Isoprenylation of Intracellular Proteins as a New Target for the Therapy of Human Neoplasms: Preclinical and Clinical Implications

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Abstract: Cell proliferation, differentiation, and survival are regulated by a number of extracellular hormones, growth factors, and cytokines in complex organisms. The transduction of the signals by these factors from the outside to the nucleus often requires the presence of small intracellular proteins (i.e. ras and other small G proteins) that are linked to the plasma membrane through a isoprenyl residue that functions as hydrophobic anchor. Isoprenylation is a complex process regulated by different enzymatic steps that could represent potential molecular targets for anti-cancer strategies. In the present paper the different transduction pathways regulated by some isoprenylated proteins such as ras and other small G proteins are described. Moreover, the molecular mechanisms of the isoprenylation process and the mode of action of the different isoprenylation inhibitors are discussed with attention to statins, farnesyltransferase inhibitors (FTI) and aminobisphosphonates. The role of different candidate targets in the determination of anti-tumour effects by FTIs is also described in order to define potential molecular markers predictor of clinical response. On the basis of several preclinical data, new strategies based on multi-step enzyme inhibition or on target prioritization are proposed in order to enhance the anti-tumour activity of agents inhibiting isoprenylation. Finally, a summary of the principal data on clinical trials based on the use of FTIs and statins is given. In conclusion, the inhibition of isoprenylation is an attractive, but still not completely investigated therapeutic alternative that requires optimization for the translation in the current treatment of neoplasms.

Key Words: Isoprenylation, farnesyltransferases, geranylgeranyltransferases, farnesyltransferase inhibitors, aminobisphosphonates, statins, ras, small G proteins.

INTRODUCTION

Cell proliferation, differentiation, and survival are regulated by a number of extracellular hormones, growth factors, and cytokines in complex organisms. These molecules serve as ligands for cellular receptors and communicate with the nucleus of the cell through a network of intracellular signaling pathways. In cancer cells, dysregulated cell signaling and proliferation may occur through overexpression or mutation of proto-oncogenes. One such proto-oncogene is ras, which functions as a molecular switch in a large network of signaling pathways, mainly controlling the differentiation or proliferation of cells. Isoprenylation process is essential for the activation of ras and of a series of ras-related proteins (other small GTP-binding proteins – G proteins) that are involved in the transduction of anti-apoptotic and proliferative signals. In fact, these molecules require the addition of a lipidic residue (isoprenyl residue) for the localization on the inner side of the plasma membrane where they can interact for co-localization with peptide growth factor and/or G coupled receptors.

RAS AND RAS-RELATED SMALL G PROTEINS: STRUCTURE AND FUNCTIONS

Oncogenic Ras Proteins

In addition to R-ras and M-ras genes, there are three potentially oncogenic ras genes in human cells, which encode four highly related proteins H-ras, N-ras, and K-ras (K4A- and K4B-) [1]. The 21-kd transforming proteins of the Harvey (H) and Kirsten (K) murine sarcoma viruses, called v-H-ras and v-K-ras, are oncogenic mutants of normal cellular ras (c-H-ras and c-K-ras). So far, the neuroblastoma (N)-ras has not been found in any retrovirus. The K-ras gene is alternatively spliced, resulting in two protein isoforms, K-rasA and K-rasB. After farnesylation, membrane anchorage of K-rasA occurs through palmitoyl moieties, whereas that of K-rasB occurs through lysine residues.

Ras is a membrane-bound guanosine triphosphate (GTP)/guanosine diphosphate (GDP)-binding (G) protein that serves as a “molecular switch,” converting signals from the cell membrane to the nucleus. These chemical signals lead to protein synthesis and regulation of cell survival, proliferation, and differentiation. However, unlike the classic heterotrimeric G proteins, Ras exists as a monomer. Each Ras protein consists of about 190-amino-acid residues that are highly conserved in the N and C termini. Most of the differences between these proteins occur in the near C-terminal hypervariable domain of about 25 amino acids,
which is presumed to be responsible for their different functions. There is a close structural and sequence homology between the monomeric Ras family G proteins and \( \alpha \)-subunits of the classic trimeric G proteins [2, 3]. Ras mutations in human cancers have been comprehensively reviewed [4]. The role of ras genes in inducing malignant transformation is supported by several lines of evidence. First, oncogenic ras but not normal ras transfected into rodent fibroblasts renders them tumorigenic [5]. Second, transgenic mice harboring oncogenic ras mutations have an increased incidence of tumor formation [6]. Finally, a high frequency of ras mutations has been found in a variety of tumor types, both naturally occurring and experimentally induced. Identified mutations are limited to a very small number of sites (amino acids 12, 13, 59, and 61), all of which abolish GAP-induced GTP hydrolysis of the Ras proteins. Such single-point mutations of the ras gene can lead to constitutive activation of Ras protein. These mutated forms of Ras have impaired GTPase activity. Although they still bind GAP, there is no “off” sign, since GTPase is no longer activated. This results in continuous stimulation of cellular proliferation. Mutations are frequently limited to only one of the ras genes, and frequency is dependent on tissue and tumor type. Thus, ras gene mutations are rare in cancers of the breast, ovary, stomach, esophagus, and prostate; however, they are present in almost all adenocarcinomas of the pancreas and in 50% of colon and thyroid cancers. Mutations in colon and pancreatic cancers are found only in the K-ras gene. In cancers of the urinary tract and bladder, mutations are primarily in the H-ras gene; mutations are in the N-ras gene in leukemia. Thyroid carcinomas are unique in having mutations in all three ras genes [7–9]. Overall, approximately 30% of all human neoplasms harbor a mutation in a ras gene. Mutations most frequently occur in K-ras and least often in H-ras. A critical experiment that underscored the importance of oncogenic ras in mammalian carcinogenesis was the demonstration that knocking out the activated ras gene in the human colon cancer cell lines DLD-1 and HCT-116 resulted in cell lines incapable of clone formation on soft agar and lacking tumorigenicity in nude mice. Thus, while human tumor cell lines may harbor multiple genetic mutations, deletion of an activated ras allele could suppress the expression of the malignant phenotype [10]. The ras oncogene has been reported to confer resistance to ionizing radiation [11, 12].

**Other Small Ras-Related G Proteins**

The Ras family includes several distinct members, such as Ras (H, K, M, N, and R), Rap (1 and 2), and Ral, that share at least 50% sequence identity. This family shares at least 30% sequence identity with several other small monomeric G protein families, such as the Rho/Rac/CDC42, Rab/Ypt, Ran, Arf, and Rad families [13, 14].

Most biologists associate Ras family proteins primarily with regulation of the actin cytoskeleton, although many other functions have been attributed to them, including membrane trafficking, transcriptional control, regulation of cell adhesion, and cell cycle progression. There are at least 20 Rho family proteins in the human genome, whereas Drosophila melanogaster has 7 and C. elegans has 5. As with many other protein families, the Rho family appears to have expanded during evolution from a small essential ‘core’ to carry out more specialized functions in higher eukaryotes.

The first thing most people do with a new Rho protein is to see what it does to the actin cytoskeleton, and most Rho proteins characterized to date have been shown to affect the organization of polymerized actin (F-actin) in some way, either when they are overexpressed or when mutant versions are expressed. Of the Rho family, RhoA and B, Rac1 and Cdc42 have been the most widely studied, primarily because they were the first Rho proteins to be characterized, and reagents are readily available. RhoA induces actomyosin-based contractility, leading to the formation of stress fibers in many types of adherent cells, and/or cell retraction. Both Rac and Cdc42 stimulate actin polymerization: Rac to induce broad plasma membrane extensions known as lamellipodia and membrane ruffles, and Cdc42 to induce the extension of finger-like plasma membrane extensions called filopodia or microspikes. Rho proteins generally cycle between an active, GTP-bound, conformation and an inactive GDP-bound conformation (with the exception of RhoE/Rnd proteins) [15]. In the GTP-bound form, they interact with downstream target proteins to induce cellular responses. Many targets for Rho proteins have been described [16], and each Rho family member can potentially interact with multiple targets. Of these, several have been shown to play a role in mediating Rho protein effects on the actin cytoskeleton. In particular, Rac and Cdc42-induced actin polymerization has been shown to be mediated by members of the WASp/WAVE family of proteins, which stimulate the Arp2/3 complex to nucleate new actin filaments [17, 18].

Rho proteins can exchange nucleotide and hydrolyse GTP at slow rates in vitro, and these reactions are catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively [16]. In addition, some but not all Rho proteins can bind to proteins known as GDIs (guanine nucleotide dissociation inhibitors), which prevent their interaction with the plasma membrane, with exchange factors and with downstream targets [16, 19]. Rho proteins are post-translationally modified at their carboxy-terminus by prenylation of a conserved cysteine, and this is required for their interaction with membranes. GEFs for Rho proteins are widely assumed to be membrane-associated (although see discussion of Rho GTPases and microtubules below), and thus Rho proteins are believed to dissociate from GDIs and associate with membranes in response to activating stimuli. Two kinds of Rho protein mutants have been used extensively to analyze their functions: activated mutants, which are constitutively GTP-bound because the GTPase activity is inhibited; and dominant-negative mutants, which have reduced affinity for nucleotides and may act by titrating out GEFs [20]. Dominant-negative proteins are selective between different subclasses of Rho proteins (e.g. Rac vs. Cdc42), but are unlikely to be selective between closely related proteins (e.g. Rac1 and Rac2) [21].

Rap proteins are of special interest because they contain the same effector domains as Ras. Rap 1 has been implicated in a number of cellular processes, such as platelet activation and T-cell anergy. The function of Rap 2 remains unclear [14].
INTRACELLULAR TARGETS OF RAS

Once activated ras has several intracellular substrates that include mainly Raf-1 and phosphatidylinositol 3 kinase (PI3-K) but also other small G proteins such as Rac and Rho.

Raf-1

Mitogen-activated protein (MAP) kinase cascades lie in a three-kinase-signaling module involved in transmitting membrane signals to the cell nucleus. A MAPK module consists of MAP kinase or extracellular signal-regulated kinase (ERK) activated by a MAP/ERK kinase (MEK or MAPKK) which, in turn, is activated by a MEK kinase (MEKK or MAPKKK). One such MEKK, which is the most well-characterized downstream effector of Ras, is the serine–threonine kinase Raf-1. This protein is recruited by Ras-GTP to the plasma membrane, where Raf is activated by an as yet unknown factor [22-30]. Localization of Raf to the plasma membrane is essential for its activation.

Thus, fusion of Raf to the C-terminal membrane-localization signal of K-ras leads to its constitutive localization to the plasma membrane and bypasses the need for Ras [22, 23]. Once activated, Raf phosphorylates MEK, which, in turn, phosphorylates ERK [29]. MAPK activation results in phosphorylation and activation of ribosomal S6 kinase and transcription factors, such as c-Jun, c-Myc, and c-Fos, resulting in the switching on of a number of genes associated with proliferation [31].

Rac and Rho

The G proteins Rac and Rho cycle between GDP- and GTP-bound forms and are regulated by factors analogous to GNEFs and GAPs [32]. These two proteins have been shown to be activated by Ras-GTP [33, 34]. Through their regulation of the active cytoskeleton, Rac and Rho are critical in cellular processes, such as formation of focal adhesions, filopodia, stress fibers, and membrane ruffling [26]. All of these processes can be activated by oncogenic ras are important for the invasive phenotype of transformed cells [35, 36].

Phosphoinositide 3-Kinase

Another ras effector is PI3-K, a complex of two distinct subunits, the catalytic subunit of 110 kd (p110) and the regulatory subunit of 85 kd (p85). Ras-GTP binds the catalytic p110. This interaction leads to its constitutive localization to the plasma membrane and bypasses the need for Ras [22, 23]. Once activated, Raf phosphorylates MEK, which, in turn, phosphorylates ERK [29]. MAPK activation results in phosphorylation and activation of ribosomal S6 kinase and transcription factors, such as c-Jun, c-Myc, and c-Fos, resulting in the switching on of a number of genes associated with proliferation [31].

