Rolle der Ras-Signaltransduktion in der Pathophysiologie von myeloischen Leukämien und potentielle Effektivität von Inhibitoren der Ras-Signaltransduktionskaskade gegenüber menschlichen Tumoren.

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We dance round in a ring and suppose,

But the Secret sits in the middle and knows.

Robert Frost

Is my understanding only blindness to my own lack of understanding?

It often seems so to me.

Ist mein Verständnis nur Blindheit gegen mein eigenes Unverständnis?

Oft scheint es mir so.

Ludwig Wittgenstein

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I declare and certify herewith, that this work has been conducted by myself, without employing unauthorized procedures or materials, and that it has not been submitted to any other university or elsewhere in order to obtain an academic grade.

Michael Alexander Morgan

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Keywords: Ras, signal transduction, farnesyltransferase inhibitors, geranylgeranyl transferase inhibitors

Schlüsselworte: Ras, Signaltransduktion, Farnesyltransferase-Inhibitoren, Geranylgeranyltransferase-Inhibitoren

1. Introduction

1.1. The Ras family of GTP-binding proteins.

The expression of many different receptors on the cell surface enables cells to respond to extracellular signals provided by the environment. After ligand binding, receptor activation leads to a large variety of biochemical events in which small GTPases (e.g. Ras) are crucial. Ras (for rat sarcoma virus) proteins are prototypical GTP-binding (G-proteins) that have been shown to play a key role in signal transduction, proliferation and malignant transformation. G-proteins are a superfamily of regulatory GTP hydrolases which cycle between an inactive, GDP-bound form and an active, GTP-bound form (Sprang 1997; Bos 1998; Rebollo & Martinez 1999; Reuter *et al* 2000) (Figure 1). Regulatory proteins which control the GTP/GDP cycling rate of Ras include GTPase activating proteins (GAPs, e.g. p120 GAP, neurofibromin-1 and GAP1m) and guanine nucleotide exchange factors (GEFs, e.g. SOS and CDC25). GAPs accelerate the rate of GTP hydrolysis to GDP, while GEFs induce the dissociation of GDP to allow association of GTP (Rebollo & Martinez 1999; Crul *et al* 2001). In the GTP-bound form, Ras couples the signals of activated growth factor receptors to downstream mitogenic effectors. Proteins that interact with the active, GTP-bound form of Ras (and thus become GTP-dependently activated) in order to transmit signals are called Ras effectors (Van Aelst *et al* 1994; Marshall 1996a,b; Wittinghofer 1998; Katz & McCormick 1997). GTP-Ras influences the activity of its effectors through : (1) direct activation (e.g. B-Raf, PI-3K), (2) recruitment to the plasma membrane (e.g. c-Raf-1), and (3) association with substrates (e.g. Ral-GDS). Additional candidates for Ras effectors include protein kinases, lipid kinases and guanine nucleotide exchange factors (Van Aelst *et al* 1994; Marshall 1996; Wittinghofer 1998; Katz & McCormick 1997; Rebollo & Martinez 1999).

The Ras-like small GTPases are a superfamily of proteins that include Ras, Rad, M-Ras, Rap1A, Rap1B, Rap2, R-Ras, TC21, RalA, RalB, Rheb, Rin, and Rit (Takai *et al* 2001). The *Ras* gene family consists of three functional genes, Harvey (H-), Kirsten (K-) and neuronal (N-) *Ras*. H-*Ras* has been assigned to the short arm of chromosome 11 (11p15.1-15.5), K-*Ras* to chromosome 12 (12p12.1-pter) and N-*Ras* to chromosome 1 (1p22-32) (Barbacid 1987). The *Ras* genes encode 21 kDa proteins which contain the carboxy-terminal sequence Cys-A-A-X-COOH (Cys, cysteine; A, aliphatic amino acid; and X, any amino acid) and are associated with the inner leaflet of the plasma membrane (H-Ras, N-Ras and the alternatively spliced K-RasA and K-RasB). The Ras proteins are all comprised of 189 amino acids, except K-RasB, which has 188 amino acids. Whereas

H-Ras, N-Ras and K-RasB are ubiquitously expressed, K-RasA is induced during differentiation of pluripotent embryonal stem cells *in vitro* (Pells *et al* 1997).

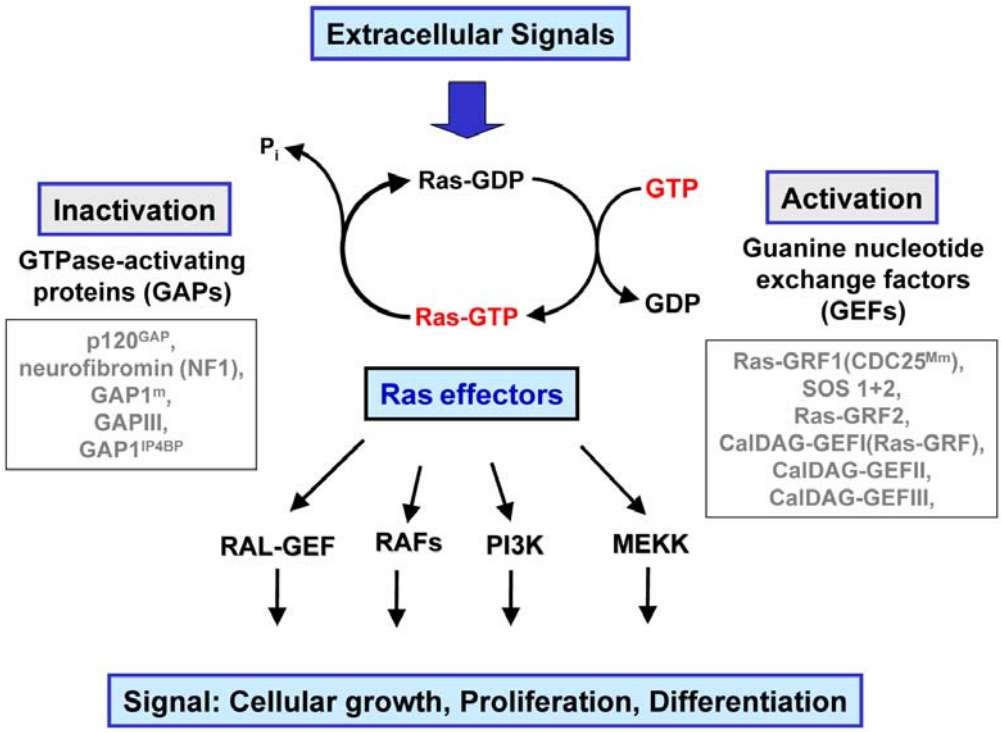

Figure 1. Schematic diagram of the switch function of Ras. Ras cycles between an active, GTP-bound and an inactive, GDP-bound state. Mitogenic signals activate guanine-nucleotide exchange factors (GEF) like SOS and CDC25. GEFs increase the rate of dissociation of GDP and stabilize the nucleotide-free form of Ras, leading to binding of GTP to Ras proteins. Ras can also be activated by the inhibition of the GTPase-activating proteins (GAPs) (modified from Reuter *et al* 2000).

1.2. Post-translational modification of Ras.

Ras proteins are produced as cytoplasmatic precursor proteins and require several post-translational modifications to acquire full biological activity. These modifications include prenylation, proteolysis, carboxymethylation and palmitoylation (Glomset & Farnsworth 1994; Zhang & Casey 1996; Casey & Seabra 1996; Gelb 1997; Mumby 1997) (Figure 2).

Protein prenylation by intermediates of the isoprenoid biosynthetic pathway is a recently discovered form of post-translational modification. At least three different

enzymes catalyze prenylation: protein farnesyltransferase (FTase), protein geranylgeranyltransferase type I (GGTase I), and geranylgeranyltransferase type II (GGTase II) (Glomset & Farnsworth 1994; Zhang & Casey 1996; Casey & Seabra 1996; Gelb 1997; Mumby 1997). Prenylated proteins share characteristic carboxy-terminal consensus sequences and can be separated into proteins with a CAAX (C, cysteine; A, aliphatic amino acid; and X, any amino acid) motif and proteins containing a CC or CXC sequence (Reiss *et al* 1990; Moores *et al* 1991; Reiss *et al* 1991; Yokoyama *et al* 1991). FTase I transfers a farnesyl group from farnesyldiphosphate (FPP) and GGTase I transfers a geranylgeranyl group from geranylgeranyldiphosphate (GGPP) to the cysteine residue of the CAAX motif (Trueblood *et al* 1993). GGTase II transfers the geranylgeranyl groups from GGPPs to both cysteine residues of CC or CXC motifs.

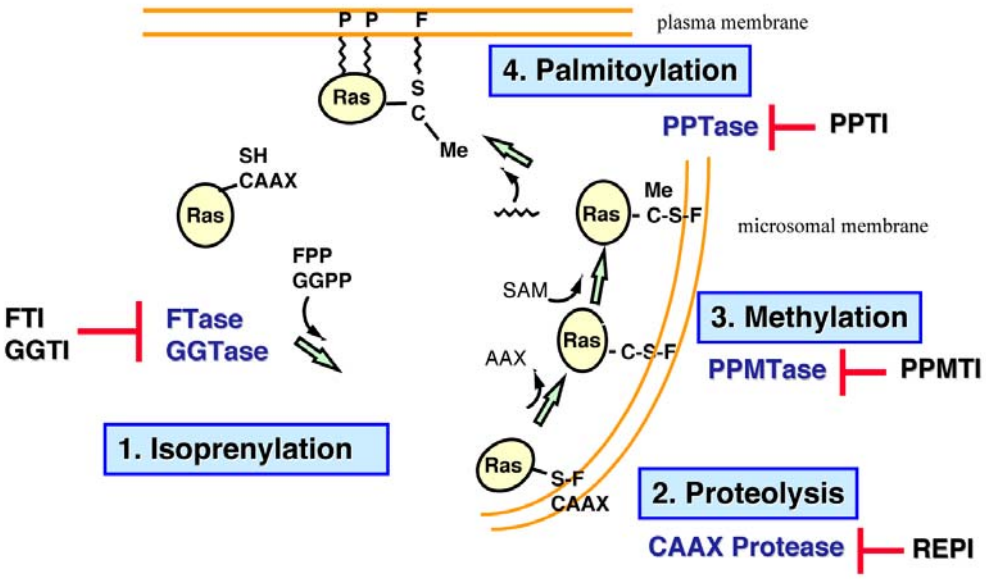

Figure 2. Overview of the post-translational modifications of Ras proteins. Protein farnesyltransferase (FTase) transfers a farnesyl group (F) from farnesyl diphosphate (FPP) to the thiol group of the cysteine residue in the CAAX motif. Alternatively, geranylgeranyltransferase type I (GGTase I) can modify K- and N-Ras with a geranylgeranyl group from geranylgeranyl diphosphate (GGPP) in the presence of farnesyl transferase inhibitors (FTIs). A CAAX-specific endoprotease removes the C-terminal tripeptide in the endoplasmatic reticulum. A prenyl protein-specific methyltransferase (PPMTase) attaches the methyl group from S-adenosylmethionine (SAM) to the C-terminal cysteine. Finally, a prenyl protein-specific palmitoyltransferase (PPTase) attaches palmitoyl groups (P) to cysteines near the farnesylated C-terminus of H- and N-Ras. FTI, farnesyltransferase inhibitor; GGTI, geranylgeranyltransferase inhibitor; REPI, Ras sequence-specific C-terminal endoprotease inhibitor; PPMTI, prenyl protein-specific methyltransferase inhibitor (modified from Reuter *et al* 2000).

The first step in the post-translational modification of Ras is farnesylation. This modification occurs by covalent attachment of a 15-carbon farnesyl moiety in a thioether linkage to the carboxy-terminal cysteine of proteins that contain the CAAX motif (Figure 3). The reaction is catalyzed by FTase, a heterodimer consisting of a 48 kDa and a 45 kDa subunit ($\alpha_{F/GGI}$ and $ß_F$). Binding sites for the substrates, farnesyldiphosphate (FPP) and the CAAX motif, are located on the α_F and $ß_F$ subunits (Pellicena *et al* 1996; Park *et al* 1997; Trueblood *et al* 1997). Substrates for FTase include all known Ras proteins, the tyrosine phosphatases PTPCAAX1 and PTPCAAX2 (Tamanoi *et al* 2001), the kinetochore-binding proteins CENP-E and CENP-F, nuclear lamins A and B, the γ subunit of the retinal trimeric G protein transducin, rhodopsin kinase, and a peroxisomal protein termed PxF (Glomset & Farnsworth 1994; Zhang & Casey 1996; Casey & Seabra 1996; Gelb 1997; Mumby 1997).

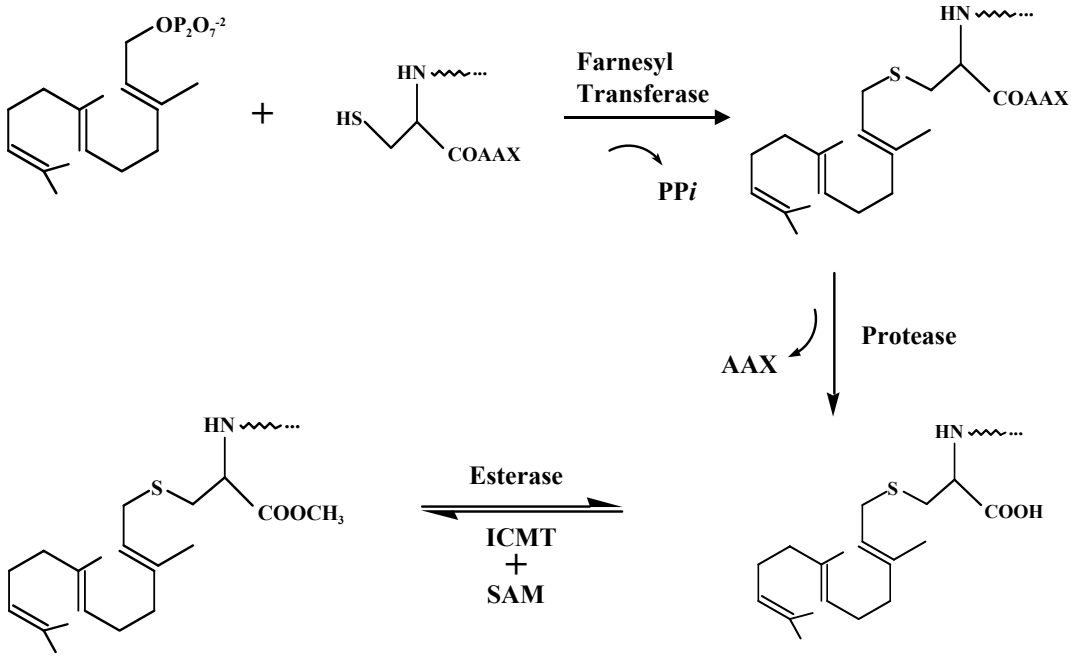

Figure 3. Farnesylation, proteolysis and reversible methylation of Ras proteins. Proteins that terminate in a CAAX motif undergo processing events including isoprenylation, C-terminal proteolytic cleavage and carboxyl methylation. In *Saccharomyces cerevisiae*, carboxyl methylation is mediated by an integral endoplasmic reticulum membrane protein named Ste14p. PPi, inorganic phosphate; ICMT, isoprenylcysteine carboxyl methyltransferase; SAM, S-adenosyl-L-methionine.

Farnesylation of Ras proteins is followed by endoproteolytic removal of the three carboxy-terminal amino acids (AAX) by a cellular thiol-dependent zinc metallopeptidase (Akopyan *et al* 1994) (Figure 3). This endoproteolytic activity (RACE for Ras and a-factor converting enzyme) is a composite of two different CAAX proteases: a zinc dependent activity encoded by AFC1 and the type IIb signal peptidase-like Rce1 (for Ras converting enzyme 1) (Boyartchuk *et al* 1997). A mammalian Rce-1 has recently been cloned and demonstrated to process Ras proteins (Otto *et al* 1999, Hollander *et al* 2000). The final step in the carboxy-terminal modification of proteins with a CAAX motif (e.g. Ras) is the methylation of the carboxyl group of the prenylated cysteine residue by the methyltransferase Icmt (Bergo *et al* 2000; Bergo *et al* 2001; Romano & Michaelis 2001; Bergo *et al* 2002).

While K-Ras contains a lysine-rich region, which is important for proper membrane localization, some Ras proteins require further modification to acquire stable membrane binding. For example, palmitoyl groups are attached to one or two cysteines near the farnesylated carboxy-termini of H-Ras, N-Ras and Ras2 (Hancock *et al* 1989; Glomset & Farnsworth 1994; Milligan *et al* 1995; Ross 1995; Zhang & Casey 1996; Casey & Seabra 1996; Dudler & Gelb 1996; Gelb 1997; Mumby 1997). Similar to farnesylation, H-Ras palmitoylation is important for signaling functions *in vivo* (Dudler & Gelb 1996). Microinjection experiments in *Xenopus* oocytes revealed that palmitoylation of H-Ras dramatically enhances its affinity for membranes, as well as its ability to activate MAP kinase and initiate meiotic maturation (Dudler & Gelb 1996; Gelb 1997). A Ras-specific protein palmitoyltransferase has been purified (Liu *et al* 1996). Additionally, a palmitoyl-protein thioesterase has been identified, purified and characterized (Camp *et al* 1994; Duncan & Gilman 1998). Recently, the crystal structure of the human putative protein acyl thioesterase (hAPT1) has been solved (Devedjiev *et al* 2000). In contrast to farnesylation and proteolysis, palmitoylation and methylation of Ras seem to be reversible and may have regulatory roles (Gelb 1997; Mumby 1997).

1.3. The Ras-to-MAP kinase signal transduction pathway.

1.3.1. ***The MAPK signaling cascades***. Mitogen-activated protein kinase (MAPK) pathways are well conserved, major signaling systems involved in the transduction of extracellular signals into cellular responses in a variety of organisms including mammals (Treisman 1996; Fanger *et al* 1997; Robinson & Cobb 1997; Elion 1998; Garrington & Johnson 1999; Schaeffer & Weber 1999). Three sequential kinases are the core

components of the MAPK signaling cascades : (1) MAP kinase (MAPK or extracellular signal-regulated kinase = ERK), (2) MAPK kinase (MAPKK, or MAPK/ERK kinase = MEK) and (3) MAPKK kinase (MAPKKK or MEK kinase = MEKK) (Figure 4). The MAPKs are activated by dual phosphorylation on tyrosine and threonine residues by upstream dual specificity MAPKKs. MAPKKs are also phosphorylated and activated by serine-/threonine-specific MAPKKKs.

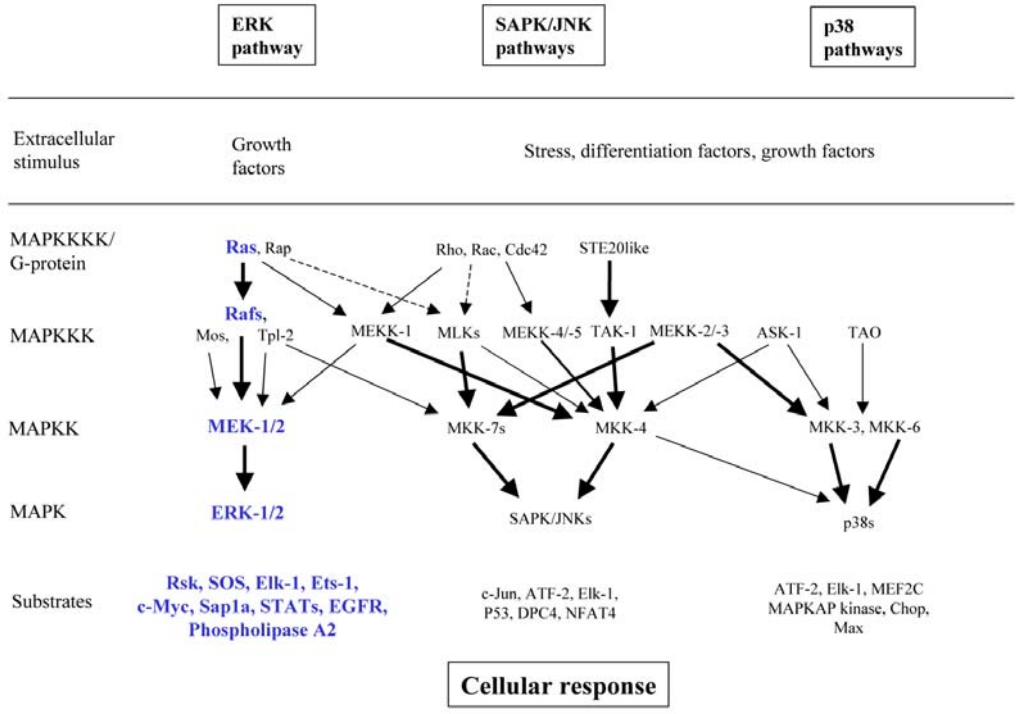

Figure 4. Mitogen-activated protein kinase modules. The MAPK cascades consist of a MAPKKK, a MAPKK and a MAPK. MAPKKK are activated through a large variety of extracellular signals like growth factors, differentiation factors and stress. The activated MAPKKK can phosphorylate and activate one or several MAPKK which, in turn, phosphorylate and activate a specific MAPK. Activated MAPK phosphorylates and activates various substrates in the cytoplasm and the nucleus of the cell including transcription factors. These downstream targets control cellular responses (e.g. apoptosis, proliferation, and differentiation) (Robinson & Cobb 1997 ; Reuter *et al* 2000).

