP. This is followed by the activation of a cascade of serine/threonine kinases, which recruits cytoplasmatic Raf-1 serine/threonine kinase to the plasma membrane. Raf kinase phosphorylates MAPK kinase (MAPKK, also known as MEK) which in turn phosphorylates a third kinase, MAPK. MAPK is also known as ERK. MAPK is then translocated to the nucleus and directly activates transcription factors including the Elk1 nuclear transcription factor, which forms a complex with serum response factor and the serum response DNA element present in many promotors. Other stimulated transcription factors are Fos and Jun, which join to form AP-1, a full nuclear transcription factor. This factor binds to specific DNA sequences near the *myc* gene, thereby initiating its transcription. The *myc* gene product is a transcription factor as well, which can activate other genes. Ultimately, these signals converge to induce the expression and activity of the D-type cyclins. D-type cyclins are proteins that form complexes with the cyclin-dependent kinases CDK4 and CDK6. The formation of these complexes enforces G_1 progression into S phase, in part by phosphorylating the retinoblastoma gene product, Rb. Another cyclin, cyclin E, is also required for S-phase entry and its expression is rate limiting for progression to the G_1 phase.[12,21,26,27] Thus, the Ras/MAPK pathway is a critical link between receptor signaling and G_1 progression. However, the Ras/MAPK pathway is not the only route to G_1 progression: Ras interaction with Raf alone does not promote the activation of the kinase function of Raf, indicating that other signals are required to converge at this point of the pathway. Furthermore, Raf can be activated by Ras-independent mechanisms, e.g. by the Src tyrosine kinase and by protein kinase C. MAPK can also be activated independently of Ras by integrin-mediated activity. In brief, each signaling protein in the cascade may be activated by multiple upstream proteins and each component is likely to have additional targets.[21,28–30] Another important effector of the Ras pathway is the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$. This protein, which is induced by Raf, inhibits the activity of cdk2–cyclin E and cdk2–cyclin A complexes,[31] leading to a blockade of DNA

synthesis.[26,32] This feedback effect limits the ability of Ras (or Raf) to transform normal cells. The best known regulator of $p21^{Waf1/Cip1}$ is p53. This protein activates $p21^{Waf1/Cip1}$ when cells are exposed to DNA-damaging agents, allowing the cells time to fix the damage before entry into S phase. In cells expressing dominant-negative p53 or large T antigen, $p21^{Waf1/Cip1}$ induction is counteracted, and proliferation of the transformed cells is unhindered.[26] These processes are depicted in Figure 2. Another protein influencing the expression of $p21^{Waf1/Cip1}$ is Rho, which will be described in the next section. Oncogenic transformation by Ras depends on many physiological events, e.g. activation of PI-3K by Ras[33] and the suppression of cdk inhibitor proteins.[26] *In vitro* studies have demonstrated that activation of the Raf/MAPK pathway alone is not sufficient to cause transformation of cells. Hence, there must also be Raf-independent pathways. Up-regulation of an EGFR-dependent autocrine loop has been identified as one possibility.[20] Potential downstream targets of Ras additional to those from the Raf/MAPK pathway are given in Table 1. Some of them are Raf-independent. As yet, the exact contribution of most of these candidate effectors to Ras signal transduction and transformation remains to be determined.

In addition to the Ras/MAPK pathway, Ras transformation requires the cooperation of the Rho/Rac pathway (Figure 3).[50,51] Rho family proteins are regulators of the organization of the actin cytoskeleton and are involved in signaling pathways that activate kinase cascades, regulate gene expression, and control cell cycle progression and cell proliferation.[51–54] Rho family proteins are members of the Ras superfamily of small GTPases as is shown by their amino acid sequences, approximately 30% is identical to the Ras proteins.[55] The three best known Rho proteins are Cdc42, Rho and Rac. Cdc42 can stimulate Rac, which in turn will stimulate Rho. However, this linear model is probably an oversimplification of the actual signal transduction pathway, because evidence for multiple crosslinks exists as well. For example, Cdc42 can influence the activity of Rho without intercession of Rac.[56] Downstream targets include Rho kinase α (ROK), whose activation leads to actin reorganization, and p21-activated serine/threonine kinase (PAK), which is involved in disassembling stress fibers (reviewed in Lim *et al.*[57]). Hence, antagonism between the different effectors of the Rho/Rac pathway is possible. Proteins acting on phospholipids are downstream targets for Rho as well. Finally, Rac and Cdc42 can utilize MAPK to signal to the nucleus.[58,59] Rho is implicated in transcriptional regulation through stimulation of Src proteins[60] and through stimulation of the *fos* promotor.[61] In addition, Rac and Cdc42 can activate Jun N-terminal kinase (JNK), which can bind to the transcription factors c-Jun, Elk-1 and ATF-2.[58] This is a possible mechanism by which Rho proteins can play a role in oncogenic transformation. Another important downstream target for Rho is $p21^{Waf1/Cip1}$: Rho suppresses the induction of this protein, thereby allowing Ras to drive cells into the S phase (Figure 4).[62] $p21^{Waf1/Cip1}$ negative cells do not require Rho proteins for Ras-activated DNA synthesis, which stresses the importance of induction of this protein by Rho in Ras transformation. Ras is capable of activating the Rho/Rac pathway.[53] In general, Rho proteins pass on proliferative signals, probably by activating multiple pathways; the signaling cascade involved in cell proliferation is distinct from the one controlling stress fibre formation. However, there appear to be crosslinks between these pathways: the proliferation pathway can influence the pathway controlling stress fiber formation, by providing an, as-yet unidentified, signal that downregulates this stress fiber formation.[50,63] Rho proteins have been implicated in *ras* transformation based on the observation that some of the key features of this *ras* transformation are Rho-regulated processes such as alterations in the actin cytoskeleton.[63]

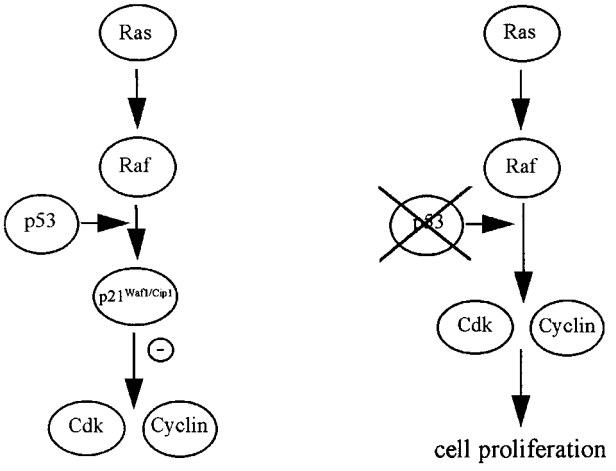


Figure 2. Signaling by cooperating oncogenes to regulate cycline/Cdk complexes. Ras stimulates cyclin/cdk activity through Raf. In cells expressing wild-type p53, excessive cell proliferation is counteracted by the inhibitor $p21^{Waf1/Cip1}$. In p53-negative cells, this inhibitory mechanism is absent.