Serine–Threonine Kinase MEKK1

The serine–threonine kinase MEKK1 is implicated in the stress-response pathway, whose downstream targets include the MAP kinases c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) [53, 54]. The MEKK1/JNK/SAPK cascade has been shown to induce apoptosis under certain circumstances [55]. MEKK1 is activated by Ras-GTP through the Ras/Calcium pathway [56]. Even though the primary target of MEKK1 appears to be JNK, recent evidence suggests that MEKK1 can activate the MEK/ERK pathway independent of Raf-1. JNK may be the target of Ras in an alternate pathway involving Rac/Rho/CDC42 and not the MAP kinase pathway. Cross-talk, however, almost certainly exists between these two pathways [28]. Fig. (1) summarizes the pathways regulated by ras.
POST-TRANSLATIONAL MATURATION: ISOPRENYLATION AND METHYLATION

The addition of a lipidic residue to all the small GTP binding proteins is essential for their correct location on the inner side of the plasma membrane and for their consequent activation by external signals. In fact, they must co-localize with their effectors that are all placed on the inner side of plasma membrane where they found also their substrates that are necessary to mediate the different functions of this class of molecules.

Mevalonate Pathway

In human cells isoprenoids are derived from the mevalonate pathway that starts from reaction catalyzed by the 3-hydroxy-3-methylglutaryl CoA (HMGCoA) reductase (the rate-limiting reaction in cholesterol biosynthesis) which catalyzes the conversion of HMGCoA to mevalonic acid. The pathway triggered by this reaction can lead to the synthesis of a key isoprenoid molecule, the farnesyl-pyrohosphate (FPP) whose formation is catalyzed by the farnesylpyrophosphate synthase (FPPS) [57]. FPP can be either converted by a series of reactions in cholesterol or can be transferred on target cellular proteins as FPP itself (reaction catalyzed by farnesyltransferase) or firstly converted in geranyl-geranyl-pyrohosphate and then transferred on cellular proteins by type I or type II geranyl-geranyl-transferase (Fig. 2).

Farnesyltransferases and Geranyl-Geranyltransferases

The joining of the 15-carbon farnesyl group (C\(_{15}\)H\(_{30}\)) and the 20-carbon geranylgeranyl group (C\(_{20}\)H\(_{34}\)) to protein-cysteines at or near their carboxy-termini is catalyzed by...
protein farnesyltransferase (FTase) and protein geranylgeranyltransferase-I and II (GGTase-I and GGTase-II) [58]. The prenyltransferases are heterodimers consisting of $\alpha$- and $\beta$-subunits with combined molecular masses ranging from 91 to 98 kDa. The $\alpha$-subunits of FTase and GGTase-I are the same, and the $\beta$-subunits differ. The $\beta$-subunits of the three enzymes are homologous to the $\alpha$-subunits and to each other. The isoprenoid groups become linked to polypeptidic cysteines through thioether (C–S–C) bonds. Conversion of the protein–cysteine acceptor site to protein-serine in onco-
genic H-Ras prevents prenylation and abolishes its malignant transforming ability [59]. FTase and GGTase-I catalyze the prenylation of substrates with a carboxy-terminal tetrapeptide sequence called a $CA_1A_2X$ box, where C refers to cysteine, A refers to an aliphatic residue, and X typically refers to methionine, serine, alanine, or glutamine for FTase or to leucine for GGTase-I. Following prenylation of physiological substrates, the terminal three residues ($A_1A_2X$) are subsequently removed by a C $A_1A_2X$ endoprotease and the carboxyl group of the terminal cysteine is methyl esterified by a methyltransferase. At this moment ras is ready to be located on the inner side of the plasma membrane to receive signals mediated by external factors.

The joining of the 15-carbon farnesyl group ($C_{15}H_{25}$) and the 20-carbon geranylgeranyl group ($C_{20}H_{33}$) to protein-cysteines at or near their carboxy-termini is catalyzed by protein farnesyltransferase (FTase) and protein geranylgeranyltransferase-I and II (GGTase-I and GGTase-II) [58]. FTase and GGTase-I catalyze the prenylation of substrates with a carboxy-terminal tetrapeptide sequence called a $CA_1A_2X$ box, where C refers to cysteine, A refers to an aliphatic residue, and X typically refers to methionine, serine, alanine, or glutamine for FTase or to leucine for GGTase-I. Following prenylation of physiological substrates, the terminal three residues ($A_1A_2X$) are subsequently removed by a C $A_1A_2X$ endoprotease and the carboxyl group of the terminal cysteine is methyl esterified by a methyltransferase [58]. Protein GGTase-II, or Rab geranylgeranyltransferase, catalyzes the geranylgeranylation of Rab proteins that terminate in CC or CXC sequences. Rab proteins ending with CXC residues are methyl esterified; those ending with CC are not. FTase and GGTase-I can catalyze the prenylation of tetrapeptides, polypeptides, and proteins containing appropriate $CA_1A_2X$ box sequences. GGTase-II, in contrast, cannot catalyze the prenylation of peptides; it uses a Rab–Rab escort protein heterodimer as substrate [58, 60]. There are a few exceptions to the substrate specificity rules for FTase and GGTase-I noted above. K-RasB, which has a classical FTase $CA_1A_2X$ box (CVM), is a substrate for FTase. Following inhibition of cellular FTase, K-RasB becomes a substrate for geranylgeranylation by GGTase-I [61, 62]. The latter reaction is made possible by an upstream polybasic sequence that alters GGTase-I substrate specificity. Furthermore, RhoB, which contains a GGTase-I $CA_1A_2X$ box (CKVL), is found in both
farnesylated and geranylgeranylated forms in cells. This is due to the ability of GGTase-I to both geranylgeranylate and farnesylate this substrate [63]. It appears that upstream sequences (as yet uncharacterized) are responsible for this altered substrate specificity. Moreover, Cdc42, which contains a carboxyterminal CCIF sequence, undergoes geranylgeranylation. Ordinarily GGTase-I substrates contain leucine in the X position of the CaaX box, but Cdc42 represents an exception to the leucine rule [64]. All three prenyltransferases require Zn2+, and FTase and GGTase-II require Mg2+ for activity [60, 62, 65-69]. Both protein geranylgeranylation and farnesylation are followed by the cleavage of the terminal tripeptide A1A2X, catalyzed by a specific peptidase, and by the subsequent methylation of the terminal cysteine catalyzed by a methyl-transferase. Finally, the protein is ready to be translocated on the cellular membranes to receive extra or intra-cellular signals. After methylation a palmitoylation on the -SH group of the penultimate cysteine residue can occur. This last reaction is reversible and occurs only for Ras proteins with a cysteine residue upstream of the CAAX motif (namely, H-Ras, N-Ras, and K-Ras4A), whereas the other CAAX-triggered events are irreversible [58, 70-71] (Fig. 2). The list of the intracellular proteins that can be isoprenylated is summarized in Table (1).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-, K- and N-Ras</td>
<td>Signalling for growth, differentiation and apoptosis</td>
</tr>
<tr>
<td>Rho-B and -E</td>
<td>Cytoskeletal organization, Cell cycle regulation</td>
</tr>
<tr>
<td>PTP-CAAX1 and 2</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>Rap2A GTPase, HDJ2/dj2/HSD1/rdj1/hsj2</td>
<td>Platelet function, Chaperone protein</td>
</tr>
<tr>
<td>Lamin A and B</td>
<td>Nuclear membrane structure</td>
</tr>
<tr>
<td>PxF</td>
<td>Peroxisomal protein</td>
</tr>
<tr>
<td>cGMP</td>
<td>Visual signal transduction</td>
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<tr>
<td>Transducin</td>
<td>Visual protein</td>
</tr>
<tr>
<td>Rhodopsin kinase</td>
<td>Visual protein</td>
</tr>
<tr>
<td>Phosphorylase kinase a and b</td>
<td>Skeletal muscle function</td>
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<table>
<thead>
<tr>
<th>Protein</th>
<th>Functions</th>
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</thead>
<tbody>
<tr>
<td>G-proteins g-subunits</td>
<td>Signaling for growth, differentiation, apoptosis</td>
</tr>
<tr>
<td>Rap1</td>
<td>Competes with Ras for various effectors</td>
</tr>
<tr>
<td>Rho A, B, C, and G</td>
<td>Cytoskeletal organization; cell cycle control</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Rho family; cytoskeletal organization</td>
</tr>
<tr>
<td>Rac 1 and 2</td>
<td>Membrane ruffling; actin reorganization</td>
</tr>
<tr>
<td>R-Ras I and R-Ras 2/TC21</td>
<td>Binds to bcl-2, which regulates apoptosis</td>
</tr>
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STRATEGIES FOR THE INHIBITION OF PRENYLATION

HMG CoA Inhibitors

HMG-CoA reductase inhibitors are a class of drugs that inhibits the rate-limiting step of the mevalonate pathway [72], essential for the synthesis of various compounds, including cholesterol and FPP as described above. Since the discovery of the first HMG-CoA reductase inhibitor (ML-236A) from penicillium citrinum and its cholesterol-lowering properties in rats [73], these agents have emerged as the dominant class of compounds for the treatment of hypercholesterolemia. Among them, lovastatin, pravastatin, simvastatin, fluvastatin, and atorvastatin are currently commercially available. Rouvastatin is the newest agent in this class but awaits approval for use in the United States. HMG-CoA reductase inhibitors decrease hepatic cholesterol production, which in turn leads to increased LDL receptor turnover, enhanced hepatic LDL-cholesterol uptake, and ultimately decreased plasma LDL-cholesterol level [74]. Overall, plasma LDL-cholesterol levels are substantially decreased by 20-60%, along with mild elevation in high-density lipoprotein-cholesterol and reduction in triglyceride levels. Numerous multicentered trials have demonstrated the efficacy of HMG-
CoA reductase inhibitors in reducing mortality and morbidity in both primary [75, 76] and secondary prevention [77-79] of coronary artery disease. Furthermore, four meta-analyses also discovered their use to be associated with long-term reduction in cerebrovascular events particularly after an initial coronary event [80-82]. More recently, these agents were also shown to have pleiotropic cardiovascular and antiatherosclerotic effects, including reversal of endothelial dysfunction, inhibition of monocyte recruitment, antioxidant activity, down-regulation of angiotensin II receptors, immunomodulation, reduction in inflammatory response, plaque stabilization, reduction in ventricular arrhythmias, and decrease in thrombogenicity [84-86]. Indeed, recent clinical studies have shown that treatment with HMG-CoA reductase inhibitors in acute coronary syndrome decreases short-term recurrent ischemia [87], and similarly after a transient ischemic attack they may suppress recurrences [88]. Other proposed beneficial effects of HMG-CoA reductase inhibitors also include stimulation of bone formation and inhibition of growth of tumor cells [89]. The rate-limiting step of the mevalonate pathway is the conversion of HMG-CoA to mevalonate, which is catalyzed by HMG-CoA reductase. The mevalonate pathway produces various end products that are important for many different cellular functions. These products include isoprene units incorporated into sterol and nonsterol compounds such as cholesterol, dolichol, ubiquinone, isopentenyladenine, GGPP, and FPP [72]. Cholesterol is essential in maintaining cellular membrane structure and integrity. It also serves as a precursor for the synthesis of steroid hormones and bile acid [90]. Dolichol works as a carrier molecule of oligosaccharides in N-linked protein glycosylation for the production of glycoproteins. Ubiquinone is involved in mitochondrial respiration and may also play a significant role in the inhibition of lipid peroxidation [91]. Isopentenyladenine is an essential substrate for the modification of certain tRNAs. Geranylgeranyl transferase and farnesyl transferase use GGPP and FPP, respectively, for posttranslational modifications of cellular proteins. These include Ras, nuclear lamins, and many small GTP-binding proteins such as members of the Rab, Rac, and Rho families as described above [92]. Blockade of the rate-limiting step of the mevalonate pathway by HMG-CoA reductase inhibitors results in decreased levels of mevalonate and its downstream products and, thus, may have significant influences on many critical cellular functions.