At least six MAPK cascades have been identified in mammalian cells (Treisman 1996; Fanger *et al* 1997; Robinson & Cobb 1997; Elion 1998; Garrington & Johnson 1999; Schaeffer & Weber 1999). The best characterized MAPK signaling pathways are (1) the

Ras-to-MAP kinase signal transduction pathway (or ERK pathway) which is responsive to signals from receptor tyrosine kinases, hematopoietic growth factor receptors, and some heterotrimeric G-protein-coupled receptors which promote cell proliferation or differentiation; (2) the SAPK/JNK pathway which is activated in response to stresses such as heat, high osmolarity, UV irradiation, and proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1); and (3) the p38 pathway which is also responsive to heat shock, osmotic stress, TNF-α and IL-1 as well as lipopolysaccharide (Figure 4) (Treisman 1996; Fanger *et al* 1997; Robinson & Cobb 1997; Elion 1998; Garrington & Johnson 1999; Schaeffer & Weber 1999). Scaffolding/adapter proteins like MP-1, JSAP-1 and JIP-1 route MAPK modules in mammals by binding ERK-1 & MEK-1, JNK-3 & SEK-1 & MEKK-1, or JNK & MKK-7 & MLKs, respectively (Elion 1998; Schaeffer & Weber 1999).

1.3.2. ***Ras-to-MAPK-signaling via receptor tyrosine kinases and cytokine receptors.*** The observation that MAP kinases ERK-1 and ERK-2 are activated by various mitogens in all cells strongly supports the idea that the Ras-to-MAPK pathway is an essential shared element of mitogenic signaling. Ras functions as a membrane-associated biological switch that relays signals from ligand-stimulated receptors to cytoplasmatic MAP kinase cascades. These receptors include G-protein coupled serpentine receptors, tyrosine kinase receptors (RTKs, e.g. PDGF-, EGF-receptor) and cytokine receptors that cause stimulation of associated nonreceptor tyrosine kinases (NRTKs, e.g. src, lyn, fes). Ligand binding to the extracellular domain of receptor tyrosine kinases causes receptor dimerization, stimulation of protein tyrosine kinase activity and autophosphorylation (Schlessinger 1993; Marshall 1995; Marshall 1996a,b; Porter & Vaillancourt 1998; Pawson & Saxton 1999).

Tyrosine autophosphorylation sites in growth factor receptors (e.g. epidermal growth factor receptor, EGF-R) function as high-affinity binding sites for SH-2 (src homology) domains of signaling molecules such as PI-3 kinase, phospholipase C-γ (PLC-γ), p120-GAP, Shc, and SHP-2 tyrosine phosphatase (Porter & Vaillancourt 1998). SH-2 domains have been demonstrated to mediate interactions between proteins. Interactions of molecules that contain SH-2 domains (e.g. PLC-γ, Ras-GAP) with autophosphorylated EGF receptor indicate the importance of SH-2 domains in different tyrosine kinase signaling pathways (Margolis *et al* 1990; Moran *et al* 1990; Buday 1999).

In contrast to receptor tyrosine kinases, cytokine receptors (e.g. the prototypical IL-3, IL-5, GM-CSF-receptors) do not contain kinase domains. These receptors are heterodimers of a ligand-specific α-subunit and a β-subunit that is common to IL-3, IL-5

and granulocyte/macrophage colony stimulating factor (GM-CSF) receptors (Adachi & Alam 1998; Guthridge *et al* 1998; D'Andrea & Gonda 2000). The NRTKs Lyn and Fes and the Janus kinase JAK2 are physically associated with the β-subunit. The conserved proline-rich motifs in the α- and β-subunits (e.g. IL-3, IL-5, GM-CSF-R, IL-2-R, G-CSF-R and EPO-R) are critical for JAK2 binding and activation (Figure 5). After ligand binding and receptor dimerization, receptor-bound tyrosine kinases become activated and cause a cascade of tyrosine phosphorylations. Analogous to RTKs, these phosphotyrosines represent docking sites for many signaling molecules, including adapter proteins (e.g. PI-3K, Shc, SHP-2, Grb-2) (Adachi & Alam 1998; Guthridge *et al* 1998; D'Andrea & Gonda 2000).

It has been well established that SH3 domains, like SH2 domains, mediate protein-protein interactions. The SH3 domain of Grb-2 binds to SOS, which is a GEF for Ras and facilitates the replacement of GDP with GTP (Schlessinger 1993; Van Aelst *et al* 1994; Marshall 1995; Marshall 1996a,b; Wittinghofer 1998; Katz & McCormick 1997; Porter & Vaillancourt 1998; Pawson & Saxton 1999). When Ras becomes GTP-loaded, Ras-effectors (like Rafs, MEKK, PI-3K and Ral) bind to Ras and become activated. The Raf kinases (A-Raf, B-Raf, c-Raf-1) are important Ras effectors and have been demonstrated to act as MAPKKKs/MEKKs in the Ras-to-MAPK (or ERK) pathway by selective phosphorylation and activation of MAP kinase kinases MEK-1 and MEK-2 (Schlessinger 1993; Daum *et al* 1994; Catling *et al* 1995; Marshall 1995; Reuter *et al* 1995; Marshall 1996; Porter & Vaillancourt 1998; Pawson & Saxton 1999). Other MEK-1/MEK-2 activators include TPL-2, MEKK-1 and c-Mos (Posado *et al* 1993; Patriotis *et al* 1994; Sameron *et al* 1996). MEK-1 and MEK-2 are dual specificity kinases that activate the MAP kinases of the ERK subgroup (ERK-1 and ERK-2) (Bardwell & Thorner 1996; Crews *et al* 1992; Wu *et al* 1993; Zheng & Guan 1993; Treisman 1996; Fanger *et al* 1997; Robinson & Cobb 1997; Elion 1998; Garrington & Johnson 1999; Schaeffer & Weber 1999).

MAPK was originally described as a 42 kD insulin-stimulated protein kinase that phosphorylated the cytoskeletal protein MAP-2 (Sturgill & Ray 1986). A 44 kD MAPK isoform was identified in subsequent studies and named ERK-1 (Boulton & Cobb 1991). ERK-1 and ERK-2 are proline-directed protein kinases that phosphorylate Ser/Thr-Pro motifs in the consensus sequence Pro-Xaa_n-Ser/Thr-Pro, where Xaa is any amino acid and n=1 or 2. Several cytoplasmatic and nuclear substrates of the ERKs have been identified. The best-characterized ERK substrates are cytoplasmatic phospholipase A_2 ($cPLA_2$), the

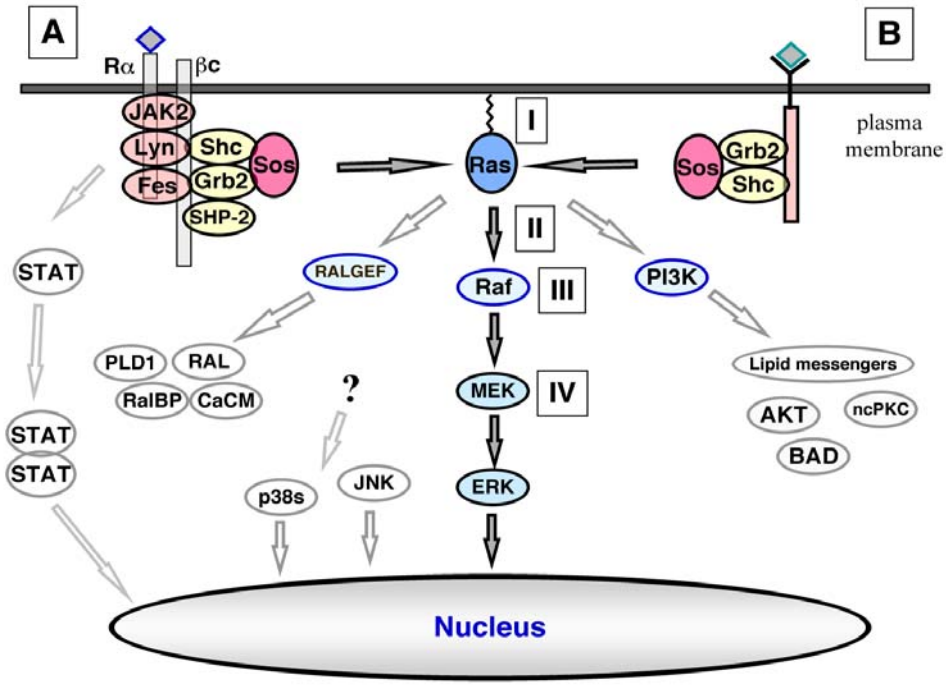

Figure 5. The classical Ras-to-MAP kinase cascade. (A) Signaling by cytokine receptors. IL-3, IL-5 and GM-CSF receptors consist of a ligand-specific α-subunit and a common β-subunit. The β-subunit binds the NRTKs Lyn, Fes and JAK2. After ligand binding, the α and β subunits are thought to dimerize, thus activating the receptor-bound NRTKs and subsequently causing a cascade of tyrosine phosphorylations. The phosphotyrosine residues represent docking sites for various signaling molecules (e.g. Shc, SHP-2). ERKs are activated via the classical Ras-to-MAPK pathway. In addition, the MAP kinases p38 and JNK become activated. The activation pathway is not completely understood but some lines of evidence support involvement of Ras and/or HPK-1 (for hematopoietic progenitor kinase, a mammalian Ste20-related protein). Activated JAK2 phosphorylates the STAT family of nuclear factors (which form hetero- and homodimers) thus causing their translocation to the nucleus and subsequent binding to γ-activating sequences of the promoter region of various genes (Adachi & Alam 1998; Guthridge *et al* 1998; D'Andrea & Gonda 2000). **(B) Signaling by RTKs.** Extracellular stimuli such as mitogens or stress cause intracellular activation of different MAP kinase cascades. The ERK1/2 pathway is activated by mitogens in all cells and is an essential part of mitogenic signaling. Nuclear translocation of activated ERKs leads to activation of transcription factors like Elk-1, CREB, SRF and fos (Pawson & Saxton 1999). Raf kinases connect upstream tyrosine kinases and Ras with downstream serine/threonine kinases. When Ras becomes GTP-loaded, Rafs bind to Ras. It is unclear if Ras-Raf binding is itself always sufficient to activate the Raf kinases, which subsequently phosphorylate and activate the downstream MEKs. GTP-Ras also binds and activates PI 3-kinase (PI 3K) and Ral-GEF. PI-3K produces lipid second messengers, which activate AKT (for Akt kinase) and non-conventional isoforms of protein kinase C (ncPKC). Ral-GEF activates Ral-GTPases by promoting the GTP-bound state of Ral. Ral-GTP binds to Ral-BP1 (a GAP for CDC42 and Rac), phospholipase D (PLD1) and calcium calmodulin-dependent protein kinase (CaCM). I, inhibitors of Ras membrane association (e.g. FTI, GGTI, PPMTI, and REPI); II, sulindac; III, Raf kinase inhibitors (e.g. Bay439006, GW5074 and ZM336372); IV, MEK inhibitors (e.g. PD098059, U0126 and Ro09-2110) (modified from Reuter *et al* 2000).

ribosomal protein S6 kinases (RSKs) and the nuclear transcription factor Elk-1 (Treisman 1996; Xing *et al* 1996; Jaaro *et al* 1997; Robinson & Cobb 1997).

Activated Elk-1 forms a complex with serum response factor and the serum response DNA element present in many promoters. Additionally, transcription factors Fos and Jun are stimulated by MAPK and join to form the full nuclear transcription factor AP-1. AP-1 initiates transcription of the *myc* gene, which leads to induction of D-type cyclin expression and activity. D-type cyclins are important for the G_1 progression into S phase of the cell cycle (Lloyd *et al* 1997; Pumiglia & Decker 1997; Khosravi-Far *et al* 1998).

1.4. The Ras-to-Ral and the Ras-to-PI-3 kinase signaling pathways.

Since the discovery of Raf as a direct Ras-effector, numerous other putative Ras-effectors have been identified. Among these, evidence to date best supports "effector" roles for the Ral-GEFs (Ral-GDS, RGL and RGF) and the p110 subunit of PI-3K (Van Aelst *et al* 1994; Carpenter & Cantley 1996; Feig *et al* 1996; Marshall 1996; Pells *et al* 1997; Wittinghofer 1998; Katz & McCormick 1997; Rebollo & Martinez 1999) (Figure 5).

Ral-GEFs are activated via binding to GTP-Ras. Ral-GEFs, in turn, activate Ral-GTPases by promoting the GTP-bound state of Ral. Since they are members of the Ras subfamily of Ras-related GTPases, Ral proteins (RalA and RalB) also cycle between the active GTP-bound states and inactive GDP-bound states. Ral-GTP binds Ral-BP1 (for Ral-binding protein1 or Rlip1 = Rip1, for Ral-interacting protein 1) which is a GAP for CDC42 and Rac. As shown in Figure 4, the GTPases CDC42 and Rac are involved in regulation of the actin cytoskeleton, the SAPK/JNK pathway and the p38 pathway.

Ras-GTP also binds to and activates the catalytic domain of PI-3 kinase. The lipid second-messenger molecules produced (e.g. phosphatidylinositol phosphates PtdIns 3,4-P_2 and PtdIns 3,4,5-P_3) activate the phosphoinositide-dependent kinases PDK1 and PDK2 which then activate Akt kinase and non-conventional isoforms of protein kinase C (ncPKC). PI-3K has been implicated in four apparently distinct cellular functions including mitogenic signaling (DNA synthesis), inhibition of apoptosis, intracellular vesicle trafficking/secretion, and regulation of actin and integrin functions. These functions are most likely mediated by distinct phosphoinositide products of PI-3K (Carpenter & Cantley 1996) (Figure 5).

1.5. Role of Ras activation in hematological malignancies.

The constitutive activation of Ras appears to be an important factor for the malignant growth of human cancer cells. While the role of R-Ras, M-Ras and TC21 in human malignancies is unclear, studies using cell lines have demonstrated that these Ras-related proteins also possess transforming activities similar to those of Ras (Cox *et al* 1994; Graham *et al* 1994; Quilliam *et al* 1999). Overexpression of normal Ras can lead to transformation, however, malignant transformation resulting from mutations is much more common (Rodenhuis 1992). Mutations of *Ras* proto-oncogenes (H-*Ras*, K-*Ras*, N-*Ras*) are frequent genetic aberrations found in 20-30% of all human tumors, although the incidences in tumor type vary greatly (Bos 1989; Clark & Der 1995; Reuter *et al* 2000). The highest rate of *Ras* mutations were detected in adenocarcinomas of the pancreas (90%), the colon (50%) and the lung (30%) as well as in follicular and undifferentiated carcinomas of the thyroid (50%). *Ras* mutations occur at sites critical for Ras regulation (e.g. codons 12, 13, 15, 16, 18, 31, 59 and 61) and increase the half-life of activated Ras-GTP through abrogation of normal intrinsic and/or GAP-stimulated GTPase activity of Ras (Bos 1989; Clark & Der 1995; Sprang 1997; Lin *et al* 1998; Lin *et al* 2000; Reuter *et al* 2000). While wild-type Ras-GTP has a half-life of one to five hours, the half-life of activated forms have been reported to be up to nine times longer (Gibbs *et al* 1984; Sweet *et al* 1984). Transformation results, at least in part, from unregulated stimulation of the mitogenic signal transduction pathway (Bos 1989; Clark & Der 1995).

Ras activation is frequently observed in hematological malignancies such as myeloid leukemias and multiple myelomas. *Ras* genes are mutationally activated in approximately one-third of the myelodysplastic syndromes (MDS) and acute myeloid leukemias (AML) (Bos *et al* 1987; Janssen *et al* 1987; Farr *et al* 1988; Padua *et al* 1988; Bos, 1989; Browett *et al* 1989; Browett & Norton 1989; Hirsch-Ginsberg *et al* 1990; Vogelstein *et al*, 1990; Byrne & Marshall 1998; Reuter *et al*, 2000; Schaich *et al* 2001) (Table 1). N-*Ras* is mutated and activated in the majority of the cases and the presence of the mutation is not associated with any particular FAB type, cytogenetic abnormality or clinical feature including prognosis (Byrne & Marshall 1998; Schaich *et al* 2001). *Ras* mutations occur in 40-100% of newly diagnosed multiple myeloma patients and the frequency increases with disease progression (Hallek *et al* 1998; Bezieau *et al* 2001; Kalakonda *et al* 2001). Mutations in N-*Ras* – especially codon 61 mutations – are more frequent than K-*Ras* mutations (Neri *et al* 1989; Corradini *et al* 1993; Hallek *et al* 1998; Kalakonda *et al* 2001).

In addition to activation by mutation, Ras is thought to be deregulated by constitutive activation of proto-oncogenes and inactivation of tumor suppressor genes (Sawyers & Denny 1994; Hunter 1997). Several types of human cancers show oncogenic activation of receptor and/or non-receptor tyrosine kinases. Constitutively activated versions of normal receptor tyrosine kinases contain single point mutations (e.g. colony-stimulating factor-1 (CSF-1) receptor, the Neu/Erb-B2 receptor, and the c-Kit receptor), duplications of juxtamembrane domain-coding sequences (e.g. FLT3 receptor) or deletions of the negative regulatory regions in the ligand binding or the transmembrane domains (e.g. Erb-B receptor). Point mutations of the CSF-1 receptor (c-FMS) at codons 301 and 969 were found in 10-20% of acute myeloblastic leukemia (AML) or myelodysplasia (MDS) (Tobal *et al* 1990; Padua *et al* 1998). Point mutations in the catalytic domain of the c-Kit receptor have been detected in some cases of myeloproliferative disorders and in 10% of the patients with mastocytosis (Nakata *et al* 1995; Nagata *et al* 1995; Buttner *et al* 1998). Additionally, activating tandem internal duplication of the FLT3 receptor has been reported in 20% of AML (Kiyoi *et al* 1999). Activating point mutations in the tyrosine kinase domain of the FLT3 receptor, most predominantly at position D835, have also been observed in AML patients (Abu-Duhier *et al* 2001; Thiede *et al* 2002; Gilliland & Griffin 2002). The members of the c-Kit/c-FMS receptor kinase family (e.g. c-Kit, c-FMS, FLT3) are linked with components of the Ras-to-MAPK signaling pathway (e.g. Grb-2 and Shc) suggesting that activating mutations of c-FMS and FLT3 may induce activation of Ras (Dosil *et al* 1993; Rohrschneider *et al* 1997).

Several chimeric proteins resulting from translocations involving receptor tyrosine kinases have been found in human hematological malignancies (Sawyers & Denny 1994; Hunter 1997) (Table 1). (1) Several Tel fusion proteins have been reported. (a) Tel-PDGFRß is a fusion protein consisting of the transcription factor Tel (for translocation, Ets, leukemia) and the platelet-derived growth factor receptor ß (PDGFRß), a well-known receptor tyrosine kinase (Golub *et al* 1994; Jousset *et al* 1997). It is generated by the t(5;12) translocation in a subset of chronic myelomonocytic leukemias (CMML) which results in receptor dimerization and activation, and thus leads to the constitutive activation of the Ras-to-MAP kinase pathway. (b) Tel-Abl, is generated by the t(12;9) translocation in acute myeloid leukemias (AML) (Papadopoulous *et al* 1995; Golub *et al* 1996). (c) Tel-ARG, t(1;12), consists of the oligomerization domain of Tel and all of the functional domains of ARG, including the SH2, SH3 and tyrosine kinase domains (Cazzaniga *et al* 1999; Iijima *et al* 2000). (d) Tel-TRKC is produced by the t(12;15) translocation that

Table 1. Activation of Ras in hematological malignancies.