Ras inactivation

Ras inactivation is tightly regulated. As yet, three proteins have been identified that inactivate Ras, by stimulating the hydrolysis of GTP: p120 GAP, neurofi-

bromin and GAP1m (collectively termed RasGAPs). p120 GAP possesses SH2, SH3 and PH domains, and can accelerate the intrinsic GTPase activity of Ras. Binding of Ras to p120 GAP exposes the SH2 and SH3 domains enabling p120 GAP to bind and activate other proteins. Hence, GAP also functions as a downstream effector of Ras.[38] Furthermore, p120 GAP is able to form complexes with the protein p190, which is a GAP for the Rho proteins.[64] As yet, the exact result of this bond is not known, but it indicates that the regulation of Ras and Rho activity is linked. The second RasGAP is neurofibromin, a 2818 amino acid protein encoded by the NF1 gene.[34,35] The affinity between Ras and neurofibromin is greater than between Ras and p120GAP, but the GTPase stimulatory activity is lower. Neurofibromin is expressed mainly in cells of the nervous system, especially neurons, Schwann cells and oligodendrocytes. As well as p120GAP, neurofibromin may also be an effector of Ras, involved in growth inhibitory signaling. Less is known about the specific functions of the third Ras inactivator, GAP1m, which contains a PH domain and a binding site specific for phosphatidylinositol 3,4,5-trisphosphate.[65,66]

Pharmacological implications

Cancer cells have amplified signaling pathways as compared to normal cells and hence interference with signal transducers will probably affect them more. In general, each tumor (type) has several activated oncogenes and mutated tumor suppressor genes. Furthermore, tumors are heterogeneous: different regions of the tumor may express different

Table 1. Potential downstream targets of Ras

Target	Full name	Function	Reference
PI3K	phosphatidylinositol-3-hydroxy kinase	suppression of Myc-induced apoptosis upstream activator of Ras	33
NF1	neurofibromatosis gene	growth inhibitor negative regulator or Ras	34–36
P120GAP	p120 GTPase-activating protein	accelerating intrinsic GTPase activity of Ras control of gene expression by Ras and of cell shape and adhesion	36–39
Bcl-2		suppression of apoptosis	40,41
RalGDS	Ral guanine nucleotide dissociation stimulator	positive regulation of Ras transformation	40,42,43
Rin-1	Ras interaction/interference gene 1	positive growth regulation	33
Byr2		regulation of agglutination, conjugation and sporulation	44,45
AF6		signal transduction at special cell–cell junctions	46
PKCδ	protein kinase C δ	positive regulation of Ras growth stimulation	40,42,43
Raf		activation of several transcription factors and their target genes	19,47–49
Rho family proteins		regulation of actin cytoskeletal organization	21,50
		critical factors in causing the invasiveness of tumor cells	
		regulation of gene expression	
		requirement for cell cycle progression through the G_1 phase	

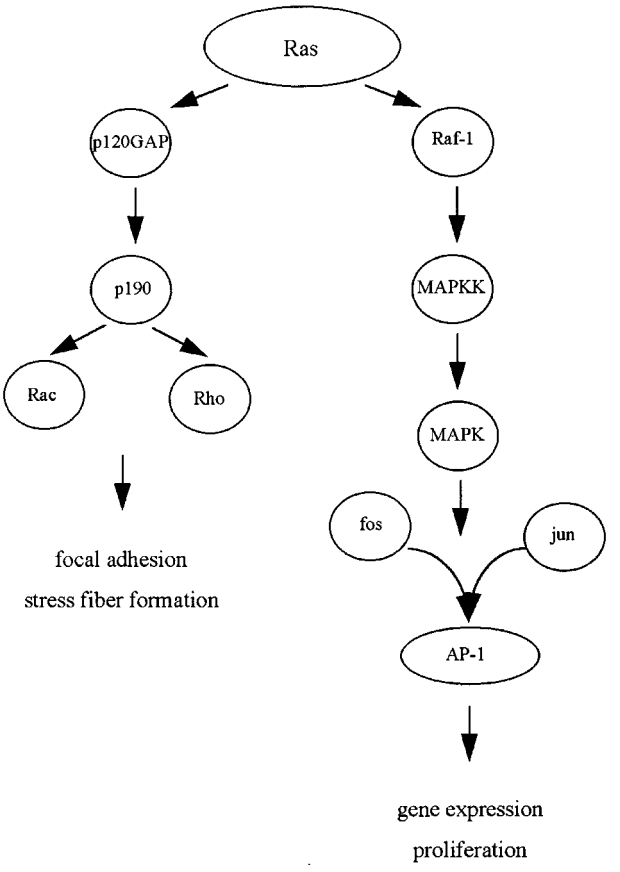


Figure 3. A two-pathway model for Ras signal transduction. Signal transduction from Ras resulting in gene expression is mediated by Raf-1 and the MAPK pathway. The Rho/Rac pathway, via p120GAP and p190, leads to focal adhesions and the formation of stress fibers.[50]

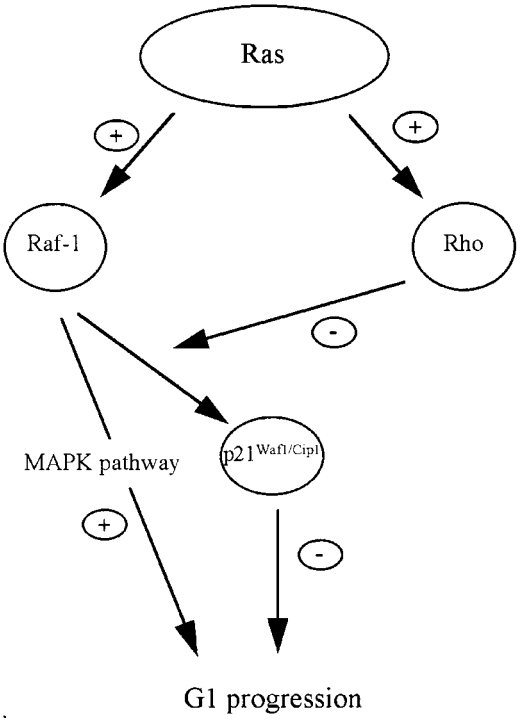


Figure 4. Rho influences Ras-driven cell proliferation. Rho decreases the induction of p21$^{Waf1/Cip1}$ (a cell cycle inhibitor) by the Ras–Raf pathway. Hence, through this mechanism, Rho acts as a stimulator of cell proliferation.

(proto-) oncogenes. The optimal treatment of a tumor will therefore most likely comprise a combination of therapeutic modalities, targeted at the different underlying (genetic) defects.[2] Most of the elements that play an important role in signal transduction are proteins that communicate with each other via specifically recognized amino acid sequences. Inhibition of signal transduction will often be directed against such protein–protein interactions. In the case of Ras, several options to block its function are possible: (i) restoration of normal GTPase activity of mutant Ras, to increase its inactivation, (ii) prevention of its post-translational modification which is required for Ras to become active, or (iii) inhibition of one or more of its downstream effectors, e.g. Raf and MAPKK or Rho and Rac. So far, the second option has been pursued the most, resulting in at least six different agents that are currently in clinical trials. The Ras protein and its inhibition will be described below.