HMG-CoA reductase inhibitors have been shown to synchronize tumor cells by blocking the transition of G1-S in the cell cycle, thereby exerting its antiproliferative effect [93]. This effect is reversed with the addition of mevalonate. In primary cultures of human glioblastoma cells, inhibition of Ras farnesylation by lovastatin is associated with reduction of proliferation and migration [94]. However, the inhibition of cell growth by lovastatin may be independent of Ras function [95]. In C6 glioma cells treated with lovastatin, free geranylgeraniol overcomes the arrest of cell proliferation, whereas the rescue effect was significantly lower with farnesol [96]. These findings suggest that geranylgeranylated proteins (but to a much lesser degree, farnesylated proteins such as Ras) are essential for progression of C6 glioma cells into the S phase of the cell cycle. In addition, N-Ras mutated, primary AML cells were no more sensitive to simvastatin than AML cells without the mutation, suggesting that the inhibition of AML cell proliferation by HMG-CoA reductase inhibitors may be independent of the Ras signaling pathway [97]. On a murine prostate tumor cell line, it was also shown that H-Ras is capable of only inducing cell spreading but incapable of supporting cell proliferation in the absence of geranylgeranylated proteins such as RhoA [98]. Recently, the antiproliferative effects of HMG-CoA reductase inhibitors on G1-S arrest are thought to be attributable to an increase in p21WAF1/CIP1 and p27KIP1, two cyclin-dependent kinase inhibitors [99-101]. Rho small GTPase(s), geranylgeranylated by GGPP, were shown to be important for the degradation of p27KIP1 [102].

The mechanism of HMG-CoA-induced apoptosis also appears to be mediated predominantly through depletion of geranylgeranylated proteins [103]. Add-back experiments of downstream products of the mevalonate pathway were conducted on lovastatin-pretreated human AML cells. Apoptosis induced by lovastatin was abrogated by mevalonate and GGPP and was partially reversed by FPP. However, other products of the mevalonate pathway, including cholesterol, squalene, lanosterol, desmosterol, dolichol, dolichol phosphate, ubiquinone, and isopentenyladenine, did not affect lovastatin-induced apoptosis in AML cells. Furthermore, the use of a geranylgeranyl transferase inhibitor mimicked the effect of lovastatin on apoptosis, whereas the use of a farnesyl transferase inhibitor was much less effective in triggering apoptosis in AML cells in vitro. These findings are also supported by a study in colon cancer cells, which showed that addition of GGPP prevented lovastatin-induced apoptosis, whereas cotreatment with FPP had no effect [104]. This study also showed that lovastatin treatment resulted in decreased expression of the antiapoptotic protein Bcl-2 and increased the expression of the proapoptotic protein Bax.

HMG-CoA reductase inhibitors generally are well tolerated and have a safe side effect profile. The most concerning adverse effects include hepatotoxicity and myotoxicity. Increases in serum liver enzymes are dose dependent and occur at a reported frequency of 1-33% [105]. The majority of cases of clinically significant transaminitis occur within the first 3 months of therapy and therefore monitoring of liver enzymes is required [106]. Myotoxicity, including myalgia and elevated serum creatine kinase (about 10 times the upper limit of normal), occurs in 0.5% of patients treated with lovastatin [107]. Rhabdomyolysis occurs in almost 0.1% of patients who receive HMG-CoA reductase inhibitor monotherapy [106]. In its extreme form, rhabdomyolysis can lead to myoglobinuria and acute renal failure [108].

Farnesyltransferase and Geranyl-Geranyltransferase Inhibitors

Several strategies were used to develop FTase inhibitors: screening of natural and chemical libraries, chemical rational design of FPP analogues or CAAX peptidomimetics [109]. To summarize, FTIs fall into four main classes: (1) FPP analogues that compete with the substrate FPP for FT such as hydroxyfarnesyl phosphonic acid; (2) CAAX peptidomimetics that compete with the CAAX box of Ras for FT, such as FTI-277 or L-774, 832; (3) the bisubstrate inhibitors that combine the properties of a farnesyl diphosphate analogue
together with a peptidomimetic, such as BMS-186511 and (4) compounds discovered by throughput screening of libraries such as SCH66336 and R115777. In Fig. (3) are shown the different classes of FTIs are shown in Fig. (3). Most FTIs selectively inhibit FTase, and do not affect GGTTase I in vitro and in vivo [110-114]. Initial studies with KRAS showed that it is an in vitro substrate for both GGTTase I and FTase [115], and in human cancer cells treated with FTIs, KRAS, but not HRAS, also becomes geranylgeranylated. Consequently, KRAS retains its membrane association and function [116, 117]. Consistent with this, inhibition of KRAS prenylation in several human cancer cell lines was resistant to FTIs or GGTTase I inhibitors (GGTIs) when used alone, and required co-treatment with FTIs and GGTIs [118, 119]. This indicates that inhibition of the growth of human tumours that contain mutated KRAS might require co-treatment with both FTIs and GGTIs. However, surprisingly, FTIs and GGTIs alone are highly potent antitumour agents [118, 119], indicating that inhibition of KRAS prenylation is not necessary for the antitumour activity of FTIs and that this activity might be mediated through farnesylated proteins other than KRAS.

Initial studies on the effects of FTIs on cell division-cycle progression showed that FTI treatment caused numerous human cancer cells to accumulate at the G2–M transition [120], mediated by a prometaphase arrest [121, 122]. Consistent with this finding, FTI-2153 was shown to inhibit bipolar spindle formation and chromosome alignment at the metaphase plate [124]. The FTI-mediated inhibition of bipolar spindle formation and accumulation of monoasters at prometaphase is not dependent on the mutation status of RAS or p53 [123]. It has been hypothesized that the farnesylated centromere-associated proteins CENPE and CENPF might be involved [121, 124].

Support for this involvement is provided by the recent observations that ectopic expression of a C-terminal fragment of CENPF (C630), that showed the same regulated localization as the authentic protein, caused inhibition of cell proliferation, a delay in progression through G2/M, as well as increased apoptosis [124]. Mutation of the CAAX motif of C630 to prevent farnesylation caused a partial loss of this inhibitory activity, arguing that loss of farnesylation will prevent proper localization of this putative dominant-negative of CENPF. Furthermore, treatment with FTIs (SCH66336) impaired CENPF localization to both the nuclear envelope and to kinetochores, as well as the degradation of CENPF hat is seen normally after mitosis. Whereas these observations support CENPF as an important target for FTI-induced G2–M arrest, the absence of clear information on the function of CENPF in kinetochore function during mitosis prevents a more definitive validation of this possibility [125].

Several groups have shown that FTIs can induce apoptosis, but only under certain circumstances. Suzuki et al. found that attached cells can only be induced to undergo FTI-mediated apoptosis when deprived of serum [126] and, conversely, Lebowitz et al. showed that FTIs induce apoptosis in the presence of serum, but only when cells are deprived of substratum attachment [127]. Integrin and growth-factor-receptor signalling pathways therefore seem to rescue cells from FTI-induced apoptosis [126, 127]. By contrast, Jiang et al. showed that in some human cancer cell lines, FTIs can induce apoptosis in the presence of serum and under attached conditions, and that apoptosis could be prevented by ectopic expression of a constitutively activated form of the AKT2 serine/threonine kinase [128]. AKT2 is activated by PI3K, a lipid kinase that stimulates the formation of phosphoinositide lipids that promote AKT activation. The PI3K–AKT pathway serves a pro-survival function for many physiological situations. These studies indicate that in human cancer cells that overexpress AKT2, an unidentified farnesylated protein that functions as an upstream activator of AKT2 mediates survival of these tumours, and that FTIs induce apoptosis by inhibiting the farnesylation and function of such a protein. Consistent with this, FTIs block growth factor- and integrin-stimulated PI3K and AKT2 activation and subsequent phosphorylation of the proapoptotic protein BAD [128]. So, in human cancer cells that overexpress AKT2, FTIs induce apoptosis by a mechanism that involves inhibition of the PI3K–AKT2 pathway. This mechanism shows cell-type differences, and is operational in some [129], but not other, cell lines such as RAS-transformed rodent fibroblasts, in which FTIs induce apoptosis by a mechanism that is independent of the PI3K–Akt pathway [130]. The ability of FTIs to induce apoptosis therefore depends on the cell type and its microenvironment, as well as the genetic alterations of such cells [125].

Fig. (3). Structures of representative agents from different classes of FTIs.
The fact that FTIs are effective in some tumors, but not in others — preclinically as well as clinically — indicates that farnesylated protein(s) are crucial to the survival and oncogenesis of some human cancers, but not all. An important unresolved mechanistic issue in the field is therefore that of identifying the farnesylated protein or proteins that are important in malignant transformation of some human cancers.

Because all RAS proteins (HRAS, NRAS and KRAS — both 4A and 4B isoforms) require farnesylation to cause malignant transformation, and because RAS is mutated in 30% of all human cancers [1], FTIs were initially developed with the hope of targeting those human tumours that are mutated for RAS [110-114]. Indeed, in HRAS transformed fibroblasts, FTIs inhibit oncogenic HRAS-dependent signalling, transformation and tumour growth in nude mice [131, 132]. However, although HRAS function is clearly blocked by FTIs, there are several observations that argue against the RAS isoforms that are most commonly mutated in human cancers (KRAS and NRAS) being the crucial target for FTIs, at least in most human tumours. First, in human cancer cell lines, the ability of FTIs to inhibit tumour growth does not correlate with the presence of a RAS mutation [133]. Second, in human cancer cells that are treated with FTIs, KRAS and possibly NRAS, but not HRAS, become geranylgeranylated, retain function, and consequently escape inhibition [116-118, 134], yet the growth of these cancer cells in soft agar and nude mice was still inhibited [135, 118, 119]. Third, although FTI treatment did block KRAS-induced tumour growth in transgenic mice, this was not associated with the accumulation of unprocessed KRAS protein [136]. These observations indicate that farnesylated proteins other than KRAS are targets for FTIs. What is clear is that FTIs are very effective inhibitors of HRAS farnesylation and oncogenesis, and, consequently, cancers in which HRAS is mutated represent strong candidates for FTI treatment.

Unfortunately, HRAS mutations are not prevalent in our most clinically important cancers. However, inhibition of the prenylation of wild-type forms of RAS could also contribute to FTI antitumour activity in human cancers that have an activated RAS pathway due to upstream signal stimulation — for example, by overexpression of ERBB1/epidermal growth factor receptor (EGFR) and ERBB2/HER2 — as wild-type RAS is still needed to mediate malignant transformation by these receptor tyrosine kinases. Inhibiting HRAS would be sufficient in tumours that depend on the PI3K–AKT pathway for survival. Indeed, it has been shown by two independent labs that HRAS preferentially drives the PI3K–AKT pathways, whereas KRAS preferentially drives the RAF–MEK–ERK pathway. Therefore, FTI inhibition of HRAS farnesylation could preferentially inhibit the PI3K–AKT pathway [137, 138]. In conclusion, although considerable evidence argues against RAS being a key target, there is also evidence that supports RAS being an FTI target in some human cancers [125].