Malignancy	Type of Ras Activation	Frequency	References
A.	***Ras* Point Mutations**		
Acute myeloid leukemia (AML)	K-, N-*Ras*	20-30%	Bos *et al* 1987; Janssen *et al* 1987; Bos 1989; Byrne & Marshall 1998; Reuter *et al*, 2000; Schaich *et al* 2001
Childhood AML	K-, N-*Ras*	20-40%	Farr *et al*, 1988; Vogelstein *et al*, 1990
Acute lymphoblastic leukemia (ALL)	K-, N-*Ras*	20%	Neri *et al* 1988; Browett *et al* 1989; Browett & Norton 1989
Chronic myelomonocytic leukemia (CMML)	K-, N-*Ras*	50-70%	Padua *et al* 1988; Hirsch-Ginsberg *et al* 1990; Sawyers & Denny 1994
Juvenile myelomonocytic myeloid leukemia (JMML)	N-*Ras*	30%	Miyauchi *et al* 1994
Multiple myeloma	K-, N-*Ras*	30-40%	Neri *et al* 1989; Tanaka *et al* 1992; Corradini *et al* 1993; Liu *et al* 1996; Hallek *et al* 1998;
		50-80%	Bezieau *et al* 2001;
		100%	Kalakonda *et al* 2001
Plasma cell leukemia	K-, N-*Ras*	50-70%	Neri *et al* 1989; Tanaka *et al* 1992; Corradini *et al* 1993; Hallek *et al* 1998; Bezieau *et al* 2001
B.	**c-*Kit*/c-*FMS* Family Receptor Mutations**		
Acute myeloid leukemia (AML)	*CSF-1* (*c-FMS*)	10-20%	Tobal *et al* 1990; Padua *et al* 1998
	FLT-3	20-34%	Dosil *et al* 1993;Stirewalt *et al* 2001; Yamamoto *et al* 2001
Myeloproliferative disorder, Mastocytosis	c-*kit*	10%	Nagata *et al* 1995; Nakata *et al* 1995; Buttner *et al* 1998
C.	**Fusion Tyrosine Kinases**		
Acute myeloid leukemia (AML)	Tel-Abl, t(12;9)		Papadopolous *et al* 1995; Golub *et al* 1996
	Tel-ARG, t(1:12)		Cazzaniga *et al* 1999; Iijima *et al* 2000
	Tel-TRKC, t(12;15)		Eguchi *et al* 1999; Liu *et al* 2000
	CEV14-PDGFR-β, t(5;14)		Abe *et al* 1997
Anaplastic large cell lymphoma	Npm-Alk, t(2;5)	30-40%	Elmberger *et al* 1995; Waggott *et al* 1995
Chronic myeloid leukemia (CML)	Bcr-Abl, t(9;22)	95%	Kurzrock *et al* 1988; Faderl *et al* 1999; Zou & Calame 1999
	Bcr-FGFR1, t(8:22)		Demiroglu *et al* 2001
Chronic myelomonocytic leukemia (CMML)	Tel-PDGFR-β, t(5;12)		Golub *et al* 1994; Jousset *et al* 1997
	HIP1-PDGFR-β, t(5;7)		Ross *et al* 1998; Ross & Gilliland 1999
D.	**Inactivation of Tumor Suppressors**		
Juvenile myelomonocytic myeloid leukemia (JMML)	Inactivation of NF-1 (Ras-GAP)		Kalra *et al* 1994; Bollag *et al* 1996; Largaespada *et al* 1996; Side *et al* 1997

includes the pointed domain of Tel and the protein tyrosine kinase domain of TRKC, a receptor tyrosine kinase that is activated by neurotrophin-3. Tel-TRKC variants have been reported to be potent activators of the MAP kinase pathway (Eguchi *et al* 1999; Liu *et al* 2000). (2) The Npm-Alk fusion protein, a fusion of the N-terminal portion of Npm with the entire cytoplasmatic domain of the receptor tyrosine kinase Alk, is generated by the t(2;5) chromosomal translocation in anaplastic large cell lymphoma (Elmberger *et al* 1995; Waggott *et al* 1995). (3) Abl is a non-receptor tyrosine kinase that is also mutated and activated in chronic myelogenous leukemia (CML) (Kurzrock *et al* 1988; Faderl *et al* 1999; Zou & Calame 1999). In Bcr-Abl, the product of the t(9;22) translocation, the N-terminal Bcr portion serves as an oligomerization domain. Bcr-Abl is a constitutively activated cytosolic tyrosine kinase that causes abrogation of growth factor dependence, blockade of differentiation and direct inhibition of apoptosis. Although Ras mutations are extremely rare in CML, the involvement of Ras has been demonstrated in Bcr-Abl positive cells by the presence of increased levels of GTP-Ras, which leads to the activation of the Raf kinases and other Ras effectors (Kurzrock *et al* 1988; Faderl *et al* 1999; Zou & Calame 1999). Thus the deregulation of Ras function appears to be a common theme in the transformation by activated receptor and non-receptor tyrosine kinases. Ras activation may cause elevated cell cycle progression and inhibition of apoptosis (Kurzrock *et al* 1988; Sawyers & Denny 1994: Hunter 1997; Byrne & Marshall 1998; Faderl *et al* 1999; Zou & Calame 1999).

In addition to oncogenes, tumor suppressor genes have also been found to be involved in the deregulation of Ras. The product of the NF-1 tumor suppressor gene, neurofibromin, encodes a Ras-GTPase activating protein (GAP) and is mutated in the autosomal dominant type 1 neurofibromatosis which is associated with an increased tendency to develop myeloid leukemias, especially JMML (DeClue *et al* 1992; Kalra *et al* 1994; Bollag *et al* 1996; Largaespada *et al* 1996). About 15% of children with JMML have clinical neurofibromatosis (Niemeyer *et al* 1997). Additionally, inactivating mutations of the NF1 gene have been found in 15% of JMML without clinical diagnosis of neurofibromatosis suggesting the existence of NF1 mutations in approximately 30% of all JMML cases (Shannon *et al* 1994; Side *et al* 1997). Ras involvement is demonstrated by the observation of moderately elevated percentages of GTP-Ras in leukemic cells from children with neurofibromatosis type 1 (DeClue *et al* 1992; Kalra *et al* 1994; Bollag *et al* 1996; Largaespada *et al* 1996). Furthermore, 15-30% of JMML cases lacking the NF1 mutation have activating *Ras* mutations (Miyauchi *et al* 1994). The observation that

human JMML cells exhibit hypersensitivity to granulocyte/macrophage colony-stimulating factor suggests a common pathophysiological mechanism involving downstream Ras signaling (Miyauchi *et al* 1994; Largaespada *et al* 1996; Birnbaum *et al* 2000).

The pathophysiological importance of the Ras-to-MAPK signaling pathway is underscored by the positioning of several oncogene and tumor suppressor gene products on this pathway. Furthermore, it has recently been demonstrated that mutant N-*Ras* induces myeloproliferative disorders resembling human chronic myelogenous leukemia (CML), acute myeloid leukemias and apoptotic syndroms similar to human myelodysplastic syndromes (MDS) in bone marrow repopulated mice (MacKencie *et al* 1999). These observations make Ras and the Ras-to-MAP kinase pathway a rational target for the development of new anticancer agents.

1.6. Inhibitors of the Ras-to-MAP kinase pathway.

1.6.1. *Inhibitors of Ras farnesyl transferase (FTase).*

Elimination of Ras function by homologous gene recombination or antisense RNA has demonstrated that expression of activated Ras is necessary for maintaining the transformed phenotype of tumor cells (Mukhopadhyah *et al* 1991; Saison-Behmoaras *et al* 1991; Shirasawa *et al* 1993; Kashani-Sabet *et al* 1994). Inhibitors of oncogenic Ras activity may therefore prove useful as anticancer agents against Ras-induced tumors. One strategy to impede oncogenic Ras function *in vivo* is the inhibition of Ras post-translational modification. It has been demonstrated that mutation of the evolutionarily conserved CAAX motif in Ras abolishes plasma membrane binding as well as transforming activity (Gibbs 1991; Lowy & Willumsen 1995; Omer & Kohl 1997; Heimbrook & Oliff 1998). Although Ras undergoes several steps of post-translational modification, only farnesylation is necessary for its membrane localization and cell transforming activity (Kato *et al* 1992). Therefore, it has been proposed that the activity of oncogenic Ras could be blocked by inhibiting the farnesyl transferase (FTase) responsible for this modification. However, many CAAX-containing proteins need additional palmitoylation for stable membrane association.

FTase is an attractive target for the development of anticancer agents because control of Ras farnesylation can control the function of oncogenic Ras (Gibbs 1991; Lowy & Willumsen 1995; Omer & Kohl 1997; Heimbrook & Oliff 1998). Numerous inhibitors of FTase have been synthesized or identified. These FTase inhibitors can be grouped into five classes:

(1) <u>FPP analogs</u> such as (α-hydroxyfarnesyl) phosphonic acid, β-ketophosphonic and β-hydroxyphosphonic acid derivatives and J-104,871 (Kato *et al* 1992; Kang *et al* 1995; Yonemoto *et al* 1998) (Figure 6).

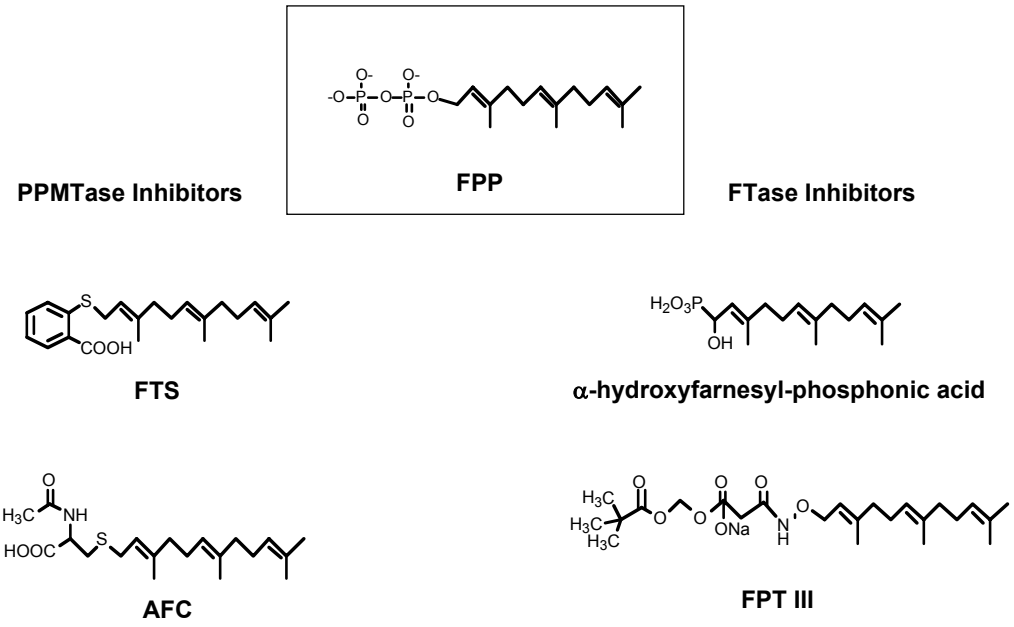

Figure 6. Chemical structures of FPP and FPP-based inhibitors of FTase and PPMTase. FPP is composed of a hydrophobic farnesyl group and a highly charged pyrophosphate moiety. The basic structural element in the FTase inhibitors is a farnesyl group, a pyrophosphate isostere and a linker.

(2) <u>CAAX peptide analogs</u> such as BZA-5B, BZA-2B (James *et al* 1993, 1995; Dalton *et al* 1995), L-731,734, L-731,735, L-739,749 (Kohl *et al* 1993, 1994; Prendergast et al 1994; Lebowitz *et al* 1997; Emanuel *et al* 2000), L-739,787 (Koblan *et al* 1995), L-739,750, L-744,832 (Kohl *et al* 1994, 1995; Barrington *et al* 1998; Sepp-Lorenzino *et al* 1995; Mangues *et al* 1998), B581 (Cox *et al* 1994), Cys-4-ABA-Met and Cys-AMBA-Met (Qian *et al* 1994), FTI-276, FTI-277 (Sun *et al* 1995; Lerner *et al* 1995; Bredel *et al* 1998; Bernhard *et al* 1998), B956 and its methyl ester B1096 (Nagasu *et al* 1995) (Figure 7).

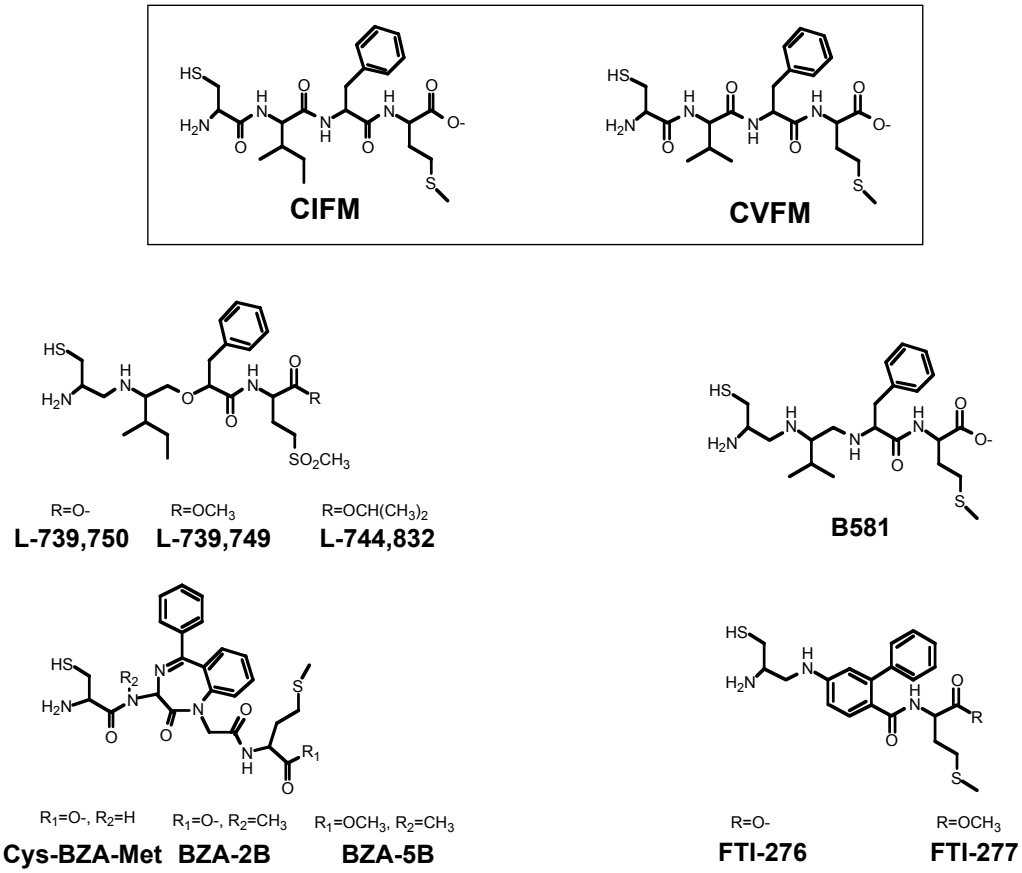

Figure 7. Chemical structures of CAAX-based FTase inhibitors. Comparison between CAAX-based FTase inhibitors of the pseudopeptide class and the CAAX tetrapeptides CIFM and CVFM. The potent, nonsubstrate FTase inhibitors CIFM and CVFM were identified by systematic amino acid replacements within the CAAX-sequence. In FTI-276 and FTI-277, the AA-residues of the CAAX-motif have been replaced by a hydrophobic linker.

(3) **<u>bisubstrate inhibitors</u>** such as phosphonic acid analogs, the phosphinate inhibitors BMS-185878 and BMS-186511, the phosphonate inhibitor BMS-184467, phosphinyl acid-based derivatives, and the hydroxamine acid analogs (Patel *et al* 1995, 1996; Manne *et al* 1995) (Figure 8).

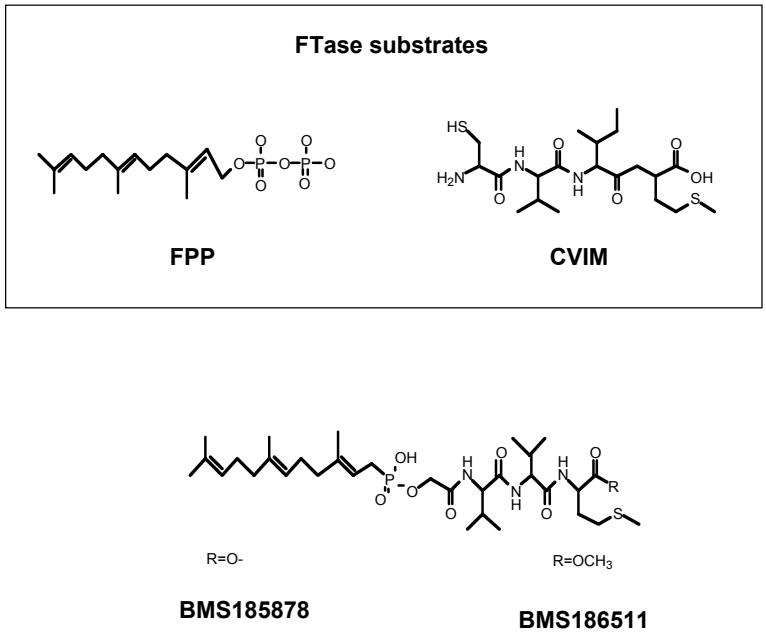

Figure 8. Structures of bisubstrate inhibitors of FTase. In bisubstrate FTase inhibitors the farnesyl group of FPP and the tripeptide group of the CAAX motif are connected via a linker.

(4) In addition, **<u>nonpeptidic, tricyclic FTase inhibitors</u>** have been developed such as SCH44342, SCH54429, SCH59228 and SCH66336 (Bishop *et al* 1995; Njoroge *et al* 1998a, 1998b; Mallams *et al* 1998; Liu *et al* 1999) (Figure 9).

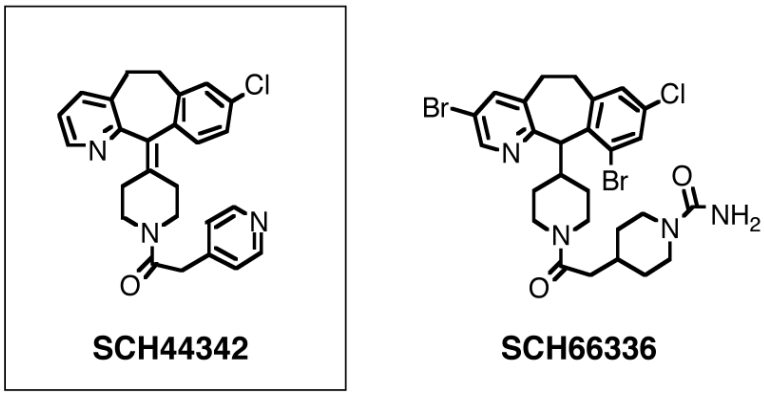

Figure 9. Structures of nonpeptidic, tricyclic FTase inhibitors. FTase inhibitor SCH44342 had no *in vivo* efficacy. Further substitutions led to SCH66336, a highly potent FTase inhibitor, which is currently being tested in several clinical phase I and II trials.

(5) Several **natural products** have also been identified as FTase inhibitors. These include limonene (Gelb *et al* 1995), manumycin (UCF1-C) and related compounds UCF1-A and UCF1–B (Hara *et al* 1993; Nagase *et al* 1997; Kainuma *et al* 1997), chaetomellic acid A and B, zaragozic acids, pepticinnamins, gliotoxin (Tamanoi 1993), barceloneic acid A (Jayasuriya *et al* 1995), RPR113228 (Van der Pyl *et al* 1995), actinoplanic acids A and B (Silverman *et al* 1995), oreganic acid (Silverman *et al* 1997), lupane derivatives (Sturm *et al* 1996), saquayamycins (Sekizawa 1996), valinoctin A and its analogs (Tsuda *et al* 1996), ganoderic acid A and C (Lee *et al* 1998).

1.6.1.1. *Effects of FTase inhibitors (FTIs) in tumor cells.*

Treatment of Ras-transformed cells with FTase inhibitors results in selective suppression of Ras-dependent oncogenic signaling. This includes the inhibition of Ras-processing which results in: (1) decreased relative amounts of fully processed Ras; (2) progressive, dose-dependent cytoplasmatic accumulation of unprocessed Ras and inactive Ras-Raf complexes; (3) inhibition of the Ras-induced constitutive activation of MAP kinase (Nagase *et al* 1997; Jayasuriya *et al* 1995; Van der Pyl *et al* 1995; Tsuda *et al* 1996; Mahgoub *et al* 1999); and (4) decreased transcriptional activity of both c-Jun and Elk-1 (Nagase *et al* 1997). Transformation by mutationally activated Raf, MEK, Mos or Fos (all of which are downstream of Ras) is not blocked by FTase inhibitors (Njorge *et al* 1998; Gelb *et al* 1995).

1.6.1.2. *Results of FTIs in animal models.*

FTIs have been demonstrated to revert Ras-dependent transformation and cause regression of Ras-dependent tumors in animal models without causing gross systemic toxicity in animals (Gibbs & Oliff 1997; Omer & Kohl 1997; Heimbrook & Oliff 1998). Some inhibitors (e.g. R115777, BMS-214662, SCH66336, and L-778,123) are presently being evaluated in phase I and II clinical trials in human cancers, including AML (Adjei *et al* 2000; Reuter *et al* 2000; Zujewski *et al* 2000; Britten *et al* 2001; Crul *et al* 2001; Eskens *et al* 2001; Karp *et al* 2001; Karp 2001; Punt *et al* 2001).

1.6.2. *Inhibitors of geranylgeranyl transferase I (GGTase I).*

Since K-*Ras* mutations are most common in human cancers (Bos 1989; Clark & Der 1995), development of inhibitors that block growth of human tumors that harbor activated K-Ras is a critical goal. Additionally, resistance of K-Ras to FTase inhibitors (James *et al* 1996), lack of potency of FTase inhibitors against K-Ras-transformed cells (Nagasu *et al* 1995) and the discovery that K- and N-Ras become geranylgeranylated in the

presence of FTase inhibitors (James *et al* 1995; Whyte *et al* 1997; Zhang *et al* 1997; Rowell *et al* 1997; Lerner *et al* 1997) demonstrate the importance of developing GGTase I inhibitors. The structures of several GGTase I inhibitors are shown in Figure 10. GGTI-279, GGTI-287, GGTI-297, GGTI-298, GGTI-2133 and GGTI-2147 are CAAL-based peptidomimetics that are selective for GGTase I over FTase (Lerner *et al* 1995; Qian *et al* 1998; Vogt *et al* 1999; McGuire *et al* 1996; Miquel *et al* 1997; Vogt *et al* 1997; Vasudevan *et al* 1999).