Ras

ras genes are expressed in most human cells, with higher levels in immature cells and certain terminally differentiated cells including epithelial cells of endocrine glands and the neurons of the central nervous system. Three functional *ras* genes have been described: H-, K- and N-*ras*. K-*ras* consists of exon 4A and 4B splice variants. Mutations of these genes may be caused by physical or chemical carcinogens and are found in many different tumors, the majority of which are derived from epithelial cells. Overall, approximately 25% of tumors express a mutated form of *ras*, with the highest frequencies found in pancreatic, colorectal and lung cancer (Table 2).[67–69] The functional differences of the four isoforms are unknown.[6] Pre-malignant cells can also harbor *ras* mutations, which has been demonstrated, for example, in human colon polyps which showed no signs of malignancy yet.[73,74] N-*ras* has been assigned to the short arm of human chromosome 1 (1p22–p32), whereas H-*ras* and K-*ras* have been assigned to the short arms of chromosomes 11 (11p15.1–p15.5) and 12 (12p12.1–pter) respectively. The p21 coding sequences of each of the genes are equally distributed

Table 2. Occurrence (%) of mutated forms of *ras* in different human tumor types

Malignancy	H-*ras* (%)	N-*ras* (%)	K-*ras* (%)	Reference
Pancreatic			90	68
Colorectal			50	67
Lung (adenocarcinoma)			30	69
Bladder	12			70,71
Leukemia (acute myeloid)		25		72

in four exons, except for the K-*ras* gene which has two alternative fourth coding exons. The introns differ widely in size and sequence.[6] Ras mutations may remain silent for substantial periods of time. Activation would follow, for example, unscheduled proliferative events or additional genetic alterations.

ras oncogenes

Oncogenes are capable of evading or are less susceptible to negative regulators from the signal transduction pathways or from external stimuli, which leads to a dysregulation of cell growth.[28] For the *ras* genes, single point mutations suffice to induce malignant transformation.[75–77] In *in vitro* experiments, cells transfected with wild-type *ras* genes grew normally, whereas those transfected with mutated *ras* genes showed malignant overproliferation.[6] The mutation(s) in *ras* inhibits the ability of the protein to interact with GAPs, resulting in continuously activated proteins.[78,79] Overexpression of normal *ras* can lead to transformation as well,[80] but appears to be rarer compared with malignant transformation resulting from mutations.[81] Mutations in *ras* oncogenes have been localized in codons 12, 13, 59 and 61, with those at 12 and 61 occurring most frequently. Wild-type *ras* codon 12 is described as GGT. Substitution of the glycine (Gly) residue at position 12 or 61 by any other amino acid residue results in oncogenic activation. Furthermore, deletion of Gly12 or insertion of additional amino acids between alanine (Ala)11 and Gly12 produces a similar effect.[6] In non-small cell lung cancer, the predominant mutation is in position 1 of codon 12, in which guanine is replaced by thymine, whereas in colon cancer a guanine replaced by adenine in position 1 of that same codon appears to have the highest frequency.[69–73] In general, *ras* oncogenes exhibit two mutations, e.g. the H- and K-*ras* strains have replaced both Gly12 and Ala59 residues by arginine (Arg)12 and threonine (Thr)59 or serine (Ser)12 and Thr59, respectively.[82] The *ras* oncogenes are necessary for initiation and maintenance of the transformed phenotype, but cannot induce a metastatic phenotype. Furthermore, in preclinical experiments, *ras* genes can transform cells only when these cells are in a proliferative stage.[82] Several suppressor genes of *ras* have been identified, the loss of which may play a role in malignant transformation as well.[83,84] Mutations in *ras* genes do not necessarily lead to malignant transformation straight away. It is possible that *ras* oncogenes remain silent until certain unscheduled proliferative conditions (e.g. exposure to growth promoting agents such as cigarette smoke, viruses, etc.) trigger neoplastic development.[82]

The Ras protein

Ras proteins act as transducers of growth and differentiation signals from receptor tyrosine kinases to the cell nucleus. They consist of 188 (K-RasB) or 189 (all other types) amino acids. The first domain is a highly conserved region of 85 amino acid residues. In the next region of 80 residues, the structure of the different p21 Ras proteins diverges slightly and a highly variable region encompasses the rest of the molecule, except for the last four amino acids, where the sequence Cys186–A–A–X–COOH is present in all members of the Ras family.[6] The A and B forms of K-Ras differ solely in their C-terminal 25 amino acids as a consequence of alternate exon use[15] and K-RasB differs from H-Ras in the terminal 24 acids.[82] Ras proteins are synthesized in the cytoplasm on free ribosomes as pro-21 and have a half-life of at least 24 h.[85] Ras proteins must be farnesylated to the cysteine residue of the latter CAAX sequence (amino acid motif where C=cysteine, A=any aliphatic amino acid and X=serine or methionine) by the enzyme farnesyl protein transferase (FPTase) to function. This farnesylation is the first and critical step in the post-translational modification of Ras. Alternatively, some Ras proteins can be prenylated by geranylgeranyl-transferase (GGTase), particularly when FPTase is pharmacologically inhibited. The introduction of the isoprenoid lipid tail anchors the protein to the cell membrane where it can play its role in growth signal transduction.[9,10] Ras proteins cycle between an inactive GDP-bound and an active GTP-bound form. Wild-type Ras has intrinsic GTPase activity, which enables the protein to rapidly hydrolyze GTP, thereby returning to its inactive state. However, mutated Ras lacks the ability to cleave GTP[79] and remains trapped in its active form, the consequence of which is that the *ras*-driven cell proliferation is continuously (over)-activated. Normal GTP-Ras has a half-life of 1–5 h,

while the half-life of activated forms can be up to 9 times longer.[86,87] Expression of activated Ras influences expression of angiogenic factors such as vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) suggesting a role in angiogenesis for Ras.[88] Inhibition of Ras activity results in inhibition of proliferating tumor cells dependent on Ras, as well as interference with anti-apoptotic signals and angiogenesis.[89]