RHOB — a member of the RAS superfamily of small GTPases — is normally both farnesylated and geranylgeranylated. Initial studies by the Goldstein and Brown groups showed that GGTase I farnesylates as well as geranylgeranylates RHOB, which would eliminate RHOB as a direct target for FTIs [63]. However, a recent study showed that RHOB can be farnesylated by FTase and its farnesylation is inhibited by FTIs [139]. Despite the fact that RHOB is farnesylated and geranylgeranylated, it is still a candidate for a target of FTIs. Evidence for inhibition of RHOB farnesylation as a mediator for FTIs’ antitumour activity stems from the following observations. First, treatment of cells with FTIs results in decreased levels of farnesylated RHOB (RHOB-F) and increased levels of geranylgeranylated RHOB (RHOB-GG) [139]. Second, a RHOB–RHOA chimeric protein that is exclusively geranylgeranylated was shown to be growth-inhibitory [140]. More recently, RHOB was proposed to be a crucial mediator of some of the effects of FTIs based on experiments using murine fibroblasts that are deleted for the RHOB gene [141]. In these experiments, the ability of FTIs to induce apoptosis was shown to be dependent on RHOB; however, the ability of FTIs to inhibit tumour growth in soft agar was completely independent of RHOB. Furthermore, the growth in nude mice of HRAS-transformed RhoB–/– fibroblasts was less sensitive but not resistant to FTIs. These observations support the suggestion that the FTI-stimulated increase in RHOB-GG protein levels might be important for FTI-induced apoptosis, but not anchorage-independent growth. This might explain the partial dependence of FTIs on RHOB in tumour growth inhibition in vivo, which might require both the ability of FTI to induce apoptosis and to inhibit anchorage-independent growth. This gain of function for RHOB-GG is believed to be a consequence of the subcellular location differences of RHOB when modified by distinct isoprenoids. Furthermore, the gain-of-function suggestion relies on the premise that RHOB-GG and RHOB-F have distinct biological functions.

So far, no clear evidence exists for differences of localization or function between endogenous RHOBF and RHOB-GG. Therefore, the genetic experiments discussed above give support for RHOB (both RHOB-F and RHOB-GG), not inhibition of RHOB-F farnesylation, as a mediator of some FTI effects. These experiments, and other related studies showing the increased sensitivity of RhoB–/– mice to carcinogen-induced tumours [142], would support a role for RHOB in tumour suppression, but is not necessarily evidence for RHOB-F as a relevant FTI target, because genetic loss of RHOB expression is not equivalent to FTI-mediated formation of RHOB-GG (see more below). Although there is some evidence that RHOB is an important target for FTIs, there are several key observations that argue for the importance of other FTase substrates in mediating FTI antitumour activity.

So, whereas some facets of FTI activation might involve targeting farnesylated RHOB, it seems unlikely that RHOB will be the crucial or only target. Therefore, for the successful clinical development of FTIs, it will be essential that the key FTase substrate(s) be identified [125].

The similar biological activities of structurally diverse FTIs argue that their antitumour activities are due to inhibition of FTase — although some FTIs do seem to possess non-FTI activities. For example, BMS-214662 is a potent and selective FTI that has FTase-independent apoptotic activity when evaluated in cell-culture assays [143]. As other equally potent FTIs lack this activity, the activity of this compound is not likely to be due solely to inhibition of
FTase. As many of the activities ascribed to FTIs are seen with multiple, structurally distinct FTIs, the antitumour activity of FTIs is anticipated to be primarily caused by inhibition of FTase activity. The different toxicities that are seen with the FTIs that have been evaluated in clinical trials might suggest off-target activities [144], although these differences might also reflect pharmacokinetic and/or pharmacodynamic differences. As inhibition of RAS-protein function cannot explain the inhibitory activities of FTIs, it is generally accepted that there must be other farnesylated proteins that are important targets for FTIs. Current efforts to identify these targets involve an evaluation of known farnesylated proteins, as well as genome-wide searches for candidate targets. For example, database searches for proteins terminating in CAAX motifs that might signal for farnesylation identify dozens of proteins, some of which possess activities that make them intriguing candidates for FTI targets. These include several bone morphogenetic proteins, transforming growth factor-α precursors, serine/threonine kinase-11 and inositol-1, 4, 5-trisphosphate 5-phosphatases (I and IV), all of which are proteins with potential roles in growth regulation [134]. Whether these proteins are farnesylated and are relevant targets for FTIs remains to be determined. Proteomic approaches might also be useful for identifying FTI targets [145]. Several farnesylated proteins have properties that support their possible involvement in FTI action. The PRL family — PRL1, PRL2 and PRL3; also called PTP(CAAX) — of protein tyrosine phosphatases are farnesylated proteins [146, 147], and overexpression of PRL1 and PRL2 can cause weak tumorigenic transformation [146] or invasion [148] of epithelial cells. Overexpression of PRL3 has also been seen in prostate and colon cancers [149–151]. PRL is normally localized to the plasma membrane and endomembranes, and FTI treatment causes nuclear accumulation. RND proteins are farnesylated members of the RHO family of small GTPases. Similar to oncogenic RAS, RND proteins cause a disruption of actin cytoskeletal organization and cell rounding [152, 153]. RND function is mediated by disruption of RHOA function, either by activation of a RHOA GAP (GTPase-activating protein) or by blocking RHOA activation of the ROCK effector [154, 155]. Recently, RND3 (also called RHOE) expression was found to be upregulated in RAS-transformed epithelial cells [156]. Perhaps RND proteins facilitate the morphological changes that are associated with RAS transformation and, consequently, the loss of their function might account for the cell flattening and morphological reversion that is seen with FTI treatment. Another candidate FTI target is RHEB, a farnesylated RAS-related protein [157]. Recent studies established aspects of RHEB function that implicate it as a positive mediator of oncogenesis (see the Akt signalling described above). In summary, it is likely that no one farnesylated protein will be identified that can explain the antitumour activity of FTIs. Furthermore, the targets are likely to be different in distinct cancers [125].

In a slightly different way, analogues of GGPP and peptidomimetics of the CAAL-terminal sequence (the consensus sequence of precursors of several geranylgeranylated proteins, where L stands for leucine) have been designed as inhibitors of PGGT-1 [117, 119, 137]. Additionally, bisubstrate analogues, consisting of both chemical structure elements, have also been developed [138, 122]. Examples of the structures of these inhibitors can be found in several review papers as mentioned above [158]. GGTTs block all human cancer cells in the G1 phase of the cell cycle [159, 121]. The biochemical mechanism by which GGTTs block cell cycle progression was consistent with their ability to block human cancer cells in the G1 phase of the cell cycle. For example, GGTT-298 induces the expression of the cyclin-dependent kinase inhibitor p21 WAF at the transcriptional levels in a p53-independent manner and this involves an Sp1/TGF-b responsive element [121, 160]. This induction of p21waf appears to be mediated by inhibition of RhoA geranylgeranylation [159, 160]. This is consistent with the fact that Ras was shown to activate RhoA which in turn suppresses p21 transcription [161]. It was also shown that dominant negative RhoA activates whereas activated RhoA suppresses p21waf expression in human pancreatic cell line (Panc-1) [138, 122]. In addition to inducing p21waf expression, GGTT-298 also induced p21 and p27 partner switching from cyclin dependent kinase (CDK) 6 to CDK2 [162]. Furthermore, GGTT-298 inhibited the activities of CDK2 and CDK4 but not CDK6 and accumulated pRb in its hypophosphorylated form [162, 163].

More recently, bispecific inhibitors have been synthesized such as BSM-214662 with dual specificity for FT and type I GGTT [164].

Bisphosphonates as Farnesylpseudophosphate Synthase Inhibitors

Bisphosphonates (BPs) such as pamidronate (PAM) and zoledronate (ZOL) are currently used for the treatment of bone metastases and were initially thought to act via an inhibition of formation of osteoclasts from immature precursor cells or direct inhibition of resorption via induction of apoptosis in mature osteoclasts [165]. Recently, evidence accumulated that BPs including PAM and ZOL are also potent inducers of apoptosis in several cancer cell types such as myeloma [166–168], breast [169] prostate cancer [170] and pancreatic cancer [171] as well as in macrophage [172] and intestinal epithelial cell lines [173]. These data indicate that the beneficial effect of BPs on metastatic bone disease may result also from a direct anti-cancer activity that may affect a broad range of tumours. The molecular basis of nitrogen-containing BP anti-cancer action underlie on their ability of inhibiting the farnesyl diphosphate synthase probably by mimicking the diphosphatase moiety [174]. Therefore, they are inhibitors of the synthesis of farnesylgeranyl diphosphate. Differently from FTI or GGTT, BPs could inhibit both the two different mechanisms of isoprenylation of intracellular proteins. We have recently demonstrated that BPs induced apoptosis and growth inhibition in epidermoid cancer cells together to depression of ras signalling and of Erk and Akt survival pathways. These effects occurred together with PARP fragmentation and the activation of caspase 3. Moreover, the latter seemed to be essential for the apoptosis induced by BPs in this experimental model. The synthesis of isoprenoids appeared largely responsible for the biological and biochemical effects of BPs since the addition of farnesol to tumour cells completely antagonized the apoptosis and restored ras activity in tumour cells exposed to BPs. These
data suggested that the activity of BPs could be due to the inactivation of the farnesylpyrophosphate activity [175].

STRATEGIES FOR THE POTENTIATION OF THE ISOPRENYLATION INHIBITION

The initial preclinical findings on FTI antitumour activity were more promising than the data derived form clinical trials (see also the clinical section of the present review). These considerations have compelled the investigators to find new strategies in order to implement the efficacy of the inhibitors of isoprenylation.

Multi-Step Enzyme Inhibition

The prenyltransferase are not strictly specific and a small G protein can be substrate of different enzymes. In fact, K-RasB, which has a classical FTase C A1A2X box (CVIM), is a substrate for FTase, but following inhibition of cellular FTase, K-RasB becomes a substrate for geranylgeranylation by GGTase-I [61, 62]. The latter reaction is made possible by an upstream polybasic sequence that alters GGTase-I substrate specificity as described above. Similarly, Rh0B, which contains a GGTase-I C A1A2X box (CKVIL), is found in both farnesylated and geranylgeranylated forms in cells. [63]. It appears that upstream sequences (as yet uncharacterized) are responsible for this altered substrate specificity. These events allow the alternative isoprenylation of the substrate of prenyltransferase inhibitors. Moreover, the occurrence of tumour cell resistance to FTI has been already described, based on changes in the FT expression and activity levels, or on mutational events producing insensitivity of the FT to FTI [109, 176, 177]. All these mechanisms could be responsible for the poor activity of FTI in human solid cancers even at biologically active concentrations [178]. Based on the relevance of farnesylation inhibitory effects in the BPs anti-tumour activity as suggested by previous findings and confirmed by our results, we have recently used the FTI R115777 together with PAM or ZOL and evaluated the effects of the combinatorial treatment on growth inhibition and apoptosis. BPs and FTI given in combination were strongly synergistic since a CI50 less than 0.5 was recorded with the dedicated software CalcuSyn. For instance, the DRI50 was of about 300-fold for PAM and FTI. Moreover, both PAM/FTI and ZOL/FTI combinations allowed the compounds to be active in terms of tumour cell growth inhibition in vivo achievable therapeutic concentrations (0.1 micromolar range for both drugs). Finally, a potentiation of the pro-apoptotic effects of the 2 drugs was also observed in the same experimental conditions.

Notably, low concentrations of FTI induced a strong increase of ras expression with only a moderate reduction of ras activation ratio that was, on the other hand, significantly reduced by 0.07 µM PAM. The BPs/FTI combination was able to restore the complete inactivation of ras. These data suggest that escape mechanisms to the inhibition of isoprenylation of ras might be based on the geranyl-geranylation or other prenylating processes even if further studies are needed in order to find the molecular mechanisms which actually produce such effects in these cells. It could be hypothesized that BPs, inhibiting the upstream enzyme farnesylpyrophosphate synthase, could prevent alternative pathways based on geranylgeranylation processes in tumour cells. However, a number of other proteins, such as the RhoB family, nuclear lamins and some tyrosin phosphatases, are also targets of farnesyl transferase [179] and might be involved in the observed effects.