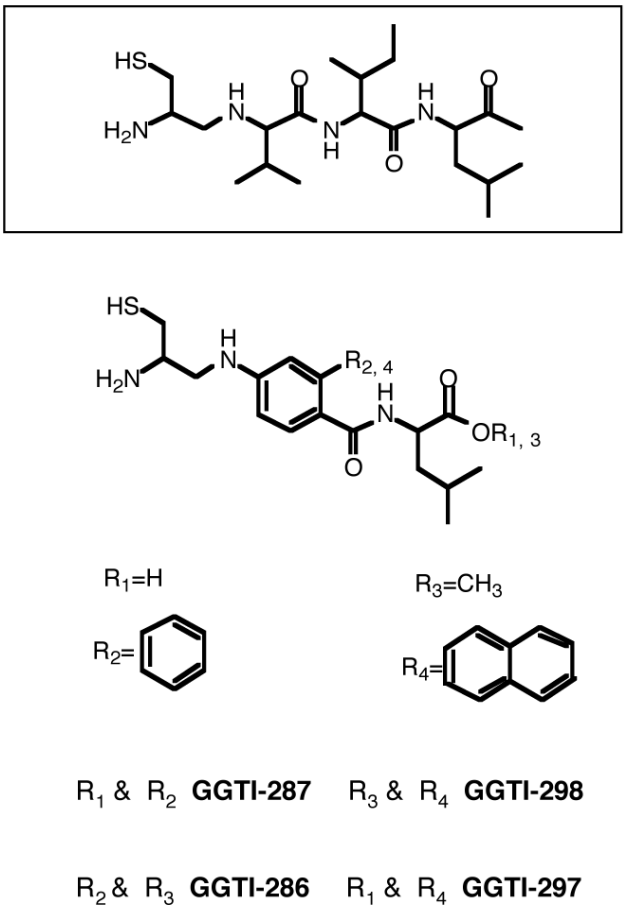

Figure 10. Structures of geranylgeranyl transferase I inhibitors. GGTase I catalyzes the geranylgeranylation of proteins terminating with CAAX sequences where X is restricted to leucine, isoleucine or to a lesser extent, phenylalanine. In cells, geranylgeranylation of proteins is far more common than farnesylation. Proteins modified by GGTase I include Rap1A, Rap1B, Rac1, Rac2, G25K, and RhoA.

1.6.2.1. ***Effects of GGTase inhibitors in tumor cells.*** H-Ras processing in human tumor cell lines was demonstrated to be highly sensitive to FTI-277 and resistant to GGTI-286,

while K-Ras4B processing was found to be more sensitive to GGTI-286 than FTI-277 (Lerner *et al* 1995a,b). While processing of H-Ras and N-Ras was inhibited by FTI-277, inhibition of K-Ras processing required both FTase and GGTase I inhibitors (Sun *et al* 1998). Furthermore, FTI-277 was demonstrated to preferentially block activation of MAP kinase by oncogenic H-Ras, while GGTase inhibitors were found to selectively inhibit the activation of MAP kinase by oncogenic K-Ras4B (Lerner *et al* 1995a,b). GGTI-298 blocks PDGF- and EGF-dependent tyrosine phosphorylation of their respective receptors and induces G_0/G_1-phase arrest and apoptosis (Vogt *et al* 1996; McGuire *et al* 1996; Miquel *et al* 1997). The GGTI-induced G_1-block has recently been shown to be due to upregulation of transcription of the CDK inhibitor $p21^{WAF1/CIP1}$ (Vogt *et al* 1997; Adnane *et al* 1998). One way GGTIs upregulate $p21^{WAF1/CIP1}$ is through inhibition of RhoA geranylgeranylation. Rho proteins facilitate progression from G_1 to S phase in growth-stimulated cells by promoting degradation of the CDK inhibitor $p27^{Kip1}$ and by downregulating the $p21^{WAF1/CIP1}$ promoter (Hirai *et al* 1997; Adnane *et al* 1998). Additionally, treatment with GGTIs results in inhibition of retinoblastoma protein (pRb) phosphorylation and partner switching of cyclin-dependent kinase inhibitors, which are important for the G_1/S transition (Sun *et al* 1999).

Recently, synergistic efficiency of a FTI/GGTI combination in adrenocortical and human colon cancer cells containing mutant K-Ras has been reported (Mazet *et al* 1999; Di Paolo *et al* 2001). In a nude mouse xenograft model, both FTI and GGTI were required to inhibit prenylation of oncogenic K-Ras, but each alone was sufficient to suppress human tumor growth (Sun *et al* 1998; 1999). Furthermore, FTI/GGTI cotreatment and treatment with a dual prenylation inhibitor (DPI) which has both FTI and GGTI activity, resulted in higher levels of apoptosis in K-Ras transformed cells relative to FTI and GGTI alone (Lobell *et al* 2001). While the CAAL-based GGTIs described by Sun *et al.* (1998) were non-toxic in mice, the chemically distinct GGTIs and DPIs used by Lobell *et al.* (2001) revealed strong toxicity in mice which may have been caused by an activity unrelated to GGTase-I inhibitory activity (Lobell *et al* 2001).

1.6.3. *Inhibitors of the prenylated protein methyltransferase (PPMTase).*

The C-terminal prenylated protein methyltransferase (PPMTase) is another potential therapeutically relevant target in the development of inhibitors against the post-translational processing of Ras. N-acetyl-*trans*,*trans* -farnesyl-L-cysteine (AFC) is a substrate for PPMTase, and acts as a competitive inhibitor (Volker *et al* 1991). Although

AFC has been shown to inhibit Ras methylation in Ras-transformed NIH3T3 fibroblasts, it does not inhibit the growth of these cells (Volker *et al* 1991). New farnesyl derivatives of rigid carboxylic acid, e.g. S-*trans*,*trans*-farnesylthiosalicylic acid (FTS), have been demonstrated to inhibit the growth of H-Ras-transformed cells and to reverse their transformed morphology by a mechanism unrelated to the inhibition of Ras methylation by PPMTase (Marciano *et al* 1995; Marom *et al* 1995). FTS is thought to interact with Ras farnesylcysteine binding domains and affect membrane-anchorage of Ras (Marciano *et al* 1995; Marom *et al* 1995). In addition, it has been reported that FTS dislodges Ras from H-Ras-transformed cell membranes and renders the Ras protein susceptible to proteolytic degradation (Haklai *et al* 1998; Jansen *et al* 1999). In contrast to FTase inhibitors (e.g. BZA-5B), FTS also inhibited the growth signaling of receptor tyrosine kinases (Marom *et al* 1995). FTS was shown to decrease total cellular Ras levels, MAPK activity, Raf-1 activity and DNA synthesis in Ras-transformed EJ-1 cells. This inhibition was also demonstrated in serum-, EGF- and thrombin-stimulated, untransformed Rat-1 cells (Gana-Weisz *et al* 1997; Haklai *et al* 1998). Recently, FTS was shown to : (1) reduce the amount of activated N-Ras and wild-type Ras isoforms in human melanoma cells and Rat-1 fibroblasts, (2) disrupt ERK-signaling, (3) revert their transformed phenotype and (4) cause a significant reduction in human melanoma growth in SCID mice (Haklai *et al* 1998). S-farnesyl-thioacetic acid (FTA), another competitive inhibitor of PPMTase, has been shown to suppress growth and induce apoptosis in HL-60 cells (Perez-Sala *et al* 1998).

The dorrigocins are novel antifungal antibiotics that were found to reverse the morphology of Ras-transformed NIH3T3 fibroblasts through inhibition of the C-terminal methylation in K-Ras transformed cells (Kadam & McAlpine 1994).

1.6.4. *Selective inhibitors of Ras C-terminal sequence-specific endoprotease.*

UM96001, TPCK and BFCCMK are Ras C-terminal sequence-specific endoprotease inhibitors (REPI) and potently inhibit ras-transformed rat kidney cell growth as well as growth of human cancer cells (Chen 1999). These compounds have been reported to almost completely block the anchorage-independent clonogenic growth of these cancer cells, possibly through selective induction of apoptosis (Chen 1999).

1.6.5. *Selective inhibitors of Raf-1 kinase.*

Bay439006, a Raf-1 inhibitor which has demonstrated efficacy in cell and animal assays, is currently being tested in Phase I trials in advanced or metastatic cancers

(Strumberg *et al* 2001). An ATP-competitive Raf-1 inhibitor with IC_{50} values between 0.3 and 2 μM against anchorage-independent growth has also been developed (Heimbrook *et al* 1998). Additionally, several compounds such as ZM 336372, SB 203580, and GW 5074 were found to block ERK1 and ERK2 activation in cells with IC_{50} values in the low micromolar range (Hall-Jackson *et al* 1999a,b; Lackey *et al* 2000).

1.6.6. *Selective inhibitors of MAP kinase kinases (MEK).*

PD098059 is a synthetic inhibitor of the Ras-to-MAP kinase pathway that selectively blocks activation of MEK-1 and, to a lower extent, activation of MEK-2 (Alessi *et al* 1995; Dudley *et al* 1995). Inhibition of MEK-1 activation was demonstrated to prevent activation of MAP kinases ERK-1/2 and subsequent phosphorylation of MAP kinase substrates both *in vitro* and in intact cells. In contrast to FTase inhibitors, PD098059 inhibited stimulation of cell growth by several growth factors (Alessi *et al* 1995; Dudley *et al* 1995). Furthermore, PD098059 reversed the transformed phenotype of Ras-transformed BALB3T3 mouse fibroblasts and rat kidney cells (Dudley *et al* 1995). PD098059 failed to inhibit the stress and IL-1 stimulated JNK/SAPK and the p38 pathways (Alessi *et al* 1995), demonstrating its specificity for the ERK pathway.

Recently, two novel inhibitors of MEK-1 and MEK-2 have been identified: U0126 (DeSilva *et al* 1998; Favata *et al* 1998) and Ro 09-2210 (Williams *et al* 1998). U0126 and PD098059 are noncompetitive inhibitors with respect to both MEK substrates (ATP and ERK) and bind to free MEK as well as MEK*ERK and MEK*ATP complexes. U0126 displays significantly higher affinity for all forms of MEK (44- to 357-fold) than does PD098059. U0126 and Ro 09-2210 have an IC_{50} of 50-70 nM, whereas PD098059 has an IC_{50} of 5 μM (DeSilva *et al* 1998; Favata *et al* 1998; Williams *et al* 1998). In contrast to U0126 and PD098059, Ro 09-2210 also inhibits other dual specificity kinases such as MKK-4, MKK-6 and MKK-7, albeit at 4-10-fold higher IC_{50} concentrations compared to its effect on MEK-1 (Williams *et al* 1998).

Another highly selective inhibitor of MEK activation, PD184352, has recently been demonstrated to potentiate apoptosis induced by Bcl-2 inhibition in AML cell lines which contain constitutively activated MAPK (Milella *et al* 2002). PD184352 is currently being evaluated in Phase I clinical oncology trials (Van Becelaere *et al* 2001).

1.6.7. *Inhibitors of Ras-transformation with unknown mechanisms of action.*

Screening tests for drugs that revert *Ras*-transformed cells to a normal phenotype led to the identification of a number of compounds such as azatyrosine, oxanosine and antipain (Cox *et al* 1991; Itoh *et al* 1989; Shindo-Okado *et al* 1989). The mechanism by which these compounds revert the *Ras*-induced phenotype is not understood. The pyrazolo-quinoline compound SCH51344 was identified based on its ability to depress human smooth muscle α-actin promoter activity in *Ras*-transformed cells. Treatment of *v-abl*-, *v-mos*-, *v-raf*-, *Ras*- and mutant active MEK-transformed NIH3T3 cells resulted in growth inhibition of these cells in soft agar (Kumar *et al* 1995). SCH51344 had very little effect on the activities of proteins in the ERK-pathway. The ability of SCH51344 to inhibit the anchorage-independent growth of RAC-V12-transformed Rat1 cells suggests that the point of inhibition is downstream from RAC (Walsh *et al* 1997).

The non-steroidal, anti-inflammatory drug sulindac has been demonstrated to attenuate the growth and progression of colonic neoplasms in animal models and in patients with familial adenomatous polyposis (Giardiello *et al* 1993; Verhuel *et al* 1999). It was recently demonstrated that sulindac sulfide (the active metabolite of sulindac) inhibits Ras signaling and transformation by non-covalent binding to the Ras protein. Furthermore, it has been demonstrated that sulindac sulfide impairs Ras-Raf binding, Raf activation, nucleotide exchange on Ras and that it accelerates the Ras-GTPase reaction (Herrmann *et al* 1998). Sulindac is currently being investigated in a randomized study for the prevention of colon cancer.

Disruption of the Ras-to-MAPK signaling pathway has also been shown for the benzoquinone ansamycin geldanamycin. Geldanamycin binds to HSP90 and disrupts the HSP90-Raf-1 multimolecular complex, which causes destabilization of Raf-1 through enhanced degradation of Raf-1 (Schulte *et al* 1996). However, the geldanamycin-HSP90 complex also causes depletion of other HSP90 substrates such as protein kinases and nuclear hormone receptors (including mutant p53 and ErbB2) (Stebbins *et al* 1997). Several NCI-sponsored clinical phase I trials are currently studying the effects of geldanamycin analogues in advanced malignancies.

1.7. Ras-signaling and effects of inhibitors of Ras-signaling in myeloid leukemias.

Based on the wealth of data reporting the effectiveness of Ras signaling inhibitors against human carcinomas harboring activated Ras, coupled with the implications of Ras in the pathophysiology of myeloid leukemias, the role of an activated Ras pathway in

myeloid leukemia cells was investigated in this study. Additionally, the effectiveness of different types of Ras-signaling inhibitors on leukemia cell growth, cell cycle progression and induction of apoptosis was studied.

2. Material and Methods

2.1. Materials

2.1.1. *Reagents and Solutions*

DNA Molecular Weight Markers	100 bp DNA Ladder (MBI Fermentas)
Protein Molecular Weight Markers	BenchmarkTM Prestained Protein Ladder (Invitrogen, Life Technologies)
0.5 M EDTA	186 g $Na_2EDTA{\cdot}2H_2O$ in 800 mL H_2O pH 8 with NaOH add H_2O to 1 L
50 X TAE	2M Tris 0.57 % acetic acid 50 mM EDTA, pH 8
6X Agarose gel loading buffer	30 % Glycerol 0.25 % (w/v) Bromophenol blue 0.25 % (w/v) Xylenecyanol
10 X TBS	24.2 g Tris 80 g NaCl pH to 7.6 with 0.1 M HCl Add H_2O to 1 L
4 X SDS-PAGE loading buffer	200 mM Tris-HCl, pH 6.8 400 mM DTT 8 % SDS 0.4 % Bromophenol blue 40 % Glycerol
SDS-PAGE stacking gel (30 mL)	17.4 mL H_2O 5.1 mL 30 % Acrylamide (29:1) 7.5 mL 0.5 M Tris, pH 6.8, containing 0.1 % SDS 150 µL 25% APS 20 µL TEMED
SDS-PAGE separating gel (50 mL)	20.8 mL H_20 16.6 mL 30 % Acrylamide (29:1) 12.5 mL 1.5 M Tris, pH 8.8, containing 0.4 % SDS 125 µL 25% APS 20 µL TEMED
10 X Glycine stripping buffer	2 M Glycine (75.07 g in 500 mL) pH to 2.6 with 1 M HCl

LB broth	10 g tryptone 5 g NaCl 5 g yeast extract 1 mL of 1 M NaOH Add H_2O to 1 L and autoclave.
LB agar	Add 15 g agar to 1 L LB broth and sterilize by autoclaving.
SOC medium	20 g tryptone 0.5 g NaCl 5 g yeast extract 10 mL 250 mM KCl Add H_2O to 900 mL and adjust pH to 7 with 1M NaOH. Add H_2O to 975 mL and autoclave. Let cool, then add : 5 mL 2 M $MgCl_2$ 20 mL 1M glucose

2.1.2. ***Cell lines.*** Cell lines (AML = HL-60, PLB-985, Kasumi-1, Mutz-2, NB4, THP-1, OCI-AML2, OCI-AML5, Mutz-3, ML-2, MV4-11, Mono-Mac-1, KG-1, and M-07e; CML = EM-2, K562, MEG-01, LAMA84, and JK-1) were obtained from the German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany). Growth-factor-dependent cell lines (Mutz-2, Mutz-3, M-07e, and OCI-AML5) were grown in medium supplemented with 10% supernatant of cell line 5637, which produces several growth factors (e.g. SCF, IL-1, IL-6, G-CSF, GM-CSF and others). V-12- and L-61-H-*Ras*-transformed NIH3T3 fibroblasts were a gift from M. Weber (University of Virginia, USA).

2.1.3. ***Antibodies.*** Antibodies against ERK-1/2, monophospho-ERK1/2, MEK-1/2, c-Myc, Rheb, RhoB, Rap2A, Lamin A/C and B, CENP-E and CENP-F, H-, K-, and N-Ras were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Diphospho-ERK-1/2 (PP-ERK-1/2) was purchased from Sigma-Aldrich (Deisenhofen, Germany). Diphospho-MEK-1/2 (PP-MEK-1/2), phospho-c-Myc, CREB-1 and phospho-CREB-1 antibodies were from New England Biolabs (Frankfurt, Germany).

2.1.4. ***Inhibitors of Ras processing and signaling.*** The following inhibitors were purchased from Calbiochem-Novabiochem (Bad Soden, Germany): B581, Cys-4Abs-Met, FPT2, FPT3, FTI276, FTI277, GGTI-286, GGTI-287, GGTI-297,

GGTI-298, GGTI-2133, GGTI-2147, FTS, PD098059 and U0126. FTI L-744,832 was obtained from Biomol Inc. (Plymouth Meeting, PA).

2.1.5. ***Plasmid containing the c-Raf-1 domain that binds to activated Ras.*** The pGEX 2T-RBD construct (encoding a GST fusion protein containing amino acids 51-131 of c-Raf-1) was a gift from J. Bos (De Rooij & Bos 1997).

2.2. Methods

2.2.1. ***Mammalian cell culture.*** Cell lines were grown in RPMI medium containing 10% heat-inactivated fetal calf serum, glutamine (292 μg/mL) and antibiotics (penicillin 60.2 mg/mL, streptomycin 133 μg/mL). Primary AML cells and purified CD34+ cells were grown in StemSpan^{TM} supplemented with 100 ng/mL rh SCF, 100 ng/mL rh Flt3-ligand, 20 ng/mL rh IL-3, and 20 ng/mL rh IL-6 (CellSystems Biotech., St. Katharinen, Germany). Cultures were maintained in a humidified atmosphere at 37 ˚C and 5 % CO_2.

2.2.2. ***Trypan blue exclusion assay.*** 2.5 or 6.25 x 10^4 cells in 250 μl of media were seeded in 96-well plates and incubated 4 days with either solvent control or the stated concentration of inhibitor. Cell counts were evaluated using a 1:1 dilution of cell suspension in 0.4 % trypan blue solution (Sigma-Aldrich, Deisenhofen, Germany). Viable and nonviable cells were counted in a Neubauer cell counting chamber.

2.2.3. ***Colony forming assays.***

2.2.3.1. ***Myeloid leukemia cell lines.*** Cells were seeded at 1.0-2.5 x 10^5/mL of cell suspension in 96-well plates and treated with inhibitors as indicated. After four days, aliquots of the cell suspensions were plated in 400 μL of methylcult H4230 (CellSystems Biotech., St. Katharinen, Germany) according to the manufacturer's instructions and incubated 7-14 days.

2.2.3.2. ***CD34+ cells.*** G-CSF primed CD34+ cells were harvested by leukapheresis from a healthy volunteer, purified to $\geq$98% by magnetic cell sorting (Clini MACS, Miltenyi Biotech, Germany) and cryopreserved in liquid nitrogen. CD34+ cells were seeded at 0.88 x 10^5/mL in StemSpan^{TM} SF

Expansion Medium supplemented with 100 ng/mL rhFlt3-ligand, 100 ng/mL rhSCF, 20 ng/mL rhIL-3, 20 ng/mL rhIL-6 and incubated four days with inhibitors as indicated. Aliquots of the cell suspensions were plated in 400 μL MethocultSF^{BIT} H4436 (CellSystems Biotech., St. Katharinen, Germany). Aggregates of more than 25 cells were scored as colonies.

2.2.4. *Sequencing of Ras mutations.*

2.2.4.1. ***Primers for PCR amplification of Ras codons 12, 13 and 61.*** Synthetic oligonucleotides were purchased from MWG-Biotech AG (Ebersberg, Germany) for use as amplification primers to identify mutations in codons 12, 13 and 61 of H-*Ras*, K-*Ras* and N-*Ras* (Hirsch-Ginsberg *et al* 1990; Mortazavi *et al* 2000).