The Rho protein

The Rho family proteins are members of the Ras superfamily as well. They are small GTPase-binding proteins. To date, 11 mammalian Rho proteins have been identified: RhoA–E, Rho G, Rac 1 and 2, Cdc42, TC10 and TTF. The Rho proteins Rac and Cdc42 share about 50% homology in their amino acid sequence. Rho proteins are involved in the formation of stress fibers and focal adhesions, cell morphology, aggregation and motility, membrane ruffling, smooth muscle contraction, and cytokinesis (reviewed in Kaibuchi *et al.*[90]). Furthermore, they can regulate gene transcription through stimulation of the JNK/SAPK and p83/HOG1 MAP kinase cascades, and the transcription factors NFκB and SRF. Finally, Rho proteins can trigger G_1 progression.[91] Like Ras, Rho proteins cycle between an inactive GDP-bound and an active GTP-bound state. GDP-bound Rho in the cytosol is often also bound to a second molecule, GDP dissociation inhibitor (GDI), which inhibits its ability to exchange GDP for GTP. Thus, GDIs are negative regulators of Rho.[92] The inactivation of Rho proteins is also organised analogous to that of Ras: several RhoGAPs have been identified that stimulate the intrinsic GTPase activity of Rho, fascilitating their conversion to the inactive GDP-bound state.[90] With regard to Ras transformation and FTPase inhibition, RhoB is the most interesting protein of this family. It is a very short-lived protein, with a half-life of only 2 h.[93] *In vivo*, it exists in two populations that are either farnesylated or geranylgeranylated.[94] Both these types of RhoB are functional. The balance between the two is thought to be of importance for cell proliferation and apoptosis.[11] When cells are exposed to a FTI, the farneyslated fraction of RhoB will decrease rapidly, which is accompanied by an increase in geranylgeranylated RhoB. These changes result in a block of cell proliferation and promotion of apoptosis.[95]

Apoptosis

In vertebrate tissues, two distinct forms of cell death have been described. The first of these occurs when cells are exposed to non-physiological conditions, e.g. stress or toxins. The cell swells and is rapidly lysed. This process is called necrosis. When less acute injuries occur, cells can undergo apoptosis, also known as programmed cell death. Apoptosis is characterized by cell shrinkage and condensation of chromatin.[96,97] Apoptosis plays a role in various fundamental biological processes, such as the negative selection of autoreactive T cells in the thymus and in hematopoiesis. In mammalian cells, a number of genes have been identified that either induce or inhibit apoptosis, among which are many oncogenes or tumor suppressor genes. The best known regulator of apoptosis is p53. Like *ras*, this gene is often mutated in human solid tumors. The p53 gene is required for efficient execution of apoptosis induced by damaging agents such as radiation or chemotherapeutics. However, cells lacking p53 undergo apoptosis as well, but they require higher concentrations of the damaging agent.[98] Other examples of genes that influence programmed cell death are the adenovirus early region 1A (E1A) gene, the c-*myc* gene and the Bcl-2 gene family. In general, the tendency of a cell to undergo apoptosis depends on the expression or interaction of a number of 'death genes'.[96,97] The *ras* oncogene is implicated in resistance to apoptosis: mutated *ras* genes in human tumors act to prolong survival in situations that would normally lead to cell death,[99–101] and (forced) overexpression of the *ras* oncogene leads to an increased resistance to drug- and UV-induced apoptosis.[102,103] The inhibition of mutated Ras activity by FTIs has been shown to reactivate the apoptotic response.[104–106] Secondly, treatment of tumors expressing wild-type *ras* with FTIs increases the rate of apoptosis as well.[107] Interestingly, these types of apoptosis are largely p53 independent.[103,104] The mechanism by which the *ras* oncogene mediates resistance to apoptosis probably involves increasing the cells' ability to breakdown hydrogen peroxide, which results in a decrease in oxidative stress.[100] However, this hypothesis needs further investigation.

Protein prenylation

Protein prenylation is a step in the post-translational modification of a number of cellular proteins. It is defined as the stable covalent modification through thio-ether bonds to C-terminal cysteine residues of proteins with isoprenoids of two types: the C15 farnesyl or the C20 geranylgeranyl group. The isoprenyl groups stem from pyrophosphate intermediates of the cholesterol biosynthetic pathway. The modified cysteine is located in the fourth position from the C-terminus in every target protein.[108,109] Both Ras

and Rho proteins require prenylation as part of their posttranslational modification. Without this process, they cannot associate with the inner surface of the plasma membrane, where they exert their biological functions. Three consecutive post-translational processing steps have been described for proteins having a C-terminal C186AAX sequence which are (i) addition of either a 15-carbon (farnesyl, a product in the cholesterol biosynthesis pathway) or 20-carbon (geranylgeranyl) isoprenoid to the Cys residue, (ii) proteolytic cleavage of the AAX peptide by the proteolytic enzyme CAAX protease and (iii) methylation of the new C-terminal carboxylate by a specific prenylcysteine-dependent methyl transferase (Figure 5). All Ras proteins except K4B-Ras subsequently undergo a fourth step: addition of a palmitate lipid moiety to one or more cysteine residues upstream of the CAAX cysteine.[110,111] The first step (addition of the isoprenoid) is absolutely required for activation of Ras, whereas the second, third and possible fourth step are not.[10] Depletion of intracellular farnesyl by blocking HMG-CoA reductase (which prevents the synthesis of mevalonate, a precursor in the synthesis of farnesyl) results in the accumulation of non-processed cytosolic Ras.[112] A number of proteins besides Ras and Rho are known to be farnesylated, including nuclear lamins A and B, skeletal muscle phosphorylase kinase, several retinal proteins, Rac proteins, and others yet to be identified. Many of these proteins play vital roles in the cell: Rho and Ras in signal transduction, the nuclear lamins in maintaining nuclear structural integrity and cell growth, and the retinal proteins transducin and rhodopsine kinase in the visual signal transduction system. In addition to Ras, several of these farnesylated proteins posses oncogenic properties or participate in mitogenic signaling.[5,108,113,114] By blocking Ras with a FTI, some inhibition of all other prenylated proteins will occur as well, the extent at which will depend on their ability to become post-translationally modified through other pathways, e.g. prenylation by geranylgeranyl transferase. Hence, treatment with a FTI could lead to a number of potential side effects, e.g. ocular impairment.

Three enzymes have been described that catalyze protein prenylation: FPTase, GGPTase I and GGPTase II. The first enzyme uses farnesyl pyrophosphate (FPP) as the isoprenoid donor and the latter two geranylgeranyl diphosphate (GGPP).[109] FPTase and GGPTase I are α/β heterodimeric enzymes. The α subunit (RAM2) is identical in both enzymes and the β subunits share approximately 30% amino acid similarity.[115–118] The β subunit (in case of FPTase termed RAM1 and in case of GGPTase Cdc34) participates in the binding of both CAAX from the accepting protein and the prenylgroup from the isoprenoid donor.[119–121] The function of the α subunit has not been determined. There are different binding sites for the protein substrates and the reaction proceeds by a sequential mechanism. FPTase farnesylates CAAX-containing proteins that end with Ser, Met or Gln. Proteins ending with Ser (such as H-Ras) bind more weakly to FPTase than proteins containing Met as the final amino acid (K-RasB, lamin B).[122,123] In fact, the affinity of FPTase for K-RasB is more than 20-fold higher than for the other forms of Ras. GGPTase I geranylgeranylates CAAX-containing proteins ending with Leu. The three prenylating enzymes are highly selective for their respective substrates, but the substrate specificities are not absolute. When cells are treated with a FTI, cross-prenylation by GGPTase I can occur, thereby rescuing the function of some of the proteins requiring prenylation.[124,125] Recently, the crystal structure of