The impairment of ras activity induced by the combined treatment was paralleled by a reduced stimulation of both the downstream Erk and Akt survival enzymes. Again, the addition of farnesol to cells treated with the combination abolished the effects of BPs/FTI combination on apoptosis and on the activity of the signalling molecules. These data suggest that the synergistic growth inhibitory and pro-apoptotic effects produced by the BPs/FTI combination involve the inhibition of both Erk and Akt survival pathways acting in these cells in a ras-dependent fashion (Fig. 4). The need to inhibit both farnesylation and geranyl-geranylation of small G proteins has compelled the research about bispecific compounds able to inhibit both enzymatic activities. L-778, 123, BMS-214662 and gliotoxin are some of these new discovered bispecific inhibitors that have anti-cancer activity [164, 165, 180].

Target Prioritization

Human tumour cells are characterized by the existence of multiple and often compensatory survival and proliferative signals. The existence of multifactorial survival pathways can be the cause of the limited activity of anti-cancer strategies based on the use of targeted based drugs such as the EGF-R tyrosine kinase-associated inhibitors Gefitinib and Erlotinib. On the basis of the initial clinical results it has been supposed that resistance to these agents could occur through the onset of alternative survival and proliferative pathways that overcome the inhibition of the EGFR tyrosine kinase activity and depends upon the used experimental tumour models (i.e. activation of other erbB-related receptors, insulin-like growth factor type I [IGF-IR], G-protein coupled receptors etc.) [181]. On the basis of these considerations attempts to inhibit multiple intracellular signalling targets have been recently developed [182, 183] in order to overcome the escape of tumour cells to single agent based therapeutic strategies. Moreover, it was recently demonstrated that a subgroup of patients with non-small-cell lung cancer have specific mutations in the EGFR gene, which correlate with clinical responsiveness to the tyrosine kinase inhibitor gefitinib. These mutations lead to increased growth factor signaling and confer susceptibility to the inhibitor [184]. Moreover, other recent data derived from clinical studies show that adenocarcinomas from never smokers comprise a distinct subset of lung cancers, frequently containing mutations within the tyrosine kinase (TK) domain of EGFR that are associated with gefitinib and erlotinib sensitivity [185]. These results suggest the hyperactivation of EGFR tyrosine kinase is required for the responsiveness of tumour cells to the antiproliferative activity of specific TK inhibitors. In fact, the increased activity of the receptor likely makes the tumour cells more dependent from an EGF-dependent pathway for the survival and/or proliferation. On the other hand, mutations of other members of erbB family genes, such as erbB2, have been also reported in human lung cancers suggesting the existence of multiple survival pathways that can influence the efficacy of a single target-
oriented therapeutic strategy [186]. Similarly to the inhibitors of the EGF-R-associated TK also for FTase has been described that they may be effective in tumor cells containing non-mutant Ras proteins that are activated by upstream oncoproteins [187]. Although oncogenic mutations affecting Ras are not prevalent in human malignant astrocytomas, it was investigated whether levels of activated Ras.GTP might be elevated in these tumors secondary to the mitogenic signals originating from activated receptor tyrosine kinases. In support of this hypothesis high levels of Ras.GTP, similar to those found in oncogenic Ras transformed fibroblasts, were present in four established human malignant astrocytoma cell lines which express platelet-derived growth factor receptors (PDGFRs) and EGFR, and 20 operative malignant astrocytoma specimens. Stimulation of PDGFR’s and EGFR’s induced tyrosine phosphorylation of the Shc adaptor protein and its association with Grb2, suggesting a mechanism by which Ras may be activated in human malignant astrocytoma cells. Furthermore, blocking Ras activation by expression of the Ha-Ras-Asn17 dominant-negative mutant, or by FTase inhibitors, decreased in vitro proliferation of the human astrocytoma cell lines [187]. These data suggest that the hyperactivation of the ras-dependent pathway can sensitize tumour cells to FTase. In this regard, we have reported that interferon α (IFNα) induces apoptosis and increases the expression of the EGFR on these cells [188]. We have moreover found that apoptosis induced by IFNα is completely antagonized by EGF and that IFNα enhances the activity of EGF on these cells [189]. On the bases of these findings we have hypothesized that the increased expression and function of the EGFR could represent a protective response of tumour cells (STRESS RESPONSE) to the antiproliferative effect of IFNα [190]. Indeed IFNα increases the expression of heat...
shock protein (HSP) 27, HSP90 and HSP70 inducible forms while does not change the levels of the constitutive form of HSP70 [201]. At the same time, IFNα induces an about 5-fold increase of activity of JNK-1 and p85MAPK and the transfection of KB cells with a plasmid encoding for a wild type form of JNK1 (JNK1wt) induces per se apoptosis and enhances the apoptosis induced by IFNα [191]. All these effects were antagonized by the addition of EGF to IFNα-treated cells suggesting again a counteracting role of the EGF-R-mediated pathway on the stress-activated IFNα-induced signalling. These results appear also in line with the recent findings demonstrating the involvement of growth factor-dependent pathways in the protection from caspase activation induced by Bad overexpression [192]. Moreover, it has been demonstrated that the EGF-R-dependent pathway controls keratinocyte survival and the expression of the pro-apoptotic bcl-xL expression through a MEK-dependent pathway [192].

Furthermore, the EGF- and Ras-dependent MAPK cascade is hyperactivated in IFNα-treated cells and could be further stimulated by the addition of EGF. In these experimental conditions, an increased activity and responsiveness to EGF stimulation of Ras, Raf-1 and Erk-1 and 2 was found in KB cells exposed to IFNα [193]. These findings suggest that the EGF-R function is preserved in IFNα-treated cells. We have previously described that other anti-proliferative agents, such as cytosine arabinoside, 5aza-2’ deoxycytidine and 8-chloro-cAMP (8ClcAMP), also increase EGFR expression on KB cells [194-196]. On this basis, we have hypothesized that the up-regulation of growth factor receptors is a common event in growth inhibited tumour cells and could represent a protective response towards the antiproliferative stimuli [190]. Also in the case of 8ClcAMP, the EGFinduced MAPK signalling is amplified likely as a consequence of the increased expression of EGFR [191]. However, MAPK activity is reduced in 8Cl-cAMP-treated KB cells suggesting a selective inhibition of Erks or of a still unknown upstream activator induced by the drug [196]. The involvement of the Ras->MAPK pathway in the protection of KB cells from the apoptosis induced by IFNα is further demonstrated by both Ras inactivation by RASN17 transfection and MEK-1 inhibition by exposure to PD098059 [193]. In fact, the transfection of RASN17 in KB cells caused apoptosis suggesting that the integrity of Ras function is necessary to produce an anti-apoptotic signal that mediates a survival response in cells exposed to IFNα via Erk-1 and 2 activation. In fact, we have demonstrated that Ras-dependent survival signalling targets Erk-1/2 since the reduction of MAPK activity by PD098059 enhanced apoptosis caused by IFNα. An additional important finding is that PD098059 specifically abrogated the recovery from apoptosis induced by EGF in IFNα-treated cells. Therefore, our results suggest that the activation of Ras->Raf-1->Mek1->Erk-1/2 signalling has a prominent role in the anti-apoptotic effects exerted by EGF in epidermoid cancer cells exposed to IFNα providing evidence of the potential benefits of the molecular interference with this pathway [193]. We have recently demonstrated that the FTase inhibitor R115777 synergizes with IFNα in inducing apoptosis and growth inhibition (M. Caraglia et al., not published data). In these experimental conditions a complete inactivation of ras and erk can be also observed (M. Caraglia et al., not published data). Taken altogether, these data suggest that tumour cells exposed to IFNα become highly sensitive to specific signalling inhibitors (“target prioritization”) avoiding the need of a wide inhibition of multiple survival signals [197, 198] (Fig. 4).

CLINICAL DEVELOPMENT OF FARNESYL TRANSFERASE INHIBITORS AND OTHER PRENYLATION INHIBITORS

SCH 66336

SCH 66336 (lonafarnib-Sarasar TM), a tricyclic FT inhibitor, has been the first of these compounds to start clinical development. This compound is specific for its target enzyme, is rapidly absorbed following oral administration, and has a linear pharmacokinetics. Four single-agent phase I trials, three of which published as full papers [199-201], have been carried out with this agent, using four different schedules (twice a day on days 1-7 every 21, twice a day on days 1-14 every 28, twice a day continuously, once a day continuously).

The toxicity profile observed in all different trials has been fairly similar, since gastrointestinal tract toxicity (nausea, vomiting, diarrhea) and fatigue have generally qualified as dose-limiting toxicity (DLT). Myelosuppression was not generally observed at the doses which were recommended for phase II. Since farnesylatation is a crucial reaction for photoreceptor synthesis, concern was placed on ocular function in all these trials, but no abnormalities were ever recorded. One objective response was observed in the American study using the 1-7 every 21 days schedule [199]. In this study, which had the additional value of highlighting the importance of prelamin A accumulation in buccal mucosal smears as biologic endpoint for in vivo evaluation of farnesyl transferase inhibition, a patient with pretreated nonsmall cell lung cancer (NSCLC) achieved a confirmed partial response. A number of disease stabilizations were observed in all of the four studies. Since a continuous exposure to a competitive inhibitor is the best way to achieve target enzyme inhibition, the continuous schedule has been chosen to be taken forward for further trials with this compound. A phase IB study was undertaken in patients with squamous cell carcinoma of the head and neck, who were randomized to receive either 100-300 mg lonafarnib bid or best supportive care for up to 14 days prior to surgery. Gastrointestinal side effects were observed, but no DLT occurred. Three out of 17 patients had a partial response; analysis of the surrogate marker DNA-J (HDJ-2), a farnesylated chaperone protein, in surgical samples revealed an increase in unfarnesylated protein in patients treated with lonafarnib [Kies et al., Proc ASCO 2001]. A few phase II studies have already been carried out with lonafarnib. In particular, a phase II randomized study of lonafarnib and gemcitabine in metastatic pancreatic cancer has already been concluded, and it has shown no statistically significant differences between the two treatment arms in terms of objective responses, 3-month progression-free survival, median overall survival [Lersch et al., Proc ASCO 2001]. Lonafarnib has also been used in second-line treatment of bladder carcinoma, but little activity and an unfavorable toxicity profile emerged in this study [Vinquist et al., Proc ASO 2001]. Similarly negative
results were obtained by Sharma et al. [202] in a phase II study of lonafarnib in patients with metastatic colorectal cancer refractory to 5-fluorouracil and irinotecan. In particular, no objective responses were observed in this study, while gastrointestinal toxicity was not negligible. Although it is likely that most of the future efforts with lonafarnib will be carried out in combination studies, a broad phase II program is planned/ongoing in order to identify the most chemosensitive diseases to which direct further efforts.