Primer sequences:

H-RAS 12,13; 5′: $^{5'}$GAC GGA ATA TAA GCT GGT GG
H-RAS 12,13; 3′: $^{5'}$TCC ATG GTC AGC GCA CTC TT
H-RAS 61; 5′: $^{5'}$AGA CGT GCC TGT TGG ACA TC
H-RAS 61; 3′: $^{5'}$CGC ATG TAC TGG TCC CGC AT
K-RAS 12,13; 5′ : $^{5'}$GAC TGA ATA TAA ACT TGT GG
K-RAS 12,13; 3′: $^{5'}$CTA TTG TTG GAT CAT ATT CG
K-RAS 61; 5′: $^{5'}$TTC CTA CAG GAA GCA AGT AG
K-RAS 61; 3′: $^{5'}$CAC AAA GAA AGC CCT CCC CA
N-RAS 12,13; 5′: $^{5'}$GAC TGA GTA CAA ACT GGT GG
N-RAS 12,13; 3′: $^{5'}$CTC TAT GGT GGG ATC ATA TT
N-RAS 61; 5′: $^{5'}$GGT GAA ACC TGT TTG TTG GA
N-RAS 61; 3′: $^{5'}$ATA CAC AGA GGA AGC CTT CG

2.2.4.2. ***Amplification of genomic DNA from cell lines.*** Genomic DNA samples were prepared from cultured myeloid leukemic cells using the QIAamp DNA Mini Kit from QIAgen (Hilden, Germany). PCR products of 103 and 109 base pairs (bp) containing the codons of interest were obtained by amplification (35 cycles) of 200 ng of genomic DNA in 50 μL reactions containing 200 μM each dNTP, 20 pmol 5′primer, 20 pmol 3′primer and 1 unit of AmpliTaq Gold (PE Corporation, Norwalk, CT). The

polymerase was activated by heating the samples at 94 °C for 12 min. The different *Ras* genes were amplified with the following PCR programs :

H-Ras 12, 13 and 61

Denaturation :	94 °C, 30 s
Annealing :	60 °C, 30 s
Extension :	72 °C, 1 min

	K-Ras 12, 13	K-Ras 61
Denaturation :	94 °C, 30 s	
Annealing :	55 °C, 30 s	58 °C, 30 s
Extension :	72 °C, 1 min	

	N-Ras 12, 13	N-Ras 61
Denaturation :	94 °C, 30 s	
Annealing :	56 °C, 30 s	58 °C, 30 s
Extension :	72 °C, 1 min	

2.2.4.3. ***Purification of PCR products.*** The PCR products were purified by 3% agarose gel electophoresis. Agarose gels were prepared by dissolving 3 g of agarose in 100 mL of 1X TAE buffer and adding 0.5 μg/mL of ethidium bromide to the cooled (50 °C) solution immediately prior to casting the gel. The gels were run at constant voltage (100 V) and the DNA bands were visualized by UV trans-illumination. The size of PCR products was determined by co-migration of DNA molecular weight markers (100–1000 bp, MBI Fermentas, Inc.). The bands of the correct size were excised from the gel with scalpels and the purified PCR products were recovered from the gel slices with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and eluted with 30 μL sterile water.

2.2.4.4. ***Cloning of purified PCR products.*** The TOPO-TA Cloning Kit from Invitrogen Corp. (Carlsbad, CA) was used to clone PCR products as follows :

Purified PCR product	2 μL
Sterile H_2O	2 μL
PCR-2.1-TOPO vector	1 μL

The reaction was allowed to proceed 5 min at room temperature before termination by addition of 1 μL of high salt stop buffer and vortexing. Then 2 μL of the cloning reaction were gently mixed with one half vial of one

shot competent cells (Invitrogen Corp.), incubated 30 min on ice, and heat shocked 30 sec at 42 °C. 250 μL of room temperature SOC medium were added and the cells were shaken (225 rpm^{-1}) horizontally at 37 °C. After 30 min, 30 μL of the cells were spread on LB-Agar plates (containing 50 μg/mL ampicillin and coated with 30 μL of 20 mg/mL X-Gal) and incubated overnight at 37 °C.

2.2.4.5. ***Small-scale preparation of plasmid DNA.*** Individual colonies were picked and grown in 5 mL LB-Amp medium for 12-16 hours. The bacteria were pelleted by centrifugation (2000 g, 10 min) and plasmid DNAs were purified using the QIAprep Spin Miniprep kit from Qiagen GmbH. The bacteria pellets were resuspended, lysed and the plasmid DNA was bound to the column matrix. After washing, the plasmids were eluted in sterile H_2O and quantified by spectrophotometry (1 OD_{260} corresponds to 50 μg of double stranded DNA).

2.2.4.6. ***Sequencing of PCR products inserted into plasmids.*** M13 forward and reverse primers were employed for sequencing plasmids using the Big Dye Sequencing kit from Perkin Elmer. These two primers bind to the regions of the plasmid that flank the insertion site. The 20 μL sequencing reactions contained 400-600 ng plasmids harboring Ras PCR products, 5 pmol of primer (M13 forward or reverse primer), and 4 μL of Sequenase Master Mix (PE Applied Biosystems). The first cycle of the PCR sequencing protocol included a polymerase activation step (96 °C, 2 min) and the remaining 24 cycles were run as follows :

Denaturation : 94 °C, 5 s
Annealing : 55 °C, 5 s
Extension : 60 °C, 4 min

The PCR products were precipitated for 10 min at room temperature with 50 μL absolute ethanol and 2 μL 3M NaOAc (pH 5.2), washed with 250 μL 70% ethanol, air-dried (30 min), redissolved in 20 μL HPLC-grade H_2O and analyzed by an ABI Prism Sequence Detection System (PE Corporation, Norwalk, CT).

2.2.5. ***Western blot analysis.*** Cells were collected by centrifugation, washed twice with PBS and then incubated 5-10 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 or 500 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 1 mM sodium ortho-vanadate, 1 mM dithiothreitol, and 10 µg/mL pepstatin). Cell debris and insoluble proteins were removed by centrifugation for 15 min at 12,000g and 4 ˚C. Soluble proteins were recovered by transferring the supernatants to clean microcentrifuge tubes. Cellular protein concentrations were determined using the Coomassie dye-binding assay according to Bradford (Bradford 1976) (Bio-Rad Laboratories, Hercules, CA). Equal amounts of total protein from cells were mixed with SDS-PAGE loading buffer and analyzed by SDS-PAGE. Electrophoresis was accomplished at 30 V until loading dye migrated through the stacking gel and then the voltage was increased to 100 V at room temperature or to 250 V if the cooling apparatus was used (8 ˚C). Proteins were then transferred from the gel to PVDF membranes (Immobilon-P, Millipore) by semi-dry or tank transfer.

2.2.5.1. ***Semidry Protein Transfer.*** For each gel to be transferred, six pieces of chromatography paper and one PVDF membrane were cut to the size of the gel. Two papers were soaked in anode buffer I (30 mM Tris-Base, 20 % MeOH) and placed on the anode. Air bubbles were removed. A third paper was soaked in anode buffer II (300 mM Tris-Base, 20 % MeOH) and placed on top of the other two papers. Air bubbles were removed. The activated membrane (20 seconds with MeOH and soaked 5 min in anode buffer II) was placed on top of the stacked papers. Air bubbles were carefully removed. The gel (soaked 10-15 min in cathode buffer) was placed on top of the membrane. The remaining three papers were soaked in cathode buffer (25 mM Tris-Base, 40 mM glycine) and placed over the gel. Any air bubbles were carefully removed and the cathode was connected. Semi-dry transfers were accomplished at constant power (0.8 mA/cm^2 of membrane).

2.2.5.2. ***Tank blot transfer of proteins.*** Two pieces of chromatography paper were cut for each gel to be transferred. Transfer cassette and sponges were submerged in tank blot transfer buffer (9 g Tris, 21.6 g glycine, 0.02 %

SDS, 10 % MeOH in 3 L total volume). One chromatography paper was placed on top of a sponge submerged in buffer, the membrane was centered on the paper, the gel was placed on the membrane and the second chromatography paper covered the gel. The stack was completed by covering with the second sponge, the cassette was closed and put into the transfer tank. Transfers were accomplished at constant power (0.5 A) for 3 h at 8 ˚C.

2.2.5.3. ***Incubation of membranes with antibodies.*** Membranes were blocked for 1-2 h at room temperature with 5 % (w/v) skim milk in 100 mM Tris-buffered saline (TBS), pH 7.6. After blocking, membranes were washed three times in 100 mM TBS/0.1% (v/v) Tween-20 (TBS-T). In most cases, primary antibodies were used at a dilution of 1:1000 and incubated with the membranes for 1 h at room temperature in TBS-T. Phospho-specific antibodies were incubated overnight at 4 ˚C in TBS-T. Membranes were then washed 3 times with TBS-T and incubated 1 h at room temperature with the appropriate horse raddish peroxidase-coupled secondary antibody at a 1:3000 dilution in TBS-T. The membranes were washed 3 times with TBS-T, incubated 1 min with a chemiluminescence solution (ECL kit, Amersham-Buchler, Braunschweig, Germany) and then exposed to film to visualize the protein bands.

2.2.5.4. ***PVDF membrane stripping.*** Membranes were re-soaked for 20 seconds in MeOH and then washed three times (5-10 min each wash) in TBS with 0.2 % Tween-20. The membrane was then soaked in 25 mL of 200 mM glycine (pH 2.6) for 1 h at room temperature. The stripping buffer was changed three times during the 1 h incubation. After stripping, the membrane was washed twice (10 min each wash) with TBS-T (0.1 %), blocked and reused.

2.2.6. ***MAP kinase assays***. MAP kinase assays were performed by immunoprecipitation of 1 mg total cellular protein with 4 mg of sepharose beads coupled to 10 μL rabbit αERK-2 antibodies with rotation 1-2 h at 4 ˚C in 1 mL total volume of lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 % Triton

X-100, 5 mM EDTA, 50 mM NaF, 200 μM Na_3VO_4, 100 μM PMSF, 40 mM 4-nitrophenyl phosphate disodium salt hexahydrate, 20 μg/mL leupeptin, 20 μg/mL pepstatin A, and 20 μg/mL aprotinin) (Reuter *et al* 1995 a,b). Beads were collected by centrifugation, washed twice with 1 mL of lysis buffer, and washed twice with 1 mL of 50 mM HEPES buffer, pH 7.5, containing 10 mM $MgCl_2$ and 1 mM DTT. Beads were resuspended in kinase reaction buffer (50 mM HEPES buffer, pH 7.5, containing 10 mM $MgCl_2$, 1 mM DTT, 20 μM ATP, 0.25 mg/mL myelin basic protein and 12 μL γ-32P-ATP, mixed by vortexing and incubated 30 min at 30 ˚C. The kinase reaction was terminated by addition of loading buffer and the reactions were analyzed by 15 % SDS-PAGE. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore) by semi-dry transfer method and the amount of radioactive myelin basic protein was determined by autoradiography and by scintillation counting of the membranes. After autoradiography, the membranes were subjected to Western blotting to determine relative amounts of activated, di-phospho-ERK-1/2. The membranes were subsequently stripped and probed for ERK-2 as a control for loading equal protein amounts of the samples.

2.2.7. ***Ras-GTP pulldown assay.*** RAS-GTP pulldown assays were accomplished essentially as described (De Rooij & Bos 1997). Bacteria containing the construct were used to innoculate 5 mL of LB-Ampicillin (10 g bacto-tryptone, 5g bacto-yeast extract, 10 g NaCl, 0.2 mL 5 M NaOH add water to 1 L and autoclave; when solution cools, add ampicillin to 50-100 μg/mL) medium and grown 12-16 h at 37 ˚C and 225 rpm^{-1}. Two mL of the overnight culture were diluted in 250 mL of LB-Amp medium and grown to an Abs_{600} of 0.6-1. GST-RBD expression in *E. coli* was then induced with 1 mM IPTG for 2-3 hours. Bacteria were collected by centrifugation and sonicated in 50 mL PBS containing 0.5 mM DTT and 1 μg/mL aprotinin, leupeptin and pepstatin A. After addition of 1% Triton X-100, clarified lysates were aliquoted and stored at –80 ˚C as glycerol stocks (10%). The fusion protein was purified on glutathione-Sepharose beads (35 μL per sample, Pharmacia, Uppsala, Sweden). For affinity precipitation, the beads were washed three times with Mg^{2+}-lysis buffer (1 mL), incubated with fresh cell lysates (1 mg total protein) 30 min at 4 ˚C, and collected by centrifugation (12,000g at 4 ˚C). After washing 3 times with 100-200 μL Mg^{2+}-lysis buffer and incubation in loading buffer (5 min at

95 ˚C), samples were analyzed by 15% SDS-PAGE and subjected to Western blotting using antibodies against H-, K-, and N-Ras to identify GTP-bound Ras.

2.2.8. ***Cell cycle analysis.*** Cell permeabilization (1-2 x 10^6 cells) was performed using the GAS-002 kit from Bio Research (Kaumberg, Austria) essentially according to the manufacturer`s instructions. After washing in phosphate buffered saline (PBS), cells were fixed in 100 µL buffer A (15 min, RT), washed in PBS (3mL) and permeabilized with 100 µL buffer B (10 min, RT). 1.25 ng of the primary antibodies (αMEK-1, αPP-MEK-1/2 or α-rabbit non-specific control) were added and incubated 1 h at RT. Cells were washed with 3 mL 0.1% BSA-PBS and then 3 µL of α-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary immunoglobulins were added (20 min in the dark, RT). After washing with 3 mL 0.1% BSA-PBS, cells were incubated in PI staining buffer (3.4 mM sodium citrate, pH 7.4, 0.3% Triton-X-100, 20 µg/mL RNase A, 50 µg/mL propidium iodide, 15-30 min at 4 °C in the dark). The cell-cycle profiles (20,000 cells) were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using the Modfit LT 2.0 software (Verity Software House Inc., Topsham, ME).

2.2.9. ***Immunocytochemical staining.*** Cells were washed twice in PBS and cytospin slides were prepared using standard techniques. Slides were air-dried for 2-24 hours and processed for staining or wrapped airtight and stored at -20 ˚C. Immunocytochemical staining of the slides was done using the Dako LSAB+ kit (DAKO Corp., Carpinteria, CA) according to the manufacturer`s instructions. After fixation in 3% paraformaldehyde (30 min, 4 ˚C), cells were washed three times with Tris-buffered saline containing 0.1% Tween-20 (TBS-T buffer) (5 min) and once with TBS buffer (2 min). Slides were incubated in 100% methanol (10 min, -20 ˚C), washed in TBS-T buffer (three times, each 5 min), and blocked with 5% horse serum in TBS-T buffer (1 h, RT). Incubation with primary antibodies (αMEK-1, αPP-MEK-1/2, αERK-2, αPP-ERK-1/2) or negative control antibodies was done overnight at 4 ˚C (1:400 in 5% BSA-TBS-T buffer). After washing in TBS-T (15 min) followed by 0.1% BSA-TBS-T (2 min) and incubation with appropriate biotinylated secondary immunoglobulins (DAKO Corp.) staining and counter staining with hematoxylin was performed.

2.2.10. ***Detection of apoptosis.***

2.2.10.1. ***TUNEL apoptosis assay.*** For detection and quantification of apoptosis at the single cell level, the *in situ* cell death detection kit of

Boehringer Mannheim was used. Labeling of DNA strand breaks was done according to the manufacturer's instructions applying the TUNEL method. Cells were collected by centrifugation (4 min, 300g), washed once with PBS, washed twice with 300 µL PBS/0.1% BSA and then fixed one hour at room temperature with 100 µL of 4% (w/v) paraformaldehyde in PBS, pH 7.4. Cells were washed once with 300 µL PBS/0.1% BSA, resuspended in 100 µL permeabilization fluid (0.1% v/v Triton X-100 in 0.1 % w/v sodium citrate), incubated 2 min on ice, washed twice with 300 µL PBS/0.1% BSA and resuspended in the TUNEL labeling solution. After incubating 1 hr at 37 °C in 5% CO_2, cells were washed twice with 300 µL PBS/0.1% BSA, resuspended in 400 µL PBS and analyzed by flow cytometry.

2.2.10.2. ***Annexin-V-PE/7-Amino-actinomycin apoptosis assay.*** In addition, the Annexin V-PE/7-Amino-actinomycin (7-AAD) double staining method was used to quantitatively determine the percentage of cells that are actively undergoing apoptosis. After washing once with PBS and twice with Annexin V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), cells were resuspended in Annexin V binding buffer (1 mL) and 5 µL of Annexin V-PE (Pharmingen, USA) was added. After 15 min at RT in the dark, samples were washed twice with Annexin V binding buffer (1 mL each wash) and resuspended in 200 µL of binding buffer. Nucleic acids were stained by adding 40 µL of 7-AAD solution (0.2 mg/mL PBS) and incubating 20 min at 4 °C in the dark. Finally, 300 µL of Annexin V binding buffer was added to each tube and cells were analyzed by flow cytometry.

2.2.11. ***Proliferation assay of primary cells from leukemia patients.*** All experiments using patient-derived material were approved by the ethics committees of Universität Ulm and the Hannover Medical School. Primary AML cells were obtained from 6 patients (> 18 years) with acute myeloid leukemia (de novo and secondary AML) after informed consent was received. Diagnosis was based on cell morphology according to FAB criteria complemented by cytochemistry and immunophenotyping. Mononuclear cells were purified by Ficoll-Hypaque gradient centrifugation (Pharmacia LKB, Uppsala, Sweden). Samples contained more than 90% leukemic cells at the time of analysis. Short term cultures of primary AML

cells were grown in StemSpan^{TM} serum-free medium (CellSystems Biotech., St. Katharinen, Germany) supplemented with cytokines (100 ng/mL rh Flt-3 ligand, 100 ng/mL rh stem cell factor (SCF), 20 ng/mL rh IL-3 and 20 ng/mL rh IL-6). For growth inhibition studies, 250 μL aliquots of primary AML cells (0.4-0.5 x 10^6 cells/mL) were treated with inhibitors as indicated. After three days, 100 μL aliquots of the cell suspensions were transferred to a fresh 96 well plate and 20 μL of the CellTiter 96^R AQ_{ueous} one solution reagent (Promega, Wisconsin, USA) was added to each aliquot and incubated 3-4 h at 37 °C with 5% CO_2. The proliferation of the samples was then quantified using an ELISA reader (Abs_{490}).

2.2.12. ***Analysis of combined drug effects.*** Dose-response curves were initially generated for each agent to estimate IC_{50}s for the cells under study. In subsequent experiments, cells were treated with serial dilutions of each drug individually and with both drugs simultaneously at a fixed ratio (1 : 1) of doses. Fractural survival (*f*) was calculated by dividing the number of colonies in drug-treated plates by the number of colonies in control plates. Data were analyzed by the method of Chou and Talalay (1984) using the CalcuSyn computer program (Biosoft, Cambridge UK). In brief, log [(1/f)-1] was plotted against log (drug dose) to obtain the resulting median effect curves, the X intercept (log IC_{50}) and slope *m* for each drug alone and drug combinations. These parameters were then used to calculate doses of the individual drugs and the combination required to produce varying levels of cytotoxicity according to the equation : Dose $_f$ = Dose $_{IC50}$ $[(1 - f)/f]^{1/m}$. For each level of cytotoxicity, the combination index (CI) was calculated according to the equation $CI = (D)_1/(D_f)_1 + (D)_2/D_f)_2 + \alpha\ (D)_1\ (D)_2/(D_f)_1\ (D_f)_2$, where $(D)_1$ and $(D)_2$ are concentrations of the combination required to produce survival *f*, $(D_f)_1$ and $(D_f)_2$ are the concentrations of the individual drugs required to produce *f*, and $\alpha = 1$ or 0 depending on whether the drugs are assumed to be mutually nonexclusive (totally independent modes of action) or mutually exclusive (same or similar modes of action), respectively. Synergy is indicated by CI <1, additivity by CI = 1 and antagonism by CI > 1 (Chou & Talalay 1984). Dose-reduction index (DRI) is a measure of the dose reduction of each drug in a synergistic combination at a given cytotoxic level as compared to each drug alone. For two drug combinations, $CI = (D)_1/(D_f)_1 + (D)_2/(D_f)_2 = 1/(DRI)_1 + 1/(DRI)_2$.