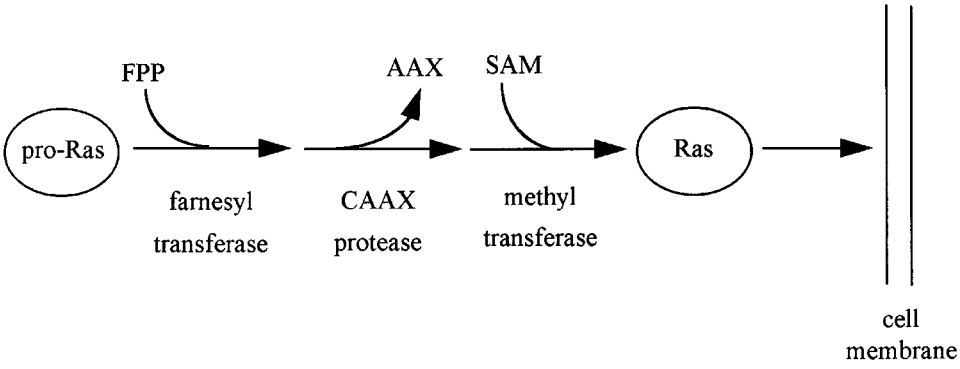


Figure 5. Post-translational modification of Ras. Ras is synthesized as an inactive pro-protein (pro-Ras). It undergoes a series of post-translational modifications that enable it to associate with the inner plasma membrane. The first step is addition of a farnesyl group to Cys186, catalyzed by the enzyme FPTase. Next, the AAX residue is cleaved off by CAAX protease and then the resulting terminal Cys186 is methylated by methyltransferase. In some Ras proteins a fourth step has been observed, involving addition of a palmitate lipid moiety to one or more cystein residues upstream of the CAAX box.

rat FPTase, which shares 97% sequence identity with the human enzyme, was determined. The active site comprises a bound Zn^{2+} ion, a pocket shaped binding site for FPP and a site where a peptide from an adjacent FPTase protein can bind.[126] FPTase requires divalent cations (Mg^{2+}, Zn^{2+}) as co-factors.[116] The farnesylation reaction can take place at a relatively wide pH range, with optimal activity between pH 6.5 and 8, and the rate of the reaction increases linearly with the concentration of substrate until it reaches saturation (Michaelis–Menten enzyme kinetics). The two substrates, FPP and the acceptor protein, bind the enzyme independently.[117,127]

GGPTase I adds a 20-carbon geranylgeranyl isoprenyl unit from its prenyl donor geranylgeranyl diphosphate, which is a product of the mevalonate biosynthetic pathway, to the cysteine residue of the CAAX motif in which X is leucine. The substrates of GGPTase I include RhoA, Rac-1 and the γ subunit of several heterotrimeric G proteins.[128] K-RasB, K-RasA and N-*ras* can be substrates for both FPTase and GGPTase I,[124] as can RhoB.[129] The third prenylating enzyme, GGPTase II or Rab geranylgeranyl transferase, catalyzes the geranylgeranylation of substrates that terminate in Cys–Cys or Cys–X–Cys sequences. Substrates for GGPTase II include several G proteins in the Rab family, but not Ras or Rho.[128] Geranylgeranylated proteins have multiple functions in the control of cellular transformation. Hence, blocking GGPTase I and/or II might exert antitumor effects, similar to those obtained by inhibition of FPTase.

FTIs

FTIs inhibit anchorage-dependent and anchorage-independent growth of a variety of transformed cells.[8,130–133] Anchorage-independent refers to growth in semisolid soft Agar matrix; the ability to grow in such a matrix is a phenotype unique to transformed cells. A survey of 42 cancer cell lines has shown that more than 70% of cells were sensitive to FTI treatment. Interestingly, the sensitive cells included both cell lines with Ras mutations and cell lines expressing the wild-type variant. Moreover, cells that overexpressed unmutated Ras were susceptible as well.[133] FTIs block farnesylation of Ras proteins, nuclear lamin A and B, skeletal muscle phosphorylase kinase, several retinal proteins (transducin, cGMP phosphodiesterase, rhodopsin), the peroxisomal protein Pxf, skeletal muscle phosphorylase kinase, the cell regulatory protein tyrosine phosphatases (PTPs) PTP-CAAX1 and PTP-CAAX2, Pex19p, and many other yet uncharacterized polypeptides. FPTase has a higher affinity for K-RasB than for H-Ras but K-RasB can be geranylgeranylated as well, especially in cells treated with a FPTase inhibitor.[134] The high affinity of K-RasB for FPTase and its ability to be a substrate for GGPTase I are due to the combination of a C-terminal methionine and a stretch of lysines that is located immediately upstream of the farnesylated cysteine.[135] N-Ras has a methionine at position X in the CAAX sequence as well and can also undergo cross-prenylation by GGPTase I, whereas H-Ras has a serine at position X and is only a substrate for FPTase. Geranylgeranylated K-Ras is at least partially functional in transforming cells[10,136] The consequences of possible cross-prenylation for the action of FTIs has not been fully elucidated. Cells with K-RasB mutations are susceptible to treatment with a FTI, but often require higher doses.[137] This suggests that FTIs might function through both *ras*-dependent and -independent mechanisms. One candidate of the latter category might be RhoB, which can also be both farnesylated and geranylgeranylated.[21] Treatment with a FTI blocks the farnesylation modification, resulting in an increase of geranylgeranylated RhoB. Loss of farnesylated RhoB limits the cell-transforming ability of Ras,[93] and the accumulation of geranylgeranylated RhoB appears to mediate growth inhibition and induce apoptosis.[95] Other proteins that might be involved are Rheb and PRL-1.[138–140] A *ras*-dependent mechanism probably involves effects on Raf: oncogenic unfarnesylated H-Ras acts as a dominant-negative inhibitor of Ras activity. This unfarnesylated Ras forms a stable complex with Raf and prevents the translocation of Raf from the cytoplasm to the membrane, thus inhibiting the Raf/MAPK pathway. Non-oncogenic Ras modified to prevent farnesylation does not interact with Raf.[141] In brief, the antineoplastic activity of FTIs may well be due to a combination of effects on multiple cellular proteins. The tumor specificity of FTIs will depend upon their ability to inhibit FPTase without inhibiting other proteinprenyl transferases. In general, FPTase inhibitors are 10- to 1000-fold more potent against farnesyl transferase than against geranylgeranyl protein transferases types I and II, which would enable drug treatment only resulting in the inhibition of FPTase.