Lonafarnib strongly synergizes in vitro with paclitaxel and in vivo evidence of synergistic interaction has been also observed, both in NCI 460 human lung carcinoma xenograft and in wap-ras (paclitaxel resistant) transgenic mouse model [203]. Additive interaction has been observed between lonafarnib and gemcitabine in 4 human tumor cell lines, and in in vivo models. Lonafarnib has been combined in vitro with cisplatin as well, and a cell line specific, sequence dependent interaction between the two drugs has been demonstrated [204]. Lonafarnib has been shown to have good in vitro antitumor activity in both human and mouse melanoma cell lines, along with the ability to enhance the level of cisplatin-induced apoptosis, an effect that was associated with enhanced G2/M cell cycle arrest [205]. Recent data show that lonafarnib synergizes with Gleevec in acute Philadelphia-positive chronic myeloid leukemia, and the combination of the two compounds might be able to reverse acquired Gleevec resistance in the acute phase of the disease [Nakajima et al., Proc AACR 2002]. A possible mechanism underlining the synergistic interaction between lonafarnib and cancer therapeutics that are MDR1 product P-glycoprotein (P-gp) substrates might lie in the potent lonafarnib inhibition of P-gp. In fact, in a test system consisting of a NIH-G185 cell line presenting an overexpressed amount of the human transporter P-gp, known P-gp inhibitors, such as cyclosporin A, verapamil, tamoxifen, and others were shown to inhibit the P-gp mediated efflux of daunorubicin. Lonafarnib significantly inhibited daunorubicin transport with an IC 50 of about 3 microM, and similarly affected the transport of rhodamine 123 with a potency similar to cyclosporin A. In addition, lonafarnib was shown to decrease P-gp mediated ATP hydrolysis by > 70% with a Km of 3 microM; this observation indicates that lonafarnib directly interacts with the substrate binding site of P-gp [206]. The possible coadministration of lonafarnib and P-gp substrates cancer therapeutics would have the additional advantage of allowing reduced chemotherapy dosage. Based on all of the above preclinical observations, a few combination clinical trials with lonafarnib have been started. In particular, lonafarnib has been combined with gemcitabine in a phase I study [Hurwitz et al., Proc ASCO 2000]. Nausea, vomiting, diarrhea, myelosuppression represented DLT in this study, in which clinical activity was observed, in particular in pancreatic carcinoma (2 partial responses and 1 minimal response), while a high percentage of patients achieved a long lasting stable disease. The encouraging results of the phase I study have led to a number of phase II studies of the combination of lonafarnib and gemcitabine, among which a study in second line advanced bladder carcinoma, in which a 32% objective response rate was recorded [Theodore et al., Proc ASCO 2003]. Paclitaxel has been studied in combination with lonafarnib in a phase I study [207]. Myelosuppres-

R115777

R115777 (tipifarnib-Zarnestra™) is another novel orally active farnesytransferase competitive inhibitor in advanced clinical development. The first phase I study has tested R115777 with an intermittent schedule (five days every 2 weeks). Neurotoxicity and fatigue qualified as DLT in this study, and a patient with metastatic colorectal cancer treated at the recommended dose for phase II, had a 46% decrease in tumor marker level, improvement in cough and radiographically stable disease for 5 months [208]. Crul et al. have recently published the results of a phase I study of continuously administered R115777 [209]. Myelosuppression and neurotoxicity were DLT in this study, while 300 mg bid qualified as RD for phase II. Antitumor activity was observed in this study. In fact, three patients with pancreatic, colon, and cervix cancer had stable disease, and one patient with colon carcinoma had a minor response accompanied by a more than 50% decrease in carcinoembryonic antigen. A fifth patient, with platinum-refractory non-small cell lung cancer, showed a partial response which lasted for 5 months. Johnston et al. [210] have recently presented data of a phase
II study of R115777 in pretreated breast cancer, in which two cohorts of patients were recruited sequentially. The 1st cohort of 41 patients received a continuous dosing (CD) regimen of 400 mg or 300 mg bid. In particular, the first 6 patients in the CD cohort treated at 400 mg bid all developed grade 3-4 neutropenia, so the subsequent 35 patients were treated at 300 mg bid. The 2nd cohort of 35 patients received 300 mg bid in a cyclical regimen of 21 days treatment followed by 7 days rest (intermittent dosing - ID). In the continuous dose cohort, 4 patients (10%) had a partial response and 6 patients (15%) had a long lasting (> 24 weeks) stable disease. In the intermittent dose cohort, 5 patients (14%) had a PR, and 3 patients (9%) had a long lasting (> 24 weeks) stable disease. Clinical activity was related neither to ras mutational status, nor to HER2 positivity. Hematologic toxicity was much lower in the ID cohort than in the 300 mg bid CD group. In fact, the incidence of grade 3-4 neutropenia and grade 3-4 thrombocytopenia was greater in the latter group (14% versus 43%, P=0.016, and 3% versus 26%, P= 0.013, respectively). Likewise, one patient in the ID cohort developed grade 2-3 neurotoxicity, compared with 15 patients in the CD cohort (3% versus 37%, P = 0.0004). The ID regimen in this study showed similar clinical efficacy, but a significantly improved toxicity profile compared to the CD regimen. Recently, a phase II study of R115777 administered at the dose of 300 mg bid on days 1-21 every 4 weeks as single agent in first line treatment of NSCLC has been reported [211]. No objective responses were observed in 44 patients treated in this study; seven patients had disease stabilization for more than 6 months. Median survival was 7.7 months, and median time to progression was 2.7 months. The most severe toxicity in this study was neutropenia, while mild peripheral neuropathy occurred in 25% of patients. Inhibition of farnesylation in vivo (buccal mucosa samples and peripheral blood mononuclear cells) was consistently documented, but, due to the lack of clinical responses, the relationship between FT inhibition and efficacy was moot. Furthermore, although FT was inhibited in the vast majority of patients, this translated into stable disease in only a few patients. Similarly, Heymach et al. [212] have recently reported a negative phase II study of R115777 administered at the dose of 400 mg bid on days 1-14 every 3 weeks in sensitive relapse small cell lung cancer. In particular, no objective responses were recorded in 22 patients; median progression-free survival was 1.4 months, and median overall survival was 6.8 months. Two phase II trials have evaluated R115777 monotherapy administered at the dose of 300 mg bid on days 1-21 every 4 weeks in newly diagnosed patients with metastatic pancreatic adenocarcinoma [213, Macdonald, et al. Proc ASCO 2002]. Although in one of these two trials, besides being well tolerated, R115777 resulted in potent FT inhibition and suppressed farnesylation of a sample target protein in peripheral blood mononuclear cells [213], it did not exhibit antitumor activity in any of the two trials. In Cohen study [213], in particular, median time to progression was 4.9 weeks, and median survival time was 19.7 weeks; the estimated 6-month survival rate was 25%, with no patients progression-free at 6 months. Cloughesy et al. [Proc ASCO 2002] have treated thirty-three patients with recurrent glioma with R115777 single agent therapy obtaining three partial responses and two long lasting stable disease. However, all of the patients enrolled in this trial were not concomitantly assuming enzyme inducing anti epileptic drugs (EIAED), which might interfere with R115777 metabolism. On the other hand, in a previous study, the same group had run a phase I trial of R115777 in patients with recurrent malignant glioma who were assuming EIAED [Kuhn et al., Proc ASCO 2002]. No objective responses were observed in this trial, and preliminary data show that R115777 pharmacokinetics can be modulated by the concomitant assumption of EIAED. Phase II single agent studies have been carried out also in hematologic malignancies. Harousseau et al. [Proc ASCO 2002] have recently reported interim results from a phase II study of R115777 at the dose of 600 mg bid on days 1-21 every 4 weeks in patients with relapsed and refractory acute myelogenous leukemia (AML). Significant reductions in bone marrow leukemic blasts in patients with relapsed AML were observed. These data follow those presented by Karp et al. [214], who performed a phase I trial of Zarnestra in adults with refractory, relapsed, or high risk leukemia, demonstrating in vivo biologic activity, dose-related accumulations in bone marrow, and clinical responses in 8 of 25 available patients. Kurzock et al. [215] have performed a phase II study in patients with myelodysplastic syndrome at tipifarnib doses of 600 mg orally bid in cycles of 4 weeks of therapy followed by a 2-week rest period. Among 28 patients, two patients had a complete response and one patient had a partial response. The activity was therefore judged modest, and the dose/schedule used, which had been recommended in a previous phase I study, were considered not well tolerated. A randomised double blind placebo controlled trial of R115777 in advanced refractory colorectal cancer has been recently published [216]. In this large international phase III trial, R115777, administered at the dose of 300 mg bid for 21 days every four weeks, was compared to placebo in patients with advanced colorectal cancer who had failed at least two prior chemotherapy regimens for metastatic disease. The study was designed to detect a 50% percent increase in overall survival with a power of 85%. A total of 368 patients were randomised (2:1 ratio) in 65 centres. The study end point was not met, since, on an intent-to-treat basis, the median overall survival was 174 days (95% CI, 157 to 198 days) for patients receiving R115777 and 185 days (95% CI, 158 to 238 days) for patients receiving placebo (P=0.376). One patient achieved a partial response in the R115777 arm. Stable disease (> 3 months) was observed in 24.3% of patients in the R115777 arm compared to 12.8% in the placebo arm. However, this did not translate into a statistically significant increase in progression-free survival. Overall, treatment was well tolerated, with an increased incidence of reversible myelosuppression, rash, and grade 1-2 diarrhea in the R115777 arm.

Key messages that come up from single agent R115777 studies are the following: recommended dose for phase II in solid tumors is 300 mg twice a day; the intermittent schedule has a better therapeutic index; dose limiting toxicities are myelosuppression and neurotoxicity; hints of antitumor activity mainly in breast cancer and hematologic malignancies have been observed; a large phase III trials in advanced refractory colorectal cancer failed to show any survival benefit for patients receiving R115777.
A huge combination study program is being carried out for R115777. A phase I study of R115777 plus docetaxel [Piccart et al., Proc ASCO 2001] has been recently carried out. Thirty-two patients were accrued in this study, the majority of whom with breast cancer. Hematologic toxicity qualified as DLT in this study, while nonhematologic toxicities were moderate, since less than 16% of patients had grade 3 toxicity, which consisted of fatigue, vomiting, diarrhea, anorexia, skin toxicity and stomatitis. One complete response in a patient with breast cancer and liver metastases was observed; furthermore, 7 patients (4 with breast cancer) had a partial response and 6 patients had a stable disease. No significant pharmacokinetic interaction between the 2 drugs was observed, and two different schedules were judged as feasible because of the low incidence or absence of DLT following first cycle: R115777 200 mg twice a day for 14 days every 21 plus docetaxel 75 mg/m², or R115777 300 mg twice a day for 14 days every 21 plus docetaxel 60 mg/m². R115777 has been combined also with other compounds, such as leucovorin-modulated 5-fluorouracil, capecitabine, irinotecan, topotecan, gemcitabine, and herceptin. In the phase I combination study of R115777 + 5-FU-leucovorin (administered according to De Gramont schedule) [Verslype et al., Proc ASCO 2001], DLT was neutropenia, maximum tolerated dose (MTD) was 200 mg twice a day for 21 days every 28. Neutropenia, along with hand and foot syndrome and diarrhea, was DLT also in the phase I combination study of R115777 and capecitabine [Holden et al., Proc ASCO 2001], in which MTDs were 400 mg twice a day and 2000 mg/m², respectively, for 14 days every 21, and a 11% partial response rate was obtained. Myelosuppression was, as expected, DLT in the phase I combination study of R115777 plus irinotecan [217]; in this study, patients were treated with escalating doses of irinotecan with interval-modulated dosing of R115777 (continuously or on days 1-14, and repeated every 21 days). In total, 35 patients were entered this trial. MTDs were 300 mg twice a day for 14 days every 21 for R115777, and 350 mg/m² every 3 weeks for irinotecan. Three patients had a partial response and 14 had stable disease. The phase I combination study of R115777 plus topotecan has been interrupted prematurely because of inability to escalate the doses as a consequence of severe myelosuppression [Liebes et al., Proc ASCO 2001]. Myelosuppression represented DLT also in the phase I study of R115777 plus gemcitabine [218], in which MTDs were 200 mg twice a day on a continuous basis for R115777, and 1000 mg/m²/week for gemcitabine. Inhibition of farnesylation of HDJ 2 was demonstrated in peripheral blood mononuclear cells at all dose levels. Partial responses were noted in a patient with advanced pancreatic cancer and in a patient with nasopharyngeal carcinoma. In all of the above mentioned phase I combination studies with R115777, no pharmacokinetic interaction between the tested compounds was observed. R115777 has already been combined with combinations of cytotoxics, such as cisplatin-gemcitabine [219]. This study has demonstrated that the combination is well tolerated, DLT is neutropenia, MTDs were 300 mg twice a day for R115777 (administered for 14 days), 1000 mg/m² for gemcitabine (administered on days 1, 8), 75 mg/m² for cisplatin administered on day 1 of a 21 day cycle. The combination showed significant antitumor activity, since one complete response and eight partial responses were observed. Five of these patients (1 complete response and 4 partial responses) had NSCLC (one previously untreated, three progressed on a regimen of paclitaxel/carboplatin, one progressed on gemcitabine). Randomized studies are warranted to clarify the role of R115777 in this combination. A phase I study of tipifarnib plus doxorubicin-cyclophosphamide in patients with metastatic breast cancer has been recently presented [Sparano et al., Proc ASCO 2004]. Eligible patients received tipifarnib plus standard doxorubicin-cyclophosphamide chemotherapy given either every 3 weeks or every 2 weeks with granulocyte-colony stimulating factor (G-CSF) support; the protocol was then modified to a 2 week schedule with G-CSF, because of evidence for a superior outcome for dose-dense therapy in operable breast cancer. The recommended dose of tipifarnib was 200 mg bid on days 1-7 when combined with doxorubicin-cyclophosphamide plus G-CSF given every 2 weeks. Objective responses have been observed in 8 out of 9 evaluable patients. A phase I trial of tipifarnib and tamoxifen has been run in patients with hormone-receptor positive metastatic breast cancer. The combined regimen of tipifarnib and tamoxifen is well tolerated (no grade 4 toxicities reported) and exhibits promising initial clinical activity. A phase II trial is currently in progress using tipifarnib 200 mg bid and tamoxifen 20 mg daily [Lebowitz et al., Proc ASCO 2004]. R115777 is being tested also in combination with Herceptin. Only toxicity data are available for the study at this stage, and they show that myelotoxicity is dose-limiting [Schwartz et al., Proc ASCO 2001].