3. Results

3.1. *Activation of the Ras-to-MAP kinase cascade.*

3.1.1. *Ras* mutations. The genomic DNA of several myeloid cell lines was analyzed for activating mutations in codons 12, 13, 15, 16, 18 and 61 of H-, K- and N-*Ras* to correlate the frequency of *Ras* mutations with the presence of constitutive activation of the ERK pathway. Four of 14 AML cell lines (28.6%) were found to contain activating *Ras* mutations (Table 2). As previously reported, a mutation of codon 12 of N-*Ras* was detected in THP-1 (GGT to GAT) and a N-*Ras* codon 61 mutation in HL-60 (CAA to CTA) replacing Gly12 with Asp and Gln61 with Leu (Lübbert *et al*, 1992). Two AML cell lines (Kasumi-1 and MV4-11) were found to harbor a K-*Ras* codon 12 mutation (GGT to GAT), which also exchanges Gly12 with Asp. Interestingly, an additional K-*Ras* codon 18 mutation was found in the second allele of MV4-11 (GCC to GAC) which leads to replacement of Ala18 with Asp. While no activating mutations of the H-*Ras* gene were detected, three cell lines (MV4-11, Kasumi-1 and NB-4) harbored silent point mutations in

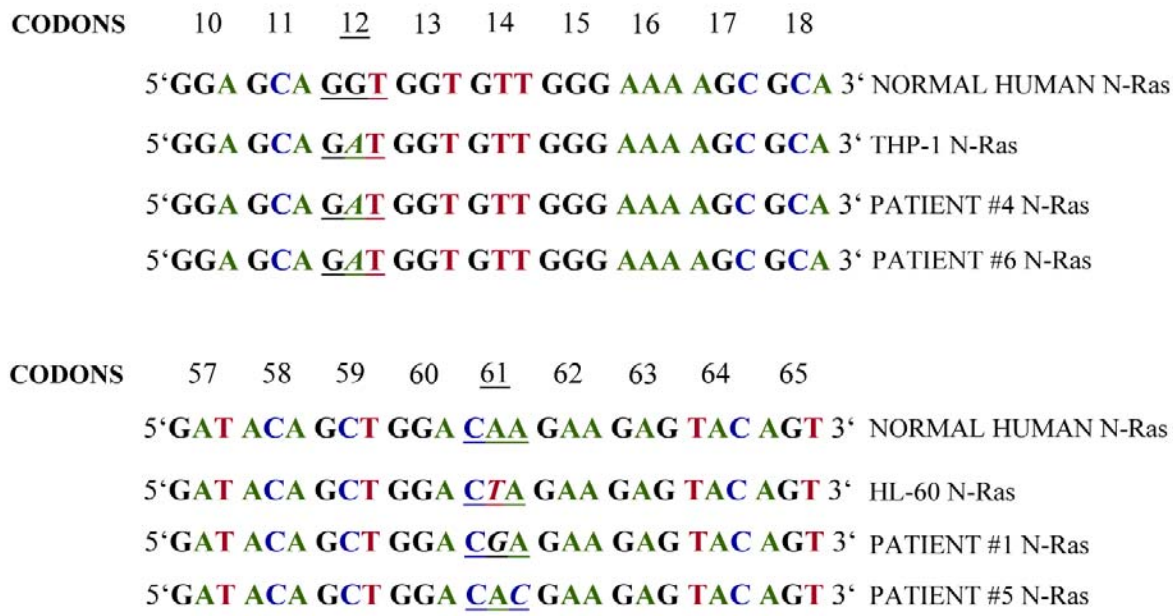

Figure 11. *Ras* mutations detected in AML cell lines and AML blasts from patients. AML cell lines HL-60 and THP-1 harbor N-*Ras* mutations in codons 61 and 12 (CAA⇒CTA and GGT⇒GAT resulting in Q61L and G12D, respectively). AML blasts from patients 1, 4, 5 and 6 were found to harbor N-*Ras* mutations (CAA⇒CGA resulting in Q61R, GGT⇒GAT resulting in G12D, CAA⇒CAC resulting in Q61H and GGT⇒GAT resulting in G12D, respectively). The codons containing mutations are underlined and the point mutations are italicized.

codon 59 (GCC to GCT). Additionally, AML blasts from 4 of 6 patients were found to harbor N-*Ras* mutations (Figure 11).

3.1.2. *Ras* activation assays. Expression of all three Ras isoforms (H-, K- and N-Ras) was detected by Western blotting the cellular lysates of all myeloid cell lines tested (not shown). To identify the presence of GTP-Ras, cell lysates were incubated with the minimal Ras-binding domain (RBD) of C-Raf-1. Western blotting with antibodies against H-, K- and N-Ras was performed to detect the binding of GTP-Ras with GST-RBD. Cellular lysates of H-*Ras* (L-61)-transformed NIH-3T3 fibroblasts were used as a positive control for activated H-Ras. As shown in Figure 12, only oncogenic H-Ras (L61) could be precipitated with GST-RBD from NIH-3T3 lysates, but not endogenous K- or N-Ras. THP-1 and HL-60, which harbor N-*Ras* mutations in codon 12 and codon 61, showed only a slight activation of N-Ras in some experiments but no activation of H-Ras or K-Ras. Surprisingly, lysates of Kasumi-1 and MV4-11, which harbor a K-*Ras* codon 12 mutation, did not contain substantial amounts of activated K-Ras. While lysates of most cell lines with wild-type Ras did not contain significant amounts of GTP-Ras, high levels of activated H-, K- and N-Ras were found in NB-4 and ML-2 cell lysates. No apparent activation of Ras was detected in the CML cell lines, although all express the activated BCR-Abl fusion tyrosine kinase, which has been reported to induce Ras activation (Kalra *et al*, 1994; Bollag *et al*, 1996; Reuter *et al*, 2000). Of the growth factor-dependent myeloid cell lines (AML-OCI-5, Mo-7e, Mutz-2 and Mutz-3), activation of H-, K- and N-Ras was observed only in AML-OCI-5 upon stimulation by IL-3 or conditioned medium of 5637 bladder carcinoma cells (not shown).

3.1.3. Activation of the MAPK cascade. To determine the frequency and the level of activation of signaling proteins downstream of Ras, lysates of myeloid cells were analyzed by Western blotting with antibodies against PP-MEK-1/2 and PP-ERK-1/2 (Figure 13). Cellular lysates of H-Ras (L61)-transformed NIH-3T3 fibroblasts were used as a positive control for activated MEK-1/2 and ERK-1/2. Significant activation of MEK-1/2 was detected in 8/14 AML cell lines and 5/5 CML cell lines. ERK-1/2 activation was observed in 9/14 AML cell lines and 2/5 CML cell lines. MEK-1/2 activation correlated with ERK-1/2 activation in 7/14 AML cell lines and 2/5 CML cell lines. Western blots for diphospho-ERK-1/2 were confirmed by immunocomplex MAPK assays using an ERK-2 antibody, which cross-reacts with ERK-1 (Figure 14). The presence of PP-ERK-1/2 in

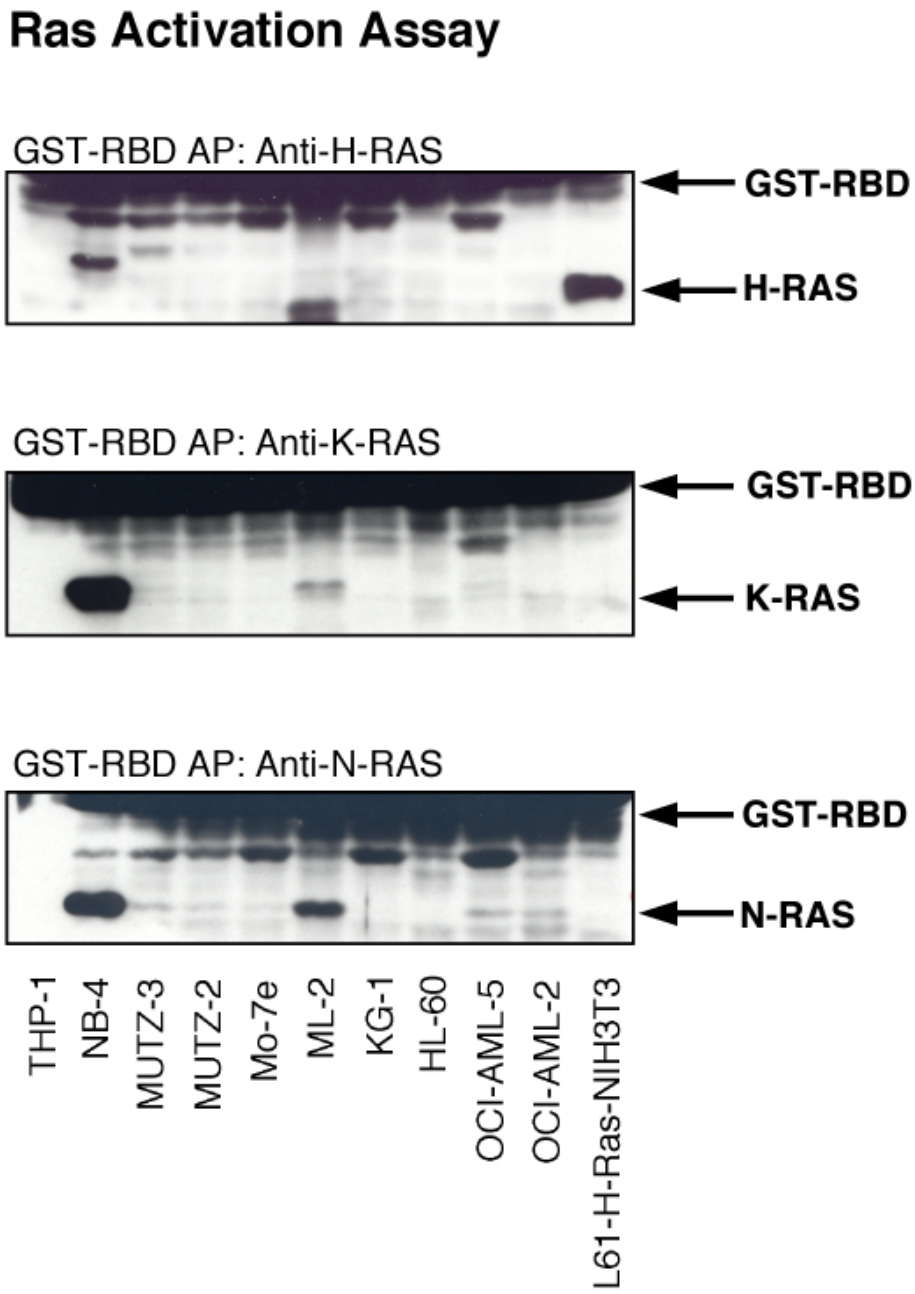

Figure 12. Activation of Ras in myeloid leukemia cell lines. Lysates of myeloid leukemia cell lines were subjected to affinity precipitation (AP) with GST-RBD as described in Methods. Ras proteins were detected by immunoblotting with H-, K-, and N-Ras antibodies. H-Ras (L61)-transformed NIH3T3 fibroblasts were used as a positive control for activated H-Ras.

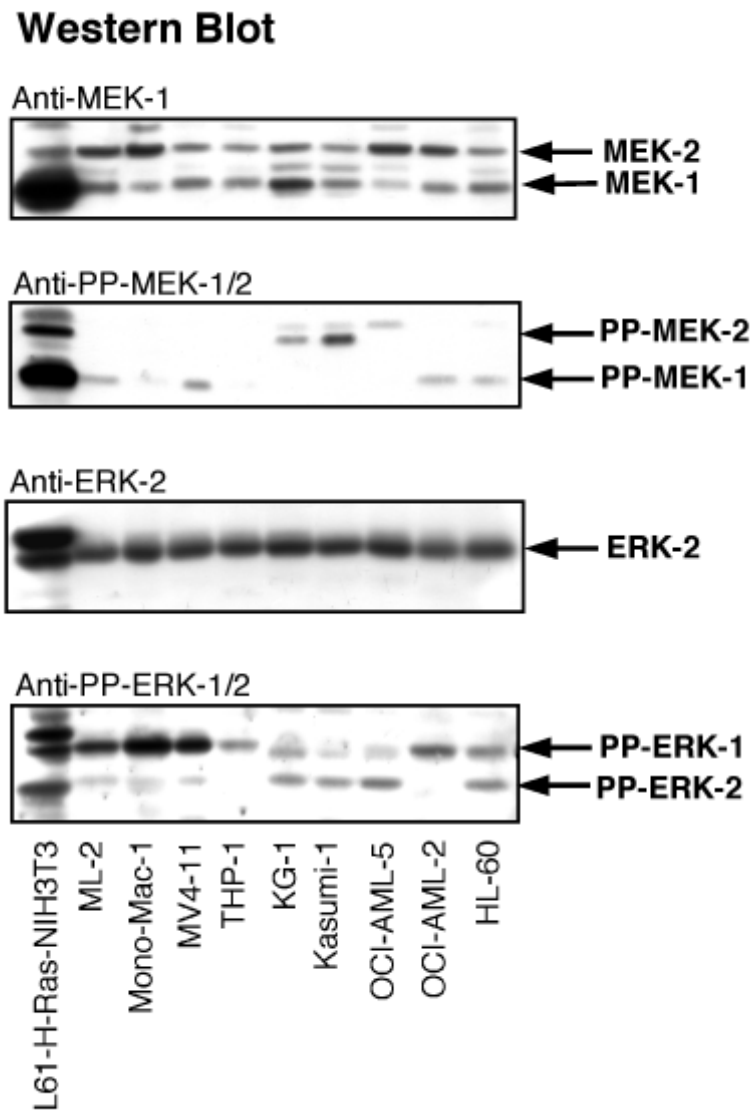

Figure 13. Activation of ERK-1/2 and MEK-1/2 in myeloid leukemia cell lines. Myeloid leukemia cell lysates were adjusted for protein concentration and equal amounts of total cell protein were subjected to SDS-PAGE. Western analysis was performed with antibodies against MEK-1, ERK-2, PP-MEK-1/2 and PP-ERK-1/2. L61-H-Ras-transformed NIH3T3 cell lysates were used as positive controls for the Ras-induced activation of ERKs and MEKs.

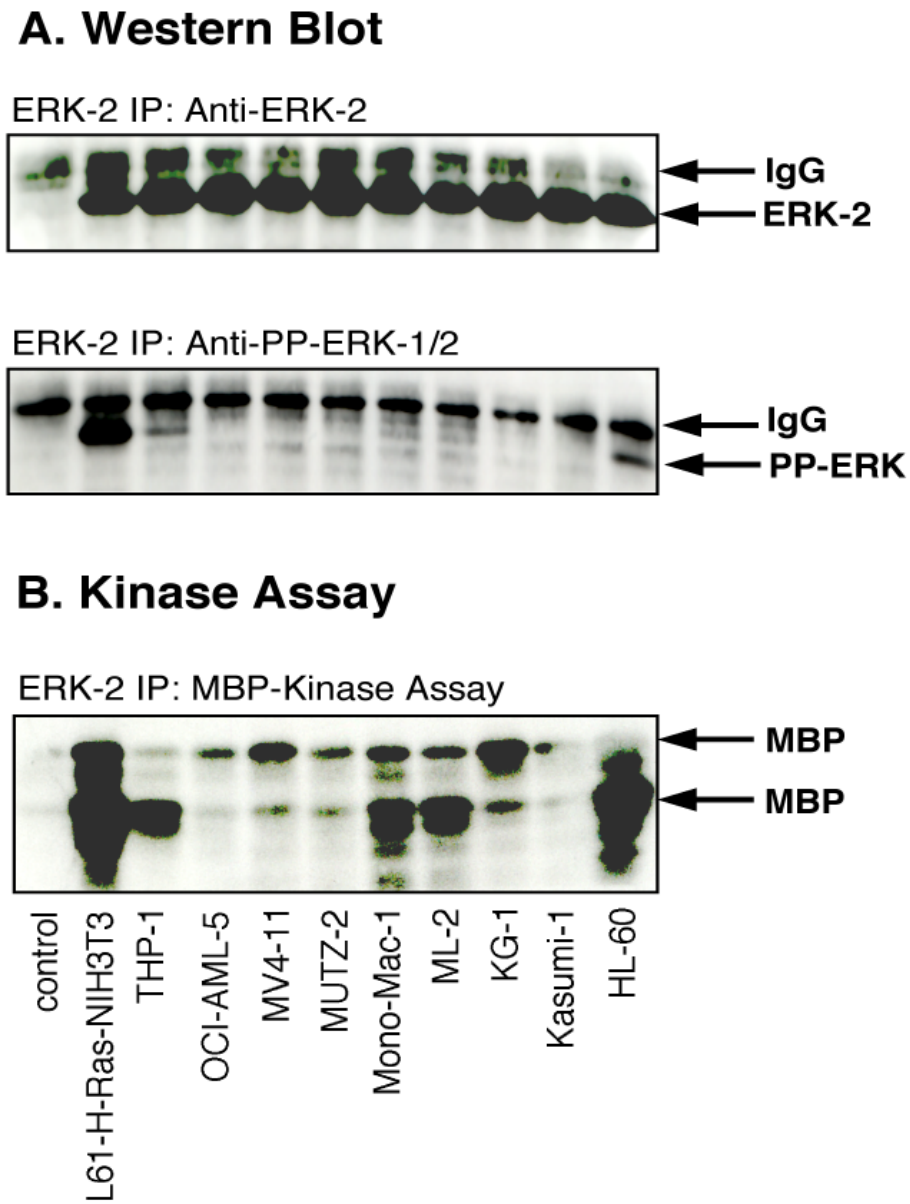

Figure 14. Activation of ERK-2 in myeloid leukemia cell lines. Lysates of myeloid leukemia cell lines were subjected to immunoprecipitation with an antibody against ERK-2 (which also cross-reacts with ERK-1). After washing of the immunoprecipitates, immunocomplex kinase assays were performed as described in Methods. (A) Western blot of the immunoprecipitates with an antibody specific for ERK-2 and an antibody specific for activated PP-ERK-1/2. (B) Autoradiogram of kinase assay demonstrating MBP phosphorylation by immunoprecipitated ERK-1/2. H-Ras (L61)-transformed NIH3T3 fibroblasts were used as a positive control for the Ras-induced activation of ERK-1/2. A non-specific antibody was used as a negative control.

Western blots of cellular lysates correlated closely with MAPK/ERK activity in immunocomplex kinase assays. Compared to leukemia cell lines, H-Ras (L61)-transformed NIH-3T3 fibroblasts were found to harbor at least 50% higher amounts of PP-ERK-1/2. The presence of *Ras* mutations or BCR-Abl did not always correlate with significant ERK-1/2 activation (Table 2).

3.1.4. Activation of transcription factors. Following activation, ERK-1/2 translocates to the nucleus resulting in phosphorylation and activation of transcription factors such as ELK-1, CREB-1 and c-Myc. To determine whether activation of ERK-1/2 induces activation of these transcription factors, myeloid leukemia cell lysates were subjected to Western blotting with specific antibodies against phospho-ELK, phospho-CREB and phospho-c-Myc. Activated CREB-1/ATF-1 and c-Myc were found in 11/14 AML cell lines and 2/5 CML cell lines and did not always coincide with the presence of phospho-ERK-1/2 (Figure 15 and Table 2).

3.1.5. Intracellular localization of PP-ERK-1/2 and PP-MEK-1/2. To determine whether activation of ERK-1/2 and MEK-1/2 was limited to subpopulations of myeloid leukemia cells, cytospins were prepared and stained with specific antibodies against ERK-2, MEK-1, PP-ERK-1/2 and PP-MEK-1/2 (Figure 16). All cells were stained with anti-ERK-2 and anti-MEK-1 antibodies. However, only 1-5% of cells showed strong staining with anti-PP-ERK-1/2 and anti-PP-MEK-1/2 antibodies. Strong nuclear staining was found in approximately 50% of PP-ERK-1/2 and PP-MEK-1/2 positive cells whereas the other portion showed mainly cytoplasmatic staining. These results were obtained for all samples tested and suggest activation of ERKs and MEKs in subpopulations (e.g. cells in certain phases of the cell cycle).

3.1.6. MEK activation during cell cycle progression. ERK-1/2 and MEK-1/2 activation during cell cycle progression was detected using a novel FACS method for cytoplasmatic staining of activated kinases of the Ras pathway. Cells were double stained with propidium iodide and antibodies specific for different members of the ERK signaling pathway (e.g. ERK-2, PP-ERK-1/2, MEK-1 and PP-MEK-1/2). As shown in Figure 17B & C, all cells were strongly positive for ERK-2 and MEK-1. Using anti-PP-ERK-1/2 and anti-PP-MEK-1/2 antibodies, most cells were only slightly positive (Figure 17D & E). Interestingly, PP-MEK-1/2 staining revealed two additional, strongly positive cell

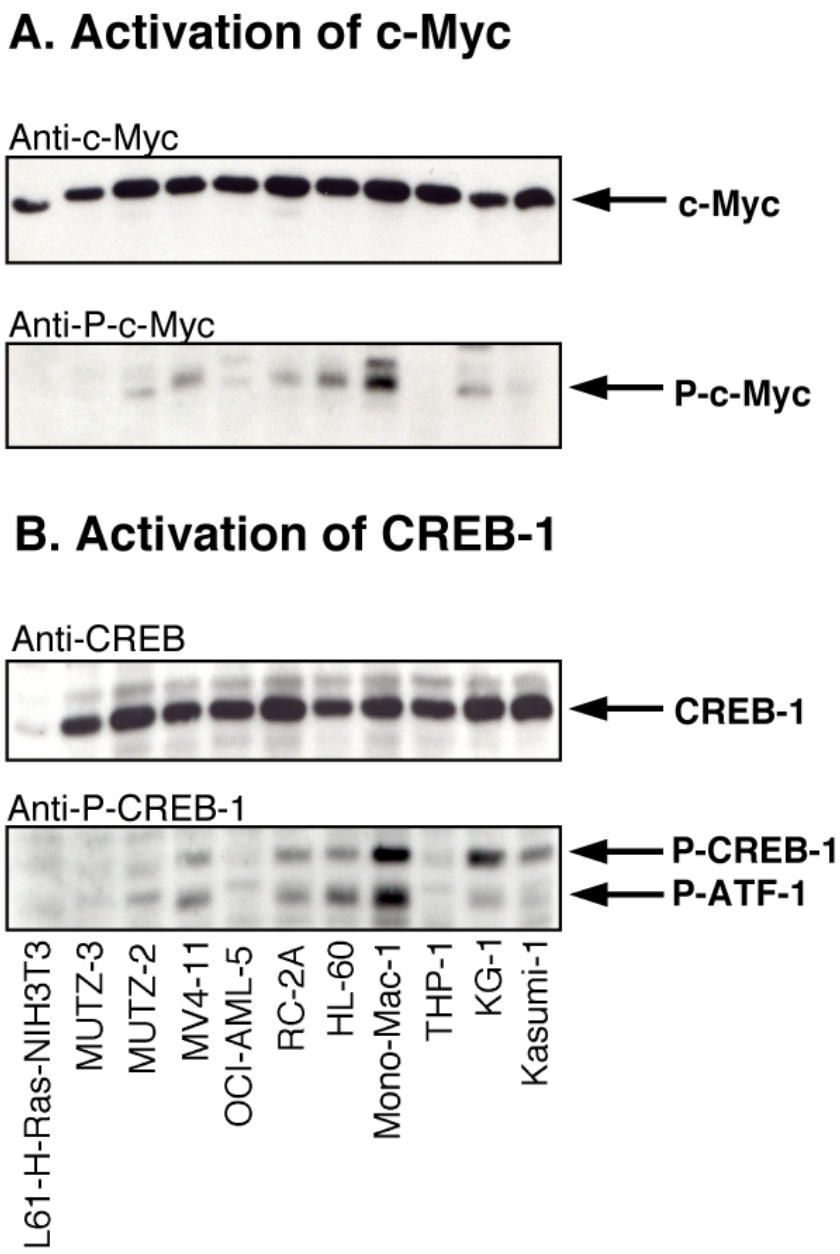

Figure 15. Activation of MAPK-dependent transcription factors in myeloid leukemia cell lines. Lysates of myeloid leukemia cell lines were subjected to SDS-PAGE followed by Western blotting with anti-c-Myc (A) and anti-CREB-1 (B) antibodies. Activated c-Myc and CREB-1/ATF-1 were detected with phospho-specific antibodies against activated c-Myc and CREB-1.