Morphological effects of FTI treatment have been studied extensively: no immediate cell cycle block is produced, but rather a gradual decrease of proliferation over 5–7 days. Eventually, cells arrest with a multiphasic cell cycle distribution, including both G_1 and G_2/M phases.[142] In KNRK cells treated with a FTI, significant changes in microtubule networks were noted. More extensive networks appeared, but the synthesis of tubulins was not affected. Furthermore, an interference with cell cycle progression was demon-

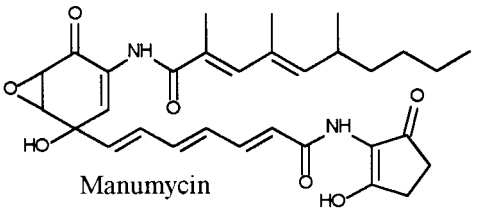
strated: treated cells had a lower percentage of cells in S phase and a higher percentage in G_1 when compared to untreated cells. This resulted in a reduction of doubling time.[101] In MMTV/N-*ras* mice with mammary carcinomas and lymphomas, treatment with a FTI also decreased mitotic activity, leading to accumulation of cells in the G_1 phase of the cell cycle.[106]

FTI classes

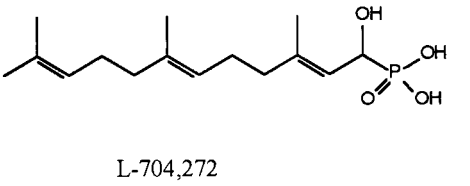
Currently known FPTase inhibitors can be subdivided into three broad categories based on their mechanism of action: compounds competitive with farnesyl pyrophosphate (FPP), compounds competitive with CAAX and bisubstrate analogs that combine features of both. This last class acts as inhibitors of the transition state of the farnesylation reaction. Some examples of compounds from these classes are given in Figure 6. Random screening of compound libraries has also resulted in the identification of FTIs, some of which are natural products. Of these FTIs, the exact mechanism of action is not always known.

An example of a FTI from the first category is L-704,272. It is a non-hydrolyzable analog of FPP, with an IC_{50} value of only 30 nM in H-*ras*-transformed cells.[131] Another compound of this class is the natural product manumycin. *In vitro*, it inhibited both human pancreatic cancer cell lines with mutant K-*ras* and human colon tumor cells, expressing wild-type *ras*, with IC_{50} values between 3.5 and 6.5 μM.[143,144] A possible disadvantage of using FPP analogs to inhibit FPTase is that other cellular enzymes utilizing FPP, such as squalene synthetase and farnesyl diphosphate synthetase, may be inhibited as well, the consequences of which need to be assessed. The second class of FTIs, those competitive with CAAX, comprises a large number of compounds. The first of these drugs were peptidomimetic agents resembling the terminal tetrapeptide in farnesylated proteins. Especially substitution of the second aliphatic amino acid by an aromatic residue converts proteins to non-substrate, competitive inhibitors.[145,146] Such substances are specific for FPTase, as compared with GGTPases. The first agent identified was the tetrapeptide CVFM, which showed strong FTI inhibition *in vitro*. However, the peptidic nature of this substance makes it very susceptible to proteolysis. Moreover, the negative charge on the C amino acid precludes cell permeability, hampering its applicability as a drug. Subsequently, several more stable compounds were developed. L-739,749 and L-744,832 were derived from the lead compound L-739,750. In these drugs the negatively charged C-terminus is masked by a methyl ester or an isopropylester, respectively. L-739,749 inhibits H-*ras* with an IC_{50} value of 0.1–1 μM and is capable of reverting H-*ras*-transformed cells back to normal.[8] L-744,832 demonstrated activity *in vitro* against a wide variety of human cell lines, including breast, colon, pancreas, lung, ovarian and hematopoeitic cancers.[133] In preclinical animal studies, several xenografts were susceptible, as were transgenic (modified to express mutated *ras*) murine tumors. Again, tumors expressing both wild-type as well as mutated or overexpressed Ras were sensitive to treatment.[104,147–149] Other interesting compounds are FTI-276 and FTI-277. In these molecules, both aliphatic amino acids from the CAAX motif are replaced. FTI-276 displayed significant antitumor activity against both xenograft and carcinogen-induced lung cancers in mice, with very little toxicity to the animals.[150,151] FTI-277 inhibited farnesylation of all forms of mutated Ras-proteins *in vitro*, but K-*ras* required 10-fold higher concentrations than H-*ras* (0.3–1.0 μM for H-Ras and 10–30 μM for K-Ras).[152] In B1086, an alkaline part replaces the two aliphatic amino acids of the CAAX motif, also yielding a more stable molecule. B1086 has shown efficacy against human fibrosarcoma, bladder and colon tumor xenografts.[153] Interestingly, the antitumor effect correlated to the degree of inhibition of *ras*-post-translational modification. Derivatives of the benzodiazepines (BZAs) can also inhibit FPTase, e.g. the compound BZA-5B.[154] The CAAX competitive FTIs thus far described all had one disadvantage: their peptidomimetic structure hinders the development of oral formulations. Because FTIs exert their antineoplastic action through interference with signal transduction, which is a continuous process, the best results of treatment can be expected from prolonged administration.[113] Hence, a convenient oral dosage form is required. To pursue this goal, several non-peptidomimetic FTIs, also competing for CAAX, have been developed. Examples include the tricyclic drugs SCH44342, SCH54429, SCH59228 and SCH66336, derivatives of an anti-histamine lead compound obtained from a compound library. These agents show very good selectivity against GGPTase I. They are very potent, inhibiting human FPTase with IC_{50}s as low as 1.9 nM. SCH66336 has been studied most extensively. *In vitro*, it blocks the growth of human neoplastic cell lines with K-*ras* mutations as well as other *ras* mutations or wild-type *ras*.[155] A phase I clinical trial has been completed with this agent as well, which will be discussed below. Another important non-peptidomimetic FTI is R115777. This compound was initially developed as an anti-fungal agent, but showed high FTI activity, based on competition for FPTase with the accepting proteins. In a panel of human tumor lines, overall sensitivity of 80% was demonstrated and