In conclusion, R115777 can be safely combined with several highly active anticancer drugs, dose limiting being mostly myelosuppression; phase III trials exploring the potential benefit from incorporation of R115777 into active chemotherapy regimens are indicated. A large phase III trial comparing gemcitabine plus R115777 versus gemcitabine plus placebo in advanced pancreatic cancer has been recently published [220]. This study enrolled 688 patients. No statistically significant differences in survival parameters were observed. The median overall survival was 193 days in the experimental arm and 182 days in the control arm (P=.75); 6-month and 1-year survival rates were 53% and 27% for the experimental arm versus 49% and 24% for the control arm; median progression-free survival was 112 days for the experimental arm and 109 days for the control arm. Ten drug-related deaths were reported for the experimental arm and seven for the control arm. Grade 3-4 neutropenia and thrombocytopenia were observed in 40% and 15% in the experimental arm versus 30% and 12% in the control arm; incidences of nonhematologic adverse events were similar in the 2 groups.

**BMS-214662**

BMS-214662 is a lead compound in the tetrahydrobenzodiazepine class of FT inhibitors discovered at Bristol Myers Squibb and is presently being evaluated in clinical trials, both alone and in combination. As compared to other classes of FT inhibitors, the enzyme inhibitory potencies and cell activities are comparable. In fact, in many cell types, low micromolar concentrations of these inhibitors block Ras farnesylation to near completion and BMS-214662, like other FTIs, is > 1000 fold selective for FT over GGT. How-
ever, unlike most of other FT inhibitors, which are known to be non toxic at high micromolar concentrations, BMS-214662 is cytotoxic at 2-10 micromolar concentrations, and this prompts to possible additional mechanisms of action besides FT inhibition. In vitro apoptosis was shown particularly evident in HCT-116 human colon cancer cells, when cells were exposed to BMS-214662 for > 24 hours. Consistent with its potent apoptotic activity, BMS-214662 demonstrated potent in vitro cytotoxicity against a wide cell line panel. Particularly sensitive to BMS-214662 were human tumor lines OVCAR-3 ovarian, HCT-116 colon, A431 squamous, and HL60 leukemia [143]. Potent in vivo antitumor activity in human tumor xenografts of different histologies has been observed with both orally and parenterally administered BMS-214662, regardless of the presence of ras mutation in the responding tumor lines. Taken as a whole, these results obtained with BMS-214662 sharply differ from those obtained with other FT inhibitors, pointing to BMS-214662 mostly as a cytotoxic compound. In particular, curative activity against well established tumors, retained also against a multidrug resistant tumor subline, looks a peculiar feature of this compound [143]. A major drawback for BMS-214662, which has come up from the phase I program, is its severe gastrointestinal and liver toxicities, which prevent the achievement of adequate systemic exposures following the oral route [Camacho et al., Proc ASCO 2001]. Therefore, it needs parenteral administration, which is a major disadvantage for a drug to be used on a long term basis. In a phase I study administering BMS-214662 as a weekly 1 hour infusion, evidence of activity was observed in a breast cancer patient refractory to high dose chemotherapy with a minor response lasting 5 months. Gastrointestinal toxicity and renal failure were dose limiting in this study [Voi et al., Proc ASCO 2001]. Ryan et al. have reported the results of a phase I study of BMS-214662 given as a one hour intravenous infusion every three weeks in patients with advanced solid tumors [221]. This study shows that BMS-214662 can be safely given with this schedule at a dose that results in pronounced inhibition of FT activity in peripheral blood mononuclear cells. However, the duration of inhibition was transient. GI tract toxicity was dose limiting and, although no objective responses were seen, one patient with pancreatic cancer continued to receive treatment for more than three years after entering the study. Mc Dermott et al. have reported the results of a phase I study of BMS-214662 administered intravenously as a one-hour infusion for five consecutive days every 21 days in patients with advanced malignancies. Diarrhea and neutropenia were the main toxicities in this study; the used schedule was safe at doses up to 81 mg/m2/day, but resulted in only transient inhibition of tissue farnesyltransferase, thus prompting to alternative schedules that maintain plasma concentration sufficient to inhibit farnesyltransferase [Proc ASCO 2004]. In keeping with the above studies, other pharmacokinetic and pharmacodynamic studies indicated that, in order to have optimal pharmacological activity, regimens of BMS-214662 that provide more sustained plasma exposures and FT inhibition are required [Tabanero et al., Proc ASCO 2001; Sonnichsen et al., Proc ASCO 2000, thus offering the basis for further patient recruitment with weekly 24 hour infusion schedule. Zhu et al. [Proc ASCO 2002] have recently presented preliminary data of a phase I study of BMS-214662 given as a 24 hour continuous intravenous infusion once weekly in patients with advanced solid tumours. Near maximum BMS-214662 plasma concentrations were rapidly approached during 24h-infusion. The degree of FT inhibition in peripheral blood mononuclear cells was sustained near the maximum effect for most of the time during which the drug was infused. An ongoing study is combining weekly BMS-214662 with paclitaxel, with minimal toxicity documented thus far, and 2 partial responses obtained in a patient with laryngeal cancer, and in a patient with prostate cancer, respectively [Bailey et al., Eur. J. Cancer 2002 S55 abstr]. In another combination study, patients were given BMS-214662 as a 1-hour infusion and cisplatin every 3 weeks. Liver toxicity, gastrointestinal toxicity and renal failure were dose limiting in this study; although no objective responses were recorded, stable disease was observed in 15 patients [222]. Dy et al. are carrying out a phase I study, in which BMS-214662 is being tested in combination with paclitaxel and carboplatin in a phase I study in patients with advanced cancers. Myelotoxicity, peripheral neuropathy, nausea, fatigue and diarrhea have emerged as main toxicities in this study; neutropenia, severe nausea and vomiting were DLT. Two PR were documented in relapsed ovarian cancer and in taxane-resistant esophageal cancer, respectively. Regression of evaluable endometrial cancer was also seen in this study. Long lasting (> 4 cycles) stable disease occurred in 10 patients. The combination was considered well tolerated, biologically effective (dose dependent reversible FT inhibition), and active [Proc ASCO 2004].

L-778, 123

L-778, 123 is a peptidomimetic farnesyl transferase inhibitor, which has been tested in a phase I study using a continuous infusion schedule [223]. This drug has been stopped in its clinical development due to its severe and unexpected toxicity. In particular, severe thrombocytopenia, a significant and possibly life-threatening QT prolongation, and profound fatigue represented the main toxicities. This study had the considerable merit of validating the importance of serial analyses of HDJ2, a chaperone protein that undergoes farnesylation, in peripheral blood mononuclear cells, as a pharmacodynamic marker of protein prenylation that might be useful in optimizing the development of drugs targeting farnesyl transferase [223]. L-778, 123 is the only FT inhibitor which has been tested in combination with radiation therapy up to now. Martin et al. [Proc ASCO 2004] have reported a phase I trial of L-778, 123 in combination with radiotherapy in locally advanced pancreatic cancer. The drug was given by continuous intravenous infusion with concomitant radiation therapy to 59.4 Gy in standard fractions. Two drug dose levels were tested: 280 mg/m2/day over weeks 1, 2, 4, and 5 (dose level 1), and 560 mg/m2/day over weeks 1, 2, 4, 5, and 7 (dose level 2). Two patients on dose level 2 experienced DLT consisting of grade 3 diarrhea, and grade 3 gastrointestinal hemorrhage, respectively. One patient on dose level 1 showed a partial response of six month duration. Five patients had stable disease, while 2 patients progressed. Dose level one was selected for further studies. The ability of ras oncogene to lead to radioresistance has been indicated through several independent lines of experimentation, and PI3K has been identified as the likely
downstream mediator of ras-induced radioresistance [224]. FT inhibitors, which block the processing of ras, result in radiosensitization and one of the possible mechanisms for that is the FT inhibitor-induced reduction of hypoxia in tumors with H-ras mutation [225]. A Rho B dependent mechanism for understanding the basis for this combination has also been described [226]. Combination studies of FT inhibitors and radiation therapy represent a well worth pursuing field for further trials.

Other Drugs Targeting Ras Signaling Pathway

Interference with the ras oncogene pathway can be achieved also in other ways than prevention of ras membrane localization, namely via inhibition of ras protein expression through antisense oligonucleotides, and inhibition of ras downstream effectors. The antisense approach involves targeting specific RNA sequences to block translation of the RNA message into protein. Oligonucleotides, which are complementary to mRNA transcripts of the activated ras oncogene, have been utilized to decrease ras protein expression. In particular, ISIS 2503, a phosphorothioate antisense oligodeoxynucleotide, is in clinical trials. Phase I studies with this compound have been completed, and they have shown that 6 mg/kg administered as a 14-day continuous infusion every 21 days, is a tolerable dose/schedule, moderate thrombocytopenia and fatigue representing the only adverse events [Dorr et al., Proc ASCO 1999]. Hints of antitumor activity observed in the above phase I study have prompted both phase II studies and phase I combination studies. Interruption of signaling pathways downstream of ras primarily involves the use of Raf kinase inhibitors. ISIS 5132 is a phosphorothiorate antisense oligonucleotide which inhibits ras kinase. Phase I studies using different schedules have been completed in absence of significant side effects [Holmlund et al., Proc ASCO 1999], and phase II studies are underway in colorectal, prostate, and ovarian cancer. Raf kinase inhibition can be achieved also with orally active compounds, which is the first to enter clinical trials was BAY43-9006. A few single agent phase I studies with BAY 43-9006, a novel, potent, orally active inhibitor of ras kinase, which is a significant contributor to the malignant phenotype driven by activated ras signalling, with either a 3 out of 4 week or a continuous schedule, are being conducted worldwide. This drug looks interesting, since it works in K-ras mutant and wild type ras models, and causes G1 arrest by downregulation of cyclin D1, cyclin D3, CDK 4, and p21. However, the drug is highly protein-bound in serum, and it inhibits a quite wide spectrum of kinases. Awada et al. [Eur J Cancer 2002 S52 abstr] have recently presented final results of a clinical and pharmacokinetic phase I study of BAY 43-9006 in refractory solid cancers. In this study BAY was given orally in an intermittent schedule. Skin toxicity was dose limiting in this study; 600 mg three weeks out of four was the recommended dose for phase II studies. One partial response was obtained in a patient with renal cell carcinoma, while two minor responses occurred in a patient with renal and in a patient with rectum adenocarcinoma, respectively. Skin toxicity was dose limiting also in Hirte study [Eur J Cancer 2002 S55 abstr]. In this trial the drug was given orally bid in escalating doses during the first 28 days of a 35-day cycle. Recommended dose for phase II was 400 mg bid.