Table 2. Activation of the Ras-to-MAPK signaling cascade in myeloid leukemia cell lines.

Cell line	Leukemia	*Ras* Mutation	Ras	ERK-1/2	MEK-1/2	CREB-1/ ATF-1
HL-60	AML M2	N-*Ras*/61	(+)	++	++	++
PLB-985	AML M2	none	-	++	-	+
Kasumi-1	AML M2	K-*Ras*/12	-	(+)	++	+
Mutz-2	AML M2	none	-	(+)	-	+
NB-4/t15;17 PML/RARA	AML M3	none	+++	+++	+++	++
THP-1	AML M4	N-*Ras*/12	(+)	++	(+)	-
OCI-AML2	AML M4	none	-	+	+	+++
OCI-AML5	AML M4	none	-	(+)	-	-
Mutz-3	AML M4	none	-	(+)	(+)	-
ML-2/t6;11, MLL/AF6	AML M4	none	+	+	+	+++
MV4-11/t4;11, MLL/AF4	AML M5	K-*Ras*/12 K-*Ras*/18	-	+++	(+)	+
Mono-Mac-1	AML M5	none	-	+++	++	+++
KG-1	AML M6	none	-	+	++	+
M-07e	AML M7	none	-	(+)	+	++
RC2A	ALL	none	-	+	+	+
EM-2	CML blast crisis	BCR-Abl	-	-	+	+
JK-1	CML erythroid blast crisis	BCR-Abl	-	-	+	
K562	CML blast crisis	BCR-Abl	-	+	+	++
MEG-01	CML megakaryocytic blast crisis	BCR-Abl	-	+	+	+
LAMA-84	CML myeloid-megakaryocytic blast crisis	BCR-Abl	-	-	+++	

Legend: Among the myeloid leukemia cell lines used in this study are 14 acute myeloid leukemia (AML) cell lines and 5 chronic myeloid leukemia (CML) cell lines (all from blast crisis). HL-60 and THP-1 harbor N-*Ras* mutations in codons 61 and 12 (CAA⇒CTA and GGT⇒GAT resulting in Gln⇒Leu and Gly⇒Asp, respectively). Kasumi-1 and MV4-11 contain a codon 12 K-*Ras* mutation (GGT⇒GAT resulting in Gly⇒Asp). An additional codon 18 mutation was detected in MV4-11 (GCC⇒GAC resulting in Ala⇒Asp). A previously unknown silent H-*Ras* mutation in codon 59 (GCC⇒GCT) was observed in MV4-11, Kasumi-1 and NB-4 (Ala). Relative activation levels of signaling proteins were determined by Western blotting and are shown by : +++ = strong signal; and - = weak or no signal. Note: PLB-985 is a clone of HL60 (DSMZ, Braunschweig, Germany).

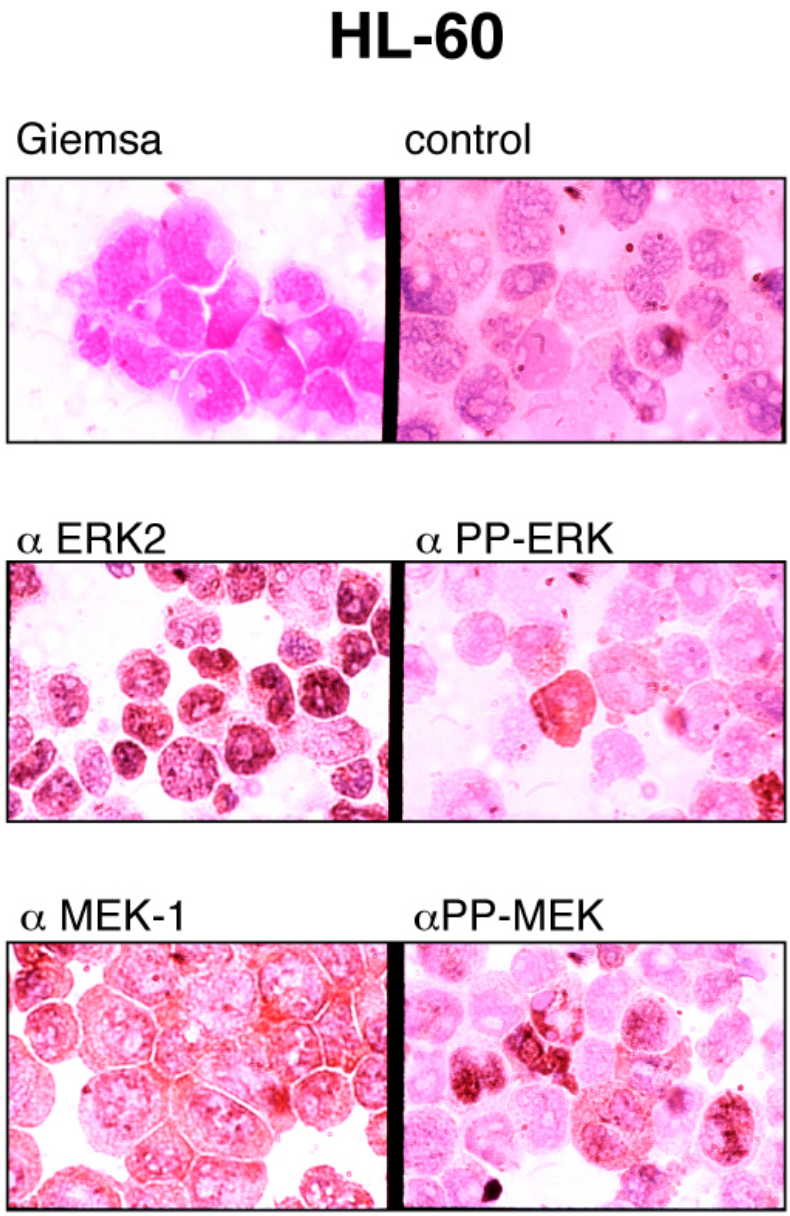

Figure 16. Immunocytostaining of myeloid leukemia cells with PP-ERK-1/2 and PP-MEK-1/2 antibodies. Cytospins of myeloid leukemia cells were stained with antibodies specific for ERK-2, PP-ERK-1/2, MEK-1 and PP-MEK-1/2 as described in methods. Approximately 1-5% of the cells were strongly stained with anti-PP-ERK-1/2 and anti-PP-MEK-1/2 antibodies. Note the nuclear staining of some cells while others are stained mainly in the cytoplasm.

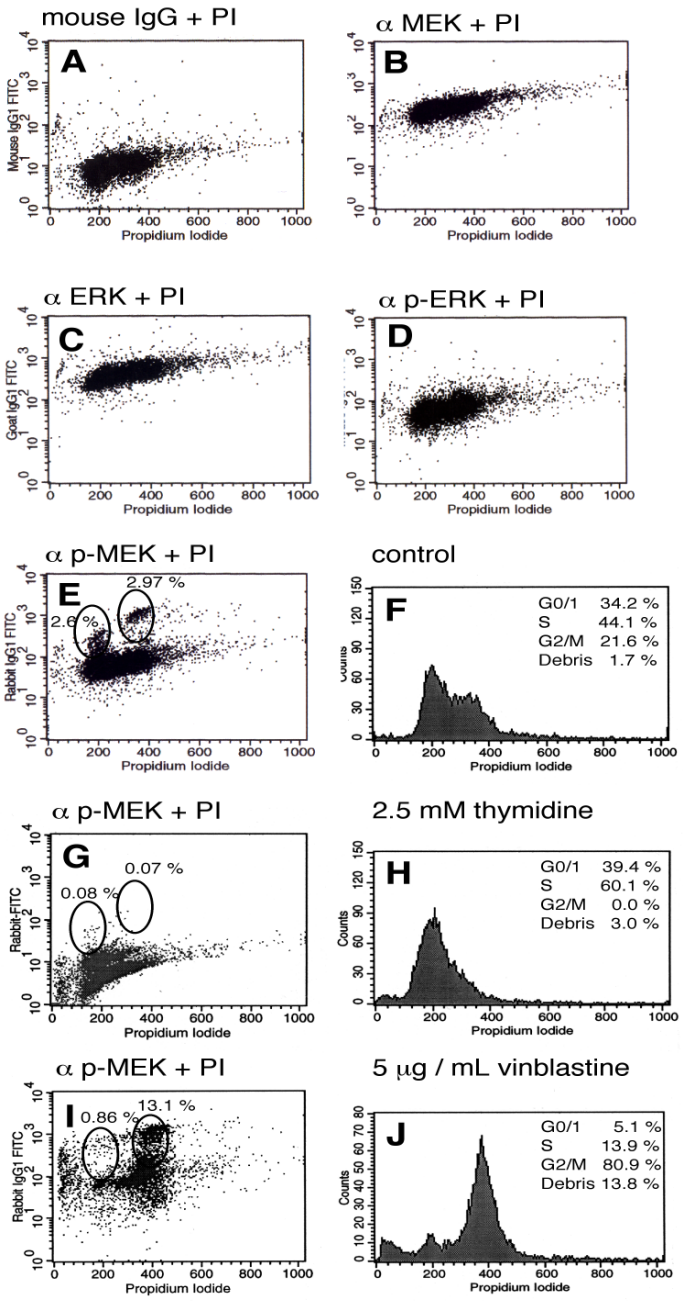

Figure 17. Activation of MEK-1/2 during cell cycle progression. HL-60 cells were prepared for FACS analysis as described in Methods. Graph A shows a representative control with unspecific mouse IgG antibodies. Similar results were obtained with rabbit and goat controls (data not shown). All cells were clearly positive when probed with ERK-2 or MEK-1 antibodies (graphs B and C), and only slightly positive when stained with phospho-specific antibodies against activated PP-ERK-1/2 and PP-MEK-1/2 (graphs D and E). Graph F shows the cell cycle profile of untreated HL-60 cells stained with propidium iodide. Additionally, PP-MEK-1/2 staining revealed two strongly positive populations, which correlated with the G_0/G_1 and G_2/M phases of the cell cycle (graphs E and F). Treatment of HL-60 cells with excess thymidine (2.5 mM) for 16 hours: G, staining with anti-PP-MEK-1/2 antibodies, H, cell cycle profile. Treatment of HL-60 cells with 5 μg/mL vinblastine for 10 hours: I, staining with anti-PP-MEK-1/2 antibodies, J, cell cycle profile.

populations (Figure 17E) which correspond to the G_0/G_1 and G_2/M phases of the cell cycle. The difference in PP-ERK-1/2- and PP-MEK-1/2-staining might be due to rapid nuclear translocation of PP-ERK-1/2. Incubation with excess thymidine (2.5 mM) resulted in a G_1/S phase block with an increase of S-phase cells to 60.1 % and a concomitant reduction of PP-MEK-1/2-positive cells in G_0/G_1 and G_2/M (<0.1% of the total cell population) (Figure 17G and H). Cell viability was not significantly affected by this treatment. Vinblastine treatment of cells (5 μg/mL, 10 hours), which induces depolymerization of mitotic interpolar microtubules and a cell metaphase block, increased the mitotic population of cells in G_2/M to 80.9% and the amount of strongly positive PP-MEK-1/2 cells in G_2/M to 13.1% (Figure 17I and J). These results demonstrate MEK-1/2 activation in the G_0/G_1 and the G_2/M phase of the cell cycle of myeloid leukemia cells. These results were further confirmed by Western blotting (Figure 18).

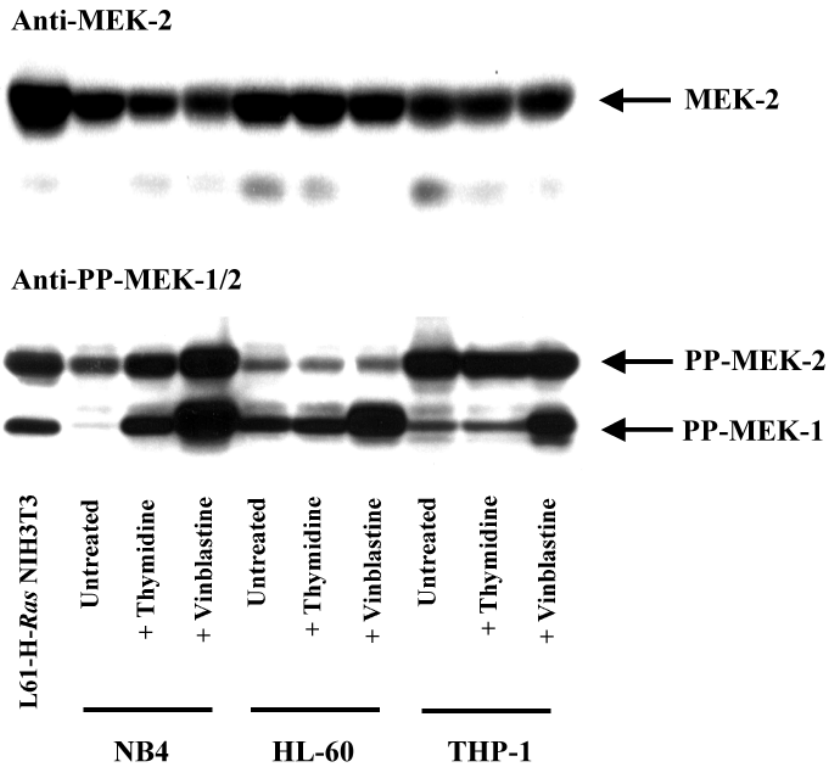

Figure 18. Cell cycle-dependent activation of MEK-1/2 in myeloid leukemia cell lines. Myeloid leukemia cells were treated 16 h with 2.5mM thymidine or 10 h with 5 μg/mL vinblastine. Lysates were adjusted for protein concentration and equal amounts of total cell protein were subjected to SDS-PAGE. Western analysis was performed with antibodies against MEK-1 and PP-MEK-1/2. Untreated, L61-H-Ras-transformed NIH3T3 cell lysates were used as positive controls for the Ras-induced activation of MEKs.

3.2. Effects of Ras-to-MAPK signaling inhibitors in myeloid leukemia cells.

3.2.1. ***Effect of inhibitors of Ras-to-MAPK signaling on myeloid leukemia cell growth.*** The effectiveness of several different types of inhibitors of Ras signaling was tested on viability and colony formation of myeloid leukemia cell lines. Inhibitors employed in this study included (1) farnesyl pyrophosphate (FPP)-based farnesyltransferase (FTase) inhibitors FPT-II and FPT-III; (2) CAAX-based FTIs FTI-276, FTI-277, B581, Cys-4-Abz-Met and L-744,832; (3) CAAL-based GGTIs GGTI-286, GGTI-287, GGTI-297, GGTI-298, GGTI-2133 and GGTI-2147; (4) MEK inhibitors U0126 and PD098059; and the (5) prenylated protein methyltransferase (PPMTase) inhibitor FTS. The compounds were initially screened for growth inhibition of myeloid leukemia cells by incubating in liquid suspension cultures containing 20 μM (FTI 744,832 and all GGTIs) or 50 μM (the remaining compounds) of inhibitor or solvent. Myeloid cell lines were preincubated in liquid suspension cultures for 96 h followed by incubation in methylcellulose to assay colony formation in the presence or absence of inhibitor. Limited growth inhibition was obtained by treatment with 50 μM of FTI-276, B-581, and Cys-4-Abz-Met (Table 3). Growth inhibition greater than 70% was observed with 50 μM of FPP-competitive FTIs FPT-3 (10/19), FPT-2 (4/19), and the CAAX-based FTase inhibitors FTI-277 (19/19) and L-744,832 (16/16) (Table 3).

Only limited growth inhibitory effects were observed in myeloid leukemia cells after treatment with 20 μM of GGTI-287 and GGTI-297 (Table 4). Growth inhibition greater than 70% was obtained with 20 μM of the methyl ester derivatives GGTI-286 (9/19) and GGTI-298 (14/19), and the non-thiol derivatives GGTI-2133 (2/19) and GGTI-2147 (16/19) (Table 4).

Growth inhibition greater than 70% was also observed with the PPMTase inhibitor FTS (3/19) and MEK inhibitors PD098059 (8/19) and U0126 (19/19) (Table 5).

3.2.2 ***Inhibition of myeloid leukemia cell growth by Ras signaling inhibitors is concentration dependent.*** To determine if the inhibition of colony formation caused by the compounds was dependent upon concentration, the most potent inhibitors were titrated against different cell lines. Purified CD-34+ cells were utilized as a control for inhibitor specificity.

Only minor growth inhibitory effects on purified CD34+ cells were observed in the presence of DMSO, or U0126 and FPT-3 at concentrations up to 50 μM (Figure 19). FTI-277 caused inhibition of stem cell colony formation with IC_{50} value of 10 μM. NB-4,

Table 3. Inhibition of colony formation and cell viability of myeloid leukemia cell lines by farnesyltransferase (FTase) inhibitors.

Cell line/ Translocation	Leukemia	Ras-to-MAPK	FPT2	FPT3	B581	Cys-4 Abs-Met	FTI-276	FTI-277	L-744,832
HL-60 N-*Ras*/61	AML M2	++	52.9±2.1	84.8	14.3±6.2	13.1±14.4	27.1±8.5	100	85±5.7
PLB-985	AML M2	+	0	32.3±11.2	0	0	0	100	n.d.
Kasumi-1 K-*Ras*/12	AML M2	+	90.9±1.0	79.1	0	n.d.	37.6±7.0	100	100
Mutz-2	AML M2	(+)	0	0	0	n.d.	15.6±2.5	83.4±1.5	100
NB-4/ t15;17; PML/RARA	AML M3	+++	97.2±1.1	100	0	15.5±12.4	0	100	100±0
THP-1 N-*Ras*/12	AML M4	++	0	100	0	0	25	87.5	100±0
OCI-AML2	AML M4	-	0	92.1±0.7	0	0	46.2±26.4	100	100
OCI-AML5	AML M4	-	37.3±6.2	72.8±4.5	22.1±6.9	39.3±9.2	32.1±4.3	100	100
Mutz-3	AML M4	-	15.1±1.3	43.2±4.5	0	n.d.	0	99.1±0.2	100
ML-2/ t6;11; MLL/AF6	AML M4	+	0	0	0	0	0	100	n.d.
MV4-11/ t4;11 K-*Ras*/12&18	AML M5	++	19.4±10.9	52.8±11.3	25.6±3.0	15.5±8.6	20.5±8.9	99.8±0.4	100±0
Mono-Mac-1	AML M5	+++	30	70	0	70	20	80	n.d.
KG-1	AML M6	+	0	0	0	0	0	87.5	100±0
M-07e	AML M7	+	0	0	0	0	0	96.4±0.9	100±0
EM-2	CML/BCR-Abl	-	80.5±1.2	100	0	n.d.	75.4±6.3	100	100
JK-1	CML/BCR-Abl	-	16.3±10.1	100	15.0±2.3	n.d.	0	100	100
K562	CML/BCR-Abl	+	0	12.6±13.5	0	0	0	100	84.2±17.7
MEG-01	CML/BCR-Abl	-	77.7±0.6	79.8±3.2	47.7±6.8	n.d.	76.8±4.8	71.6±3.8	100
LAMA-84	CML/BCR-Abl	-	17.4±10.8	40.5±3.0	0	n.d.	86.4±3.6	100	90

Legend: Cells were seeded at 1.0 to 2.5 x 10^6/mL of cell suspension in a 96-well plate and treated with 20 μM inhibitor or solvent control for 4 days. Aliquots of the cell suspensions were then plated in 400 μL methylcellulose for 7 to 14 days and cell growth was evaluated as described in "Methods". Inhibition is given in percentage of solvent control. AML indicates acute myeloid leukemia; CML, chronic myeloid leukemia; MAPK, MAP kinase; n.d., not determined. Note: PLB-985 is a clone of HL60 (DSMZ, Braunschweig, Germany).