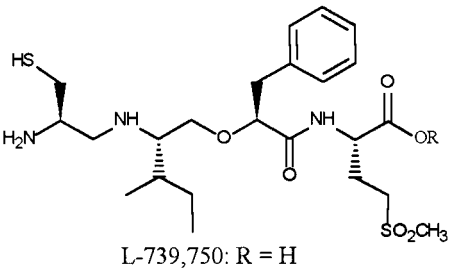
(1) Natural componds

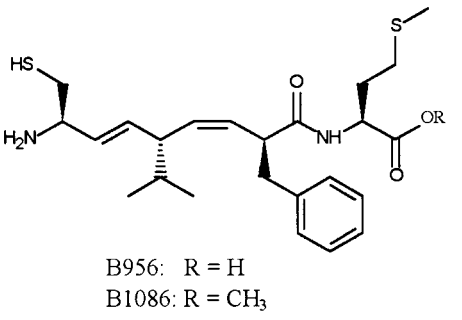


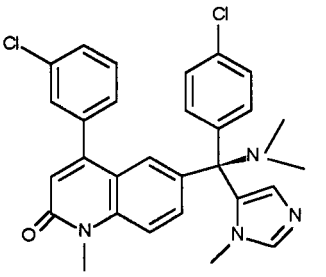
(2) Compounds competitive with FPP

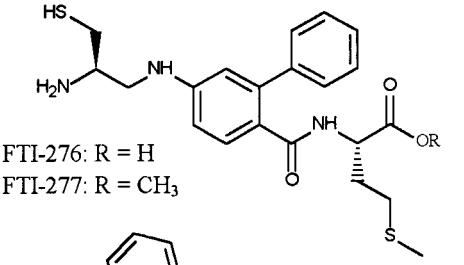


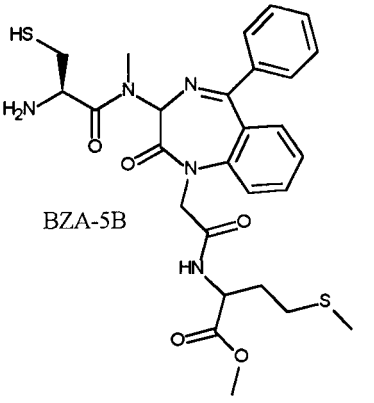
(3) Compounds competitive with CAAX











(4) Bisubstrate analogs

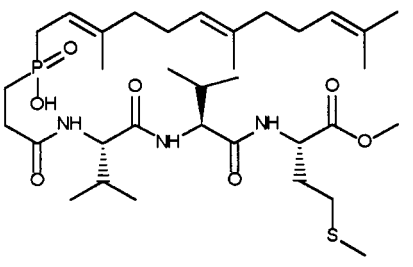


Figure 6. Representative agents from different classes of FTIs.

complete growth inhibition could be attained at concentrations below 120 nM.[156] In animal studies, several xenografts responded, including lung and pancreatic tumors. The toxicity profile was favorable.[157] R115777 has undergone several early clinical trials. The last class of FTIs are the bisubstrate analogs, which consist of a FPP analog linked to a CAAX analog. The bisubstrate compound BMS186511 inhibited Ras signaling and growth in K-*ras*B-transformed cells at concentrations of only 0.1 μM.[158] It also inhibited a neurofibromin-deficient cell line, ST88-14,[159] which holds promise for the possible treatment of patients with neurofibromatosis.

FPTase inhibitors not belonging to the three classes described above include agents that act on the mevalonate pathway and microbial products. FPP is an intermediate in the mevalonate pathway: it is converted into squalene which is the precursor of cholesterol. Like cholesterol, limonene is an end-product of this pathway and it has been shown to inhibit Ras processing.[160] It demonstrated both chemopreventative as well as chemotherapeutic activity in rodent tumors.[161] Other compounds derived from the mevalonate pathway that interfere with Ras include diallyl disulfide, compactin and lovastatin. They act by inhibiting FPP synthesis.[162,163] An example of a microbial FPTase inhibiting drug is oreganic acid, produced by an endophytic fungus isolated from the leaves of *Berberis oregana*. It has an IC_{50} of 14 nM on purified FPTase.[164]

Preclinical studies

FPTase inhibitors have demonstrated antitumor efficacy in a large number of animal studies. Initial experiments were performed using nude mice with transplanted rodent tumors that had demonstrated good sensitivity to FTIs in cell culture. As was observed in the *in vitro* studies, tumors arising from *ras*-transformed cells were inhibited, whereas tumors arising from cells transformed by *raf* showed resistance to FTI treatment.[165] Human tumor xenografts showed sensitivity to FTIs as well. Examples are studies performed with L-739,749, FTI-276, R115777 and SCH66336. The first, L-739,749, demonstrated activity against tumors bearing H-, K- and N-*ras*, but not against tumors with wild-type *ras*. It was also active against a transplanted tumor that expressed mutated p53 and *myc* in addition to mutated *ras*.[130] The FTI-276 was active against a lung cancer xenograft, and the tumor response correlated with the inhibition of *ras* processing in the tumors. This suggests that the observed efficacy was, at least in part, mechanism based.[150,153] R115777 administered orally inhibited the growth of transplanted H-*ras*-transformed NIH 3T3 cells by 56–86% at doses ranging from 6.25 to 25 mg/kg twice daily. Toxicity to the animals in this study was minimal.[166] SCH66336, finally, was capable of inhibiting the growth of a wide variety of human xenografts with and without *ras* mutations, including tumors derived from the colon, lung, pancreas, prostate and bladder.[167]

The most remarkable results of FTI treatment have been achieved in experiments with transgenic mice. In MMTV mice, which spontaneously develop salivary and mammary gland carcinomas, complete regressions were noted upon treatment with L-744,832, with response rates up to 100%, depending on the tumor size. The reference treatment with doxorubicin resulted in complete regression in 18% of the animals.[147] Transgenic mice bearing tumors with both mutated *ras* and mutated p53 responded to L-744,832 treatment as well.[104] Also, prophylactic treatment was effective: in MMTV mice treated with SCH66336, tumor onset was delayed, and the number and weight of the formed tumors was reduced.[167] Histologic examination of the treated animals showed negligible toxicity in these studies.

Clinical studies

As yet, at least six agents have been tested in phase I clinical trials: R115777, L-778,123, L-744,832, BMS-214662, SCH66336 and FTI-277. Of four of these agents, (preliminary) trial results are available. Table 3 outlines the current stage of clinical testing for each of them. Two phase I studies with R115777 have been completed which included pharmacokinetic sampling.[168,169] Dose-limiting toxicities were myelosuppression (neutropenia, CTC grade 3–4 and leukopenia, CTC grade 3–4) in the continuous treatment schedule, and neuropathy (CTC grade 3) and fatigue (CTC grade 2) in the intermittent schedule. Less severe side effects included gastrointestinal disturbances and headache. Because several proteins involved in retinal signal transduction are farnesylated, all patients underwent ophthalmic examination before study entry and regularly while on treatment. In the trial with continuous dosing, one patient showed a decrease of electroretinography values after 1 month of treatment, which normalized upon dose reduction. In the trial where R115777 was administered in cycles, two patients developed small unilateral visual field defects while on therapy. However, both patients remained asymptomatic. The maximum tolerated dose was established at 500 mg b.i.d. when given in cycles and at 300 mg b.i.d. when given continuously. Even though the primary goal of these trials was not to