Three patients had tumour shrinkage of at least 20%. Strumberg et al. [Eur J Cancer 2002 S53 abstr] have instead used BAY 43-9006 on a continuous basis. Diarrhea and skin toxicity qualified as DLT in this study in which activity in a patient with a hepatocellular carcinoma was observed; in particular, this patient had a sustained remission after 20 weeks of treatment at 400 mg bid which represented the recommended dose for phase II. Combination studies of BAY 43-9006 with cytotoxic agents, such as docetaxel and gemcitabine, are planned/ongoing. Siu et al. [Proc ASCO 2003] have presented the preliminary results of a phase I study of BAY 43-9006 plus gemcitabine in advanced solid tumors. Doses which were selected for phase II were: gemcitabine 1000 mg/m2 and BAY 43-9006 400 mg bid. One partial response in a patient with ovarian cancer, plus 11 stable diseases were observed in 16 evaluable patients.

MEK inhibitors represent a promising, non cytotoxic approach to the interruption of the Ras/MAP kinase pathway for cancer therapy. CI-1040 is an oral, highly selective small molecule inhibitor of the dual-specificity kinases, MEK 1 and MEK 2, which prevents phosphorylation and subsequent activation of mitogen-activated protein kinase (MAPK). A phase I trial of CI-1040, administered for 21 consecutive days every 4 weeks in patients with advanced cancer, is ongoing. Fatigue, skin toxicity and diarrhea have qualified as main toxicities in this trial, in which a partial response in a patient with pancreatic cancer, plus a huge number of stable diseases, were achieved. Pharmacodynamic effects were evaluated in this trial by inhibition of phosphorylation of MAPK in peripheral blood mononuclear cells, and a dose of 800 mg twice a day to be administered with food was considered as recommendable for phase II studies in order to achieve plasma concentrations necessary to inhibit the activation of MAPK in peripheral blood mononuclear cells [Proc ASCO 2002].

Statins

3-Hydroxy-3-methylglutaryl CoA reductase inhibitors, commonly referred to as statins, are a class of drugs that inhibits the rate-limiting step of the mevalonate pathway, which is essential for the synthesis of various compounds, including cholesterol [108]. These drugs have proven therapeutic and preventive effects in cardiovascular diseases; nevertheless, there are also emerging interests in their use as anticancer agents based on preclinical evidence of their antiproliferative, proapoptotic, anti-inflammatory, and radiosensitizing properties. The statin-induced inhibition of the mevalonate pathway interferes with various end products that are important for many different cellular functions; in particular, the production of FPP and GGPP is inhibited. These intermediates provide precursors for the post-translational modification known as prenylation, in which lipid moieties such as FPP and GGPP are added to intracellular proteins. Two phase I clinical trials of lovastatin in advanced malignancies have been carried out. Thibault et al. treated 88 patients with advanced solid tumors [227]. Myopathy was found to be DLT. Use of ubiquinone was associated with reversal of myopathy, and its prophylactic administration prevented the development of this toxicity in a cohort of 56 patients. One patient with anaplastic astrocytoma, who progressed after surgery, radiation therapy and two cycles of
carmustine had a minor response that was maintained for 8 months. Larmer et al. carried out a phase I-II trial of lovastatin in anaplastic astrocytoma and glioblastoma multiforme [228]. No myopathy was observed in this study. 9 out of 18 patients were concomitantly treated with radiotherapy, and no neurologic toxicity was observed. In the 9 patients treated with concurrent lovastatin and radiotherapy, there were two partial responses and two minor responses; the response duration for these patients ranged from 160 to 236 days. For the patients treated with lovastatin alone there was one partial response, which lasted in excess of 405 days, when lovastatin was discontinued because of cost, and one minor response. A phase II study of lovastatin in gastric cancer has been reported; no objective responses were reported in this study; anorexia was the most common toxicity, while two patients developed mild and reversible myalgia with elevated muscle enzymes [229]. On the other end, Kawata et al. reported positive results in a phase II randomized study of pravastatin in patients with hepatocellular carcinoma [230]. In this study, patients were randomized to receive standard treatment with or without pravastatin. The median survival was 18 months in the pravastatin group and 9 months in the control group. Finally, a phase I-II study of lovastatin in recurrent or metastatic squamous cell carcinoma of the head and neck and of the cervix is ongoing. The rationale for this study consists in the in vitro sensitivity of retinoid-responsive cancers to statins. This study explores a prolonged oral administration schedule with dose-and duration-escalation steps and includes pharmacokinetic and pharmacodynamic evaluations. The role of statins in chemoprevention of human cancer is also a matter of investigation. Graaf et al. [231] have shown a statin-induced protective effect against cancer in a large case control study. In particular, 3, 129 patients were identified and matched to 16,976 controls. Statin use was associated with a risk reduction of cancer of 20%; statins were shown to be protective when used longer than 4 years or when more than 1, 350 defined daily doses were taken. Shannon et al. [Proc ASCO 2004] have reported similar results in a case control study aimed at evaluating the protective effect of statins against the occurrence of prostate cancer. Any statin use was associated with a reduced risk of prostate cancer, with use for a longer period of time and at a higher dose conferring the greatest protection.

PERTIN тому и CONCLUSIVЕ REMARKS

The isoprenylation of intracellular proteins is an important post-translational process that is required for the activation of several signal transducers. The latter are mainly ras and ras-related small G proteins that mediate different survival and proliferative signals based on the activation of several intracellular key enzymes, such as Erk1/2, Akt and others, implicated in tumor cell growth regulation. Based on these considerations, the inhibition of isoprenylation through the targeting of its enzymatic steps is an attractive perspective for the treatment of human neoplasms. However, the mode of action of FTI and other isoprenylation inhibitors are still not completely known and initial clinical results derived from early clinical trials are not very encouraging. Pre-clinical results derived from several studies suggest the development of integrated strategies based on multi-step enzyme inhibition or on target prioritization in order to potentiate and optimize the anti-tumour activity of strategies based on the administration of inhibitors of isoprenylation. In conclusion, the inhibition of the post-translational maturation of intracellular proteins, resulting in isoprenyl residue addition, is an attractive perspective in cancer therapy, but a more detailed understanding of its physiology is required in order to obtain optimal clinical results.

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ABBREVIATION

GEF = Guanine nucleotide exchange factor
GDI = Guanine nucleotide dissociation inhibitors
FTI = Farnesyltransferase inhibitor
GTP = Guanosine triphosphate
GDP = Guanosine diphosphate
GAP = GTPase activating protein
PI3K = Phosphatidylinositol 3 kinase
MAP = Mitogen-activated protein
Erk = Extracellular signal-regulated kinase
PIP3 = Phosphatidylinositol 3, 4, 5-triphosphate
PKB = Protein kinase B
PH = Pleckstrin homology
NF-kB = Nuclear factor-kB
IKK = I-kB kinase
S6K1 = 70 kDa ribosomal S6 kinase
4E-BP1 = Eukaryotic-initiation factor 4E binding protein-1
TSC2 = Tuberous sclerosis complex-2
JNK/SAPK = c-Jun N-terminal kinases/stress-activated protein kinases
HMG-CoA = 3-hydroxy-3-methylglutaryl CoA
FPP = Farnesyl-pyrophosphate
FPPS = Farnesylpyrophosphate synthase
FTase = Farnesyltransferase
GGTase-I = Geranylgeranyltransferase-I
GGTase-II = Geranylgeranyltransferase-II
GDI = Guanine nucleotide dissociation inhibitors
CDK = Cyclin dependent kinase
BPs = Bisphosphonates
PAM = Pamidronate
ZOL = Zoledronate
IGF-IR = Insulin-like growth factor type I

\[ \text{ABBREVIATION} \]

\[ \begin{align*}
\text{GEF} & = \text{Guanine nucleotide exchange factor} \\
\text{GDI} & = \text{Guanine nucleotide dissociation inhibitors} \\
\text{FTI} & = \text{Farnesyltransferase inhibitor} \\
\text{GTP} & = \text{Guanosine triphosphate} \\
\text{GDP} & = \text{Guanosine diphosphate} \\
\text{GAP} & = \text{GTPase activating protein} \\
\text{PI3K} & = \text{Phosphatidylinositol 3 kinase} \\
\text{MAP} & = \text{Mitogen-activated protein} \\
\text{Erk} & = \text{Extracellular signal-regulated kinase} \\
\text{PIP3} & = \text{Phosphatidylinositol 3, 4, 5-triphosphate} \\
\text{PKB} & = \text{Protein kinase B} \\
\text{PH} & = \text{Pleckstrin homology} \\
\text{NF-kB} & = \text{Nuclear factor-kB} \\
\text{IKK} & = \text{I-kB kinase} \\
\text{S6K1} & = \text{70 kDa ribosomal S6 kinase} \\
\text{4E-BP1} & = \text{Eukaryotic-initiation factor 4E binding protein-1} \\
\text{TSC2} & = \text{Tuberous sclerosis complex-2} \\
\text{JNK/SAPK} & = \text{c-Jun N-terminal kinases/stress-activated protein kinases} \\
\text{HMG-CoA} & = \text{3-hydroxy-3-methylglutaryl CoA} \\
\text{FPP} & = \text{Farnesyl-pyrophosphate} \\
\text{FPPS} & = \text{Farnesylpyrophosphate synthase} \\
\text{FTase} & = \text{Farnesyltransferase} \\
\text{GGTase-I} & = \text{Geranylgeranyltransferase-I} \\
\text{GGTase-II} & = \text{Geranylgeranyltransferase-II} \\
\text{GDI} & = \text{Guanine nucleotide dissociation inhibitors} \\
\text{CDK} & = \text{Cyclin dependent kinase} \\
\text{BPs} & = \text{Bisphosphonates} \\
\text{PAM} & = \text{Pamidronate} \\
\text{ZOL} & = \text{Zoledronate} \\
\text{IGF-IR} & = \text{Insulin-like growth factor type I} \\
\end{align*} \]
EGFR = Epidermal growth factor receptor
PDGFRs = Platelet-derived growth factor receptors
TK = Tyrosine kinase
IFNα = Interferon α
JNK1α = Wild type form of JNK1
8ClcAMP = 8-chloro-cAMP
DLT = Dose-limiting toxicity
NSCLC = Non-small cell lung cancer
MDR = Multi drug resistance
P-gp = P-glycoprotein
ID = Recommended dose
AML = Acute myelogenous leukemia
MTD = Maximum tolerated dose
CI = Confidence interval
G-CSF = Granulocyte-colony stimulating factor
EIAED = Enzyme inducing anti epileptic drugs

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