Table 3. Phase I clinical trials with FTIs

Drug	Trials	Patients	Route	Schedule	MTD	Responses	Reference
R11577	monotherapy	solid tumors	oral	chronic b.i.d.	300 mg b.i.d.	1 PR in NSCLC	168
	monotherapy	solid tumors		5 days b.i.d., 10 days rest	500 mg b.i.d.	none	169
	monotherapy	advanced leukemia		chronic b.i.d.	not reached	PR in 6 patients	170
	with gemcitabine	not specified		R115777 chronic b.i.d., gemcitabine days 1, 8 and 15 Q 28 days	200 mg b.i.d.	none	171
L-778,123	monotherapy	solid tumors	i.v.	(a) 2-week continuous, 1 week rest and (b) 4-week continuous, 1 week rest	(a) 840 mg/m^2/day and (b) not reached	none none	172
	with radiotherapy	pancreatic, head & neck, NSCLC		1-week continuous 280 mg/m^2/day in weeks 1, 2, 4 and 5 of 7-week RT regimen	not reached	2 CR in head & neck, 2 PR in NSCLC	173
	with paclitaxel	not specified		1-week continuous 280 mg/m^2/day paclitaxel on day 1	280 mg/m^2/day	none	174
BMS-214662	monotherapy	solid tumors	i.v./oral	1-h infusion Q 27 days, oral single dose at course 2	not reached	1 MR in NSCLC	175
SCH66336	monotherapy	solid tumors	oral	7 days b.i.d., 2 weeks rest	350 mg b.i.d.	1 PR in NSCLC	176
	with gemcitabine	solid tumors		SCH66336 chronic b.i.d., gemcitabine days 1, 8 and 15 Q 28 days	100–150 mg (gemcitabine 1000 mg/m^2)	2 PR in pancreas, 2 MR in pancreas and mesothelioma	177
	with paclitaxel	solid tumors		SCH66336 b.i.d. (chronic or intermittent not specified)	not reached	4 PR in NSCLC and salivary gland	178
L-744,832	not published						
FTI-277	not published						

NSCLC, non-small cell lung cancer; CR, complete response; PR, partial response; MR, minor response.

evaluate antitumor efficacy, responses have been observed: one patient with an advanced, platinum-refractory non-small cell lung cancer had a partial response and several patients, with colorectal, pancreatic and cervix carcinomas, demonstrated stable disease. Pharmacokinetic studies in these trials suggested dose linearity for R115777 kinetics. Peak plasma concentrations were reached within 0.5–4 h, and elimination occurred in a biphasic mode, with sequential half-lives of about 5 and 16 h. Steady-state concentrations were reached quickly, within 2–3 days.[168,169] The other FTI for which clinical trial results have been published is SCH66336.[176] The dosing schedule used in this phase 1 trial was intermittent. Main side effects included nausea, vomiting, diarrhea and fatigue, whereas hematologic toxicity was mild. The maximum tolerated dose was established at 400 mg b.i.d. (when administered for 7 days out of every 3 weeks). One patient with metastatic NSCLC had a partial response. Interestingly, this trial also investigated the biological effectiveness of SCH66336, by measuring the farnesylation state of the protein lamin A in buccal mucosa cells. Accumulation of unfarnesylated prelamin A was detected in patients upon treatment, the amount of which appeared to increase with increasing doses of the FTI. This provided the first clinical evidence of successful inhibition of FPTase by a FTI.[176] Phase II and III trials of these agents are underway, as well as several phase I and II trials that investigated combinational therapy of FTIs with classical cytotoxic agents (Table 3).[179] Synergy with existing cytotoxic agents may well be expected from preclinical experiments. FTIs have been reported to sensitize malignant cells to cell death by irradiation or treatment with paclitaxel.[142,180] Secondly, one could anticipate that FTIs will enhance the action of DNA-damaging agents, because RhoB plays a role in DNA repair. Since FTIs block prenylation and hence activation of RhoB, DNA repair could be impaired.[89] Thirdly, combination therapy with angiogenesis inhibitors could be beneficial, because several angiogenic factors (FGF and VEGF) bind tyrosine kinase receptors, the signals of which are transduced through the Ras pathway.[108] Oncogenic Ras is known to upregulate these factors.[88,181] In fact, a reduction of VEGF upon FTI exposure has recently been demonstrated in two *in vitro* studies.[107,182]

Resistance to FTIs

As yet, intrinsic resistance of tumor cells for FTIs has been described in preclinical studies. This resistance is often but not always related to the type of *ras* mutation present, being most frequent in K-*ras*-transformed cells. However, less is known about acquired resistance. In the MMTV-v-H-*ras* oncomouse model, daily administration of L-744,832 was required to prevent reappearance of the regressed tumor. When drug treatment was discontinued, some of the reappearing tumors were unresponsive to renewed treatment with the FTI.[147] Also in a cell culture model, resistance could be induced by sustained exposition of the cells to the FTI L-739,749. The mechanism underlying this acquired resistance is P-glycoprotein-independent.[183] These results suggest that prolonged administration of FTIs may be required to elicit sustained tumor responses. It is also possible that FTI-resistant tumors may arise in some patients. Interestingly, normal cells are not inhibited by FTIs. The exact mechanism underlying this selectivity has not been unravelled yet, but several observations provide important clues. First, some proteins can be geranylgeranylated when a FTI is present, which might rescue their functioning.[124,125] Secondly, the sensitivity of farnesylated proteins can differ, e.g. much higher concentrations of the FTI BZA-5B were required to inhibit the farnesylation of the nuclear protein lamin A than to inhibit H-*ras*, suggesting the possibility to dose this agent such that vital proteins will still function.[184] Thirdly, normal cells might possess redundant pathways that compensate for the loss of farnesylated proteins.[185] This last mechanism could also explain the resistance to FTIs observed in some cell lines. Further studies investigating the frequency at which resistance occurs as well as determining the underlying mechanism are warranted.

Conclusions

Human cancer develops as a result of mutations in multiple genes that control normal cell proliferation, differentiation and apoptosis. Increased understanding of the mechanism by which mutated genes confer a neoplastic phenotype on cells can result in novel, mechanism-based, anticancer therapeutic strategies. In this perspective, the *ras* proto-oncogene has attracted much attention, for it is one of the genes most commonly found mutated in human tumors. Three *ras* oncogenes have been identified: K-, H- and N-*ras*. Ras mutations lead to an increase in cell proliferation and tumor formation. Furthermore, activation of mutated *ras* is probably associated with resistance to apoptosis. Through blocking the mutated *ras* gene product, these processes can be reversed. With this aim many different FTIs have been developed, that block farnesylation of Ras, reverse Ras-mediated cell transformation in human cell lines and inhibit the growth of

human tumor cells in nude mouse. However, Ras is not the only proposed mediator of the biological effects of these compounds and inhibition of farnesylation of other proteins, such as RhoB, may very well contribute to, or be even more important for the change in the level of tumor apoptosis and cell cycle parameters observed following treatment with a FTI. Preclinical experiments involving tumor xenograft as well as transgenic mouse models demonstrated considerable antitumor activity for a number of FTIs. Interestingly, both tumors with and without mutated *ras* seem to respond to treatment. Currently, there are at least six FTIs in phase I trials with several others reported to be in preparation for clinical studies. Preliminary results indicate possible antitumor activity in patients with pancreatic, colorectal, NSCLC and breast cancer, with relatively little reported side effects.

In summary, based on the novel mechanism of action, preclinical anti tumor activity, manageable toxicity and encouraging activity in early clinical studies, further clinical development of FTIs as a single agent as well as in combination with chemotherapy and radiotherapy should be pursued with great effort.

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