Table 4. Inhibition of colony formation and cell viability of myeloid leukemia cell lines by GGTIs.

Cell line/ Translocation	Leukemia	*Ras* Mutation	GGTI 286	GGTI 287	GGTI 297	GGTI 298	GGTI 2133	GGTI 2147
HL-60	AML M2	N-*Ras*/61	95.1±4.7	0±0	57±9.9	95.9±9.1	48.5±28.9	87.4±16.3
PLB-985	AML M2	none	100±0	79.9±28.5	1±1.3	0±0	0±0	100
Kasumi-1	AML M2	K-*Ras*/12	100	0	0±0	100±0	0±0	100±0
Mutz-2	AML M2	none	20±28.3	5.5±7.8	13±18.3	90	2.5±3.5	86.5±12
NB4/t(15;17) PML/RARA	AML M3	none	99±1.7	15	5±7.1	100±0	100±0	100±0
THP-1	AML M4	N-*Ras*/12	97.5±4.2	63	25.5±28.9	100±0	10.5±14.8	100±0
OCI-AML2	AML M4	none	94	0	2.3±4	100±0	12.7±21.9	100±0
OCI-AML5	AML M4	none	6±8.5	9±12.7	4.5±6.4	47.5±3.5	5.5±7.8	99.5±0.7
Mutz-3	AML M4	none	23±20.9	12±16.9	31±32.5	3.5±4.9	0±0	89.5±14.8
ML-2/t(6;11) MLL/AF6	AML M4	none	90	n.d.	0±0	100±0	0±0	100±0
MV4-11/t(4 ;11) MLL/AF4	AML M5	K-*Ras*/12 K-*Ras*/18	93.3±11.5	5	20±14.1	100±0	9±2.8	98.5±2.1
Mono-Mac-1	AML M5	none	2	4	6.7±11.5	100±0	0±0	93.3±5.8
KG-1	AML M6	none	38.1±14.6	0±0	0±0	97.5±3.5	0±0	88.5±12.0
M-07e	AML M7	none	0±0	7±9.9	15.5±21.9	0±0	16±22.7	100
EM-2/t(9;22)	CML, bc	BCR-Abl	98±1.4	2.5±3.5	5±7.1	100±0	100±0	100±0
JK-1/t(9;22)	CML, bc	BCR-Abl	19.5±3.5	19±19.8	0±0	42.5±19.0	16.8±23.7	74.5±31.8
K562/t(9;22)	CML, bc	BCR-Abl	67.1±22.1	0	43±26	74.3±6	29.2±26	0±0
MEG-01/t(9;22)	CML, bc	BCR-Abl	7.5±10.6	26±12.7	7±9.9	78±1.4	0±0	53.5±9.2
LAMA84/t(9;22)	CML, bc	BCR-Abl	67.5±31.8	5±7.1	14.5±7.8	96.5±4.9	13.5±9.2	83.5±9.2

Legend: Cells were seeded at 1.0 to 2.5 x 10^6/mL of cell suspension in a 96-well plate and treated with 20 μM inhibitor or solvent control for 4 days. Aliquots of the cell suspensions were then plated in 400 μL methylcellulose for 7 to 14 days and cell growth was evaluated as described in "Methods". Inhibition is given in percentage of solvent control. AML indicates acute myeloid leukemia; CML, chronic myeloid leukemia; bc, blast crisis; n.d., not determined. Note: PLB-985 is a clone of HL60 (DSMZ, Braunschweig, Germany).

Table 5. Inhibition of colony formation and cell viability of myeloid leukemia cell lines by MEK, and PPMTase.

Cell line	Leukemia	Ras-to-MAPK	U0126	PD098059	FTS
HL-60 N-*Ras*/61	AML M2	++	100	81.4±8.1	64.4±6.8
PLB-985	AML M2	+	100	54.6±7.4	54.6±6.5
Kasumi-1 K-*Ras*/12	AML M2	+	100	100	100
Mutz-2	AML M2	(+)	100	62.1±3.3	37.9±11.4
NB-4 /t15;17 PML/RARA	AML M3	+++	100	16.5±6.8	46.5±6.0
THP-1 N-*Ras*/12	AML M4	++	87.5	50	0
OCI-AML2	AML M4	-	100	100	0
OCI-AML5	AML M4	-	99.1±0.8	63.3±5.2	20.5±15.5
Mutz-3	AML M4	-	99.2±0.3	56.3±2.1	0
ML-2/ t6;11 MLL/AF6	AML M4	+	100	100	0
MV4-11/(t4;11) K-*Ras*/12&18 MLL/AF4	AML M5	++	100	100	13.4±7.2
Mono-Mac-1	AML M5	+++	90	0	20
KG-1	AML M6	+	90	50	50
M-07e	AML M7	+	100	0	0
EM-2/t(9;22)	CML	-	100	0	64.4±12.5
JK-1/t(9;22)	CML	-	100	70.5±3.9	26.1±12.0
K562/t(9;22)	CML	+	100	30.4±25.7	53.3±22.1
MEG-01/t(9;22)	CML	-	99.6±0.6	79.4±1.8	97.8±1.6
LAMA-84/t(9;22)	CML	-	100	87.6±1.0	100

Legend: Cells were seeded at 1.0 to 2.5 x 10^6/mL of cell suspension in a 96-well plate and treated with 20 μM inhibitor or solvent control for 4 days. Aliquots of the cell suspensions were then plated in 400 μL methylcellulose for 7 to 14 days and cell growth was evaluated as described in "Methods". Inhibition is given in percentage of solvent control. AML indicates acute myeloid leukemia; CML, chronic myeloid leukemia; MAPK, MAP kinase; '+' and '-' indicate activation of the Ras-to-MAPK pathway as determined by Western blotting. Note: PLB-985 is a clone of HL60 (DSMZ, Braunschweig, Germany).

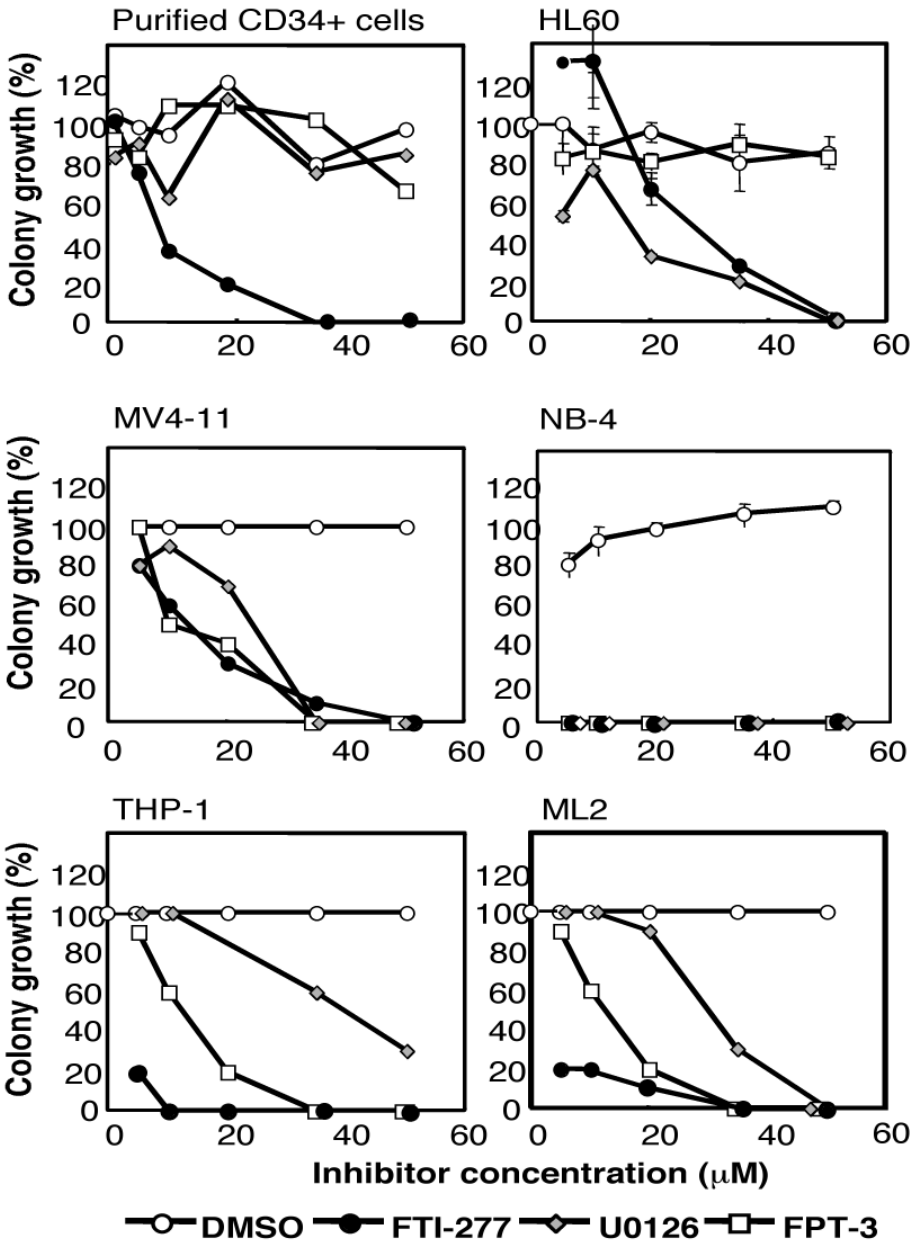

Figure 19. Inhibition of colony growth by U0126, FPT-3, and FTI-277. Purified human CD34+ cells and myeloid leukemia cell lines were incubated in liquid suspension cultures with increasing concentrations of the MEK inhibitor U0126, the FPP-based FTI FPT-3, and the CAAX-based FTase inhibitor FTI-277. After four days, viability of the cells was determined by trypan blue dye assays. Aliquots of the samples were incubated in methylcellulose for an additional 7-14 days in the presence of freshly added inhibitors. Representative experiments are shown. Results are expressed in percentage inhibition of maximal spontaneous colony growth. Error bars indicate the standard margin of error.

ML-2 and THP-1 cells showed significant leukemia selective inhibition of colony formation in the presence of FPT-3, U0126 and FTI-277. The IC_{50} values for FTI-277 were <5 μM for these cell lines, suggesting that FTI-277 may elicit leukemia specific toxicity in some cell lines. However, HL-60 and MV4-11 cells revealed IC_{50} values for FTI-277 similar to the IC_{50} values determined for purified CD34+ cells. HL-60 and MV4-11 colony formation was significantly inhibited in the presence of U0126, but HL-60 cells were resistant to FPT-3 (Figure 19). FTI L-744,832 caused inhibition of stem cell colony formation with an IC_{50} value of <1 μM (Figure 20). At >10-15 μM GGTI-286, GGTI-298, and GGTI-2147 elicited substantial toxicity towards purified CD34+ human stem cells. Only minor growth inhibitory effects on purified CD34+ cells were observed in the presence of DMSO or GGTI-2133 at concentrations up to 20 μM (Figure 20). Most cell lines (e.g. NB-4, HL-60 and THP-1) exhibited significant inhibition of colony formation in the presence of GGTI-286, GGTI-298, and GGTI-2147. At concentrations below 10 μM, GGTI-2147 elicited leukemia specific toxicity in some cell lines, e.g. HL-60 (IC_{50} 12.6μM), ML-2 (IC_{50} 8.2μM), MV4-11 (IC_{50} 4.2μM), NB4 (IC_{50} <1μM) and THP-1 (IC_{50} <1μM), as colony formation of human CD34+ cells (IC_{50} 15-20 μM) was largely unaffected (Figure 20).

3.2.3. ***Effect of inhibitors of Ras-to-MAPK signaling on cell cycle progression.*** To elucidate the effects of the most potent inhibitors on cell cycle progression of leukemia cells, NB-4 cells were incubated 18 h and 36 h in the presence of 50 μM FPT-3, 50 μM U0126 and 20 μM FTI-277 (Figures 21 and 22). NB-4 cells were studied because they showed strong activation of the Ras pathway and revealed high sensitivity towards inhibitors of Ras signaling. After incubation with FPT-3, U0126 and FTI-277, double staining with propidium iodide and anti-P-MEK-1/2 antibodies was performed and cells were analyzed by flow cytometry. Incubation with DMSO for 18 hours elicited no substantial changes in NB-4 cell cycle progression (Figure 21). However, incubation with DMSO for 36 h resulted in an increase of the G_0/G_1 fraction to 63.5% compared to 48.5% in untreated controls (Figure 22A and B). In DMSO-treated control cells, 0.8% showed strong activation of MEK-1/2 in the G_0/G_1 phase and 2.4% in the G_2/M phase of the cell cycle. These subpopulations were slightly reduced after 36 h (Figure 22A and B).

After 18 h incubation with 50 μM FPT-3, a strong increase of the sub-G_0/G_1 fraction (debris) was observed suggesting rapid induction of apoptotic DNA fragmentation (increase of debris from 13.5 to 35.8%) (Figure 21C). Furthermore, the G_0/G_1 fraction

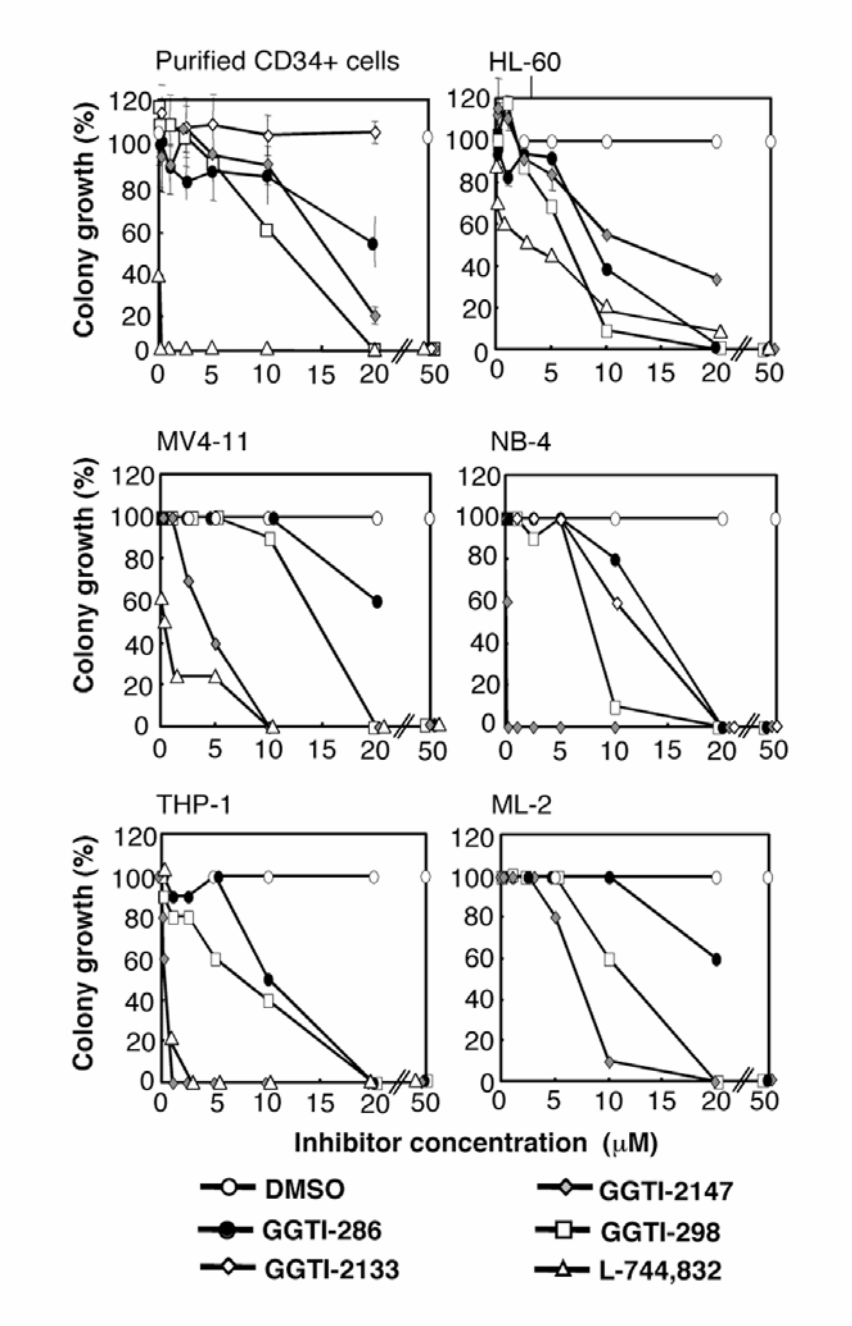

Figure 20. Inhibition of myeloid leukemia cell and colony growth upon treatment with GGTI-286, GGTI-298, GGTI-2147 and GGTI-2133. Purified human CD34+ cells and myeloid leukemia cell lines were incubated in liquid suspension cultures with increasing concentrations of the CAAL-based inhibitors of geranylgeranyltransferase-1 GGTI-286, GGTI-298, GGTI-2147 and GGTI-2133, of the CAAX-based farnesyltransferase inhibitor L-744,832 or with DMSO as solvent control. After four days, viability of the cells was determined by trypan blue dye assays. Aliquots of the samples were incubated in methylcellulose for an additional 7-14 days in the presence of freshly added inhibitors. Representative experiments are shown. Results are expressed in percentage inhibition and are normalized to solvent (DMSO) controls. Error bars indicate the standard margin of error.

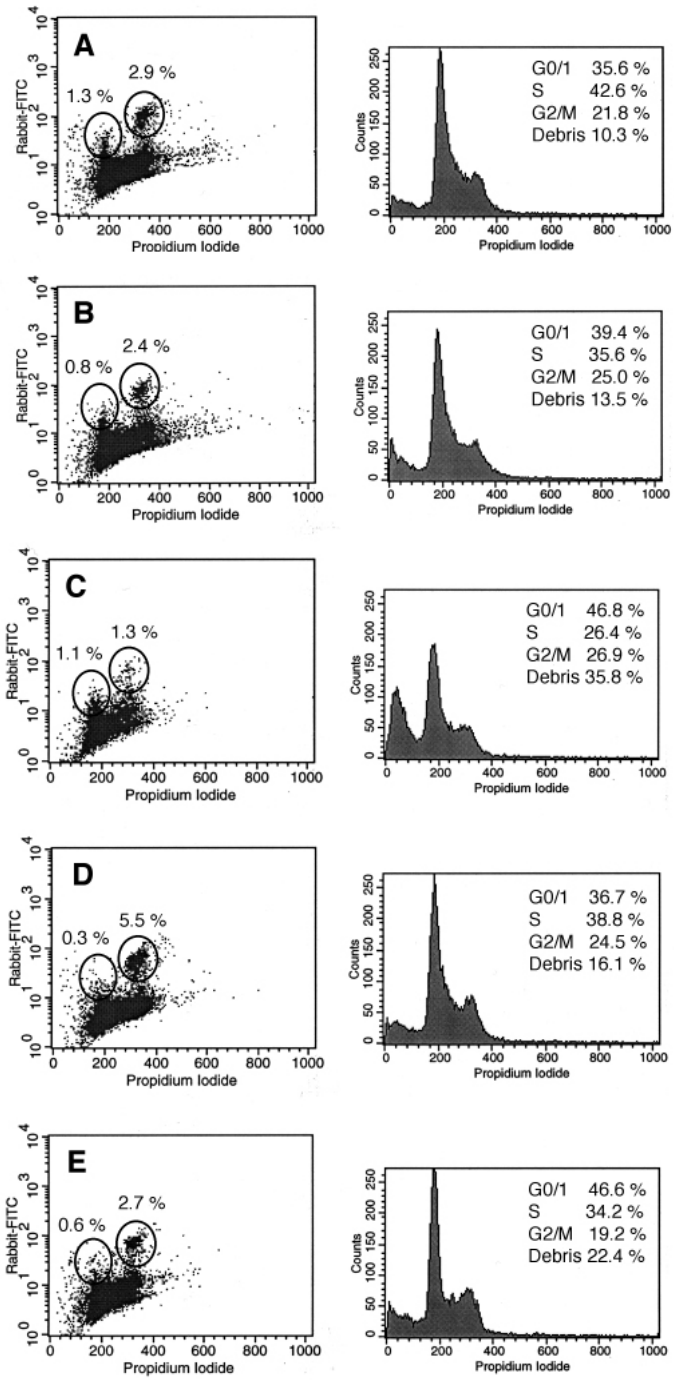

Figure 21. Effects of U0126, FPT-3 or FTI-277 treatment for 18 hours on the cell cycle dependent activation of MEK-1/2. NB-4 cells were incubated in the absence or the presence of 50 μM FPT-3, 50 μM U0126 or 20 μM FTI-277. After 18 hours, double staining with propidium iodide and an antibody specific for activated PP-MEK-1/2 was performed as described in Methods. Representative cell cycle profiles (left) and FACS profiles of the double staining (right) are shown. A, untreated NB-4 cells; B, solvent-treated (DMSO) NB-4 cells; C, NB-4 cells treated with 50 μM FPT-3; D, NB-4 cells treated with 20 μM FTI-277; E, NB-4 cells treated with 50 μM of U0126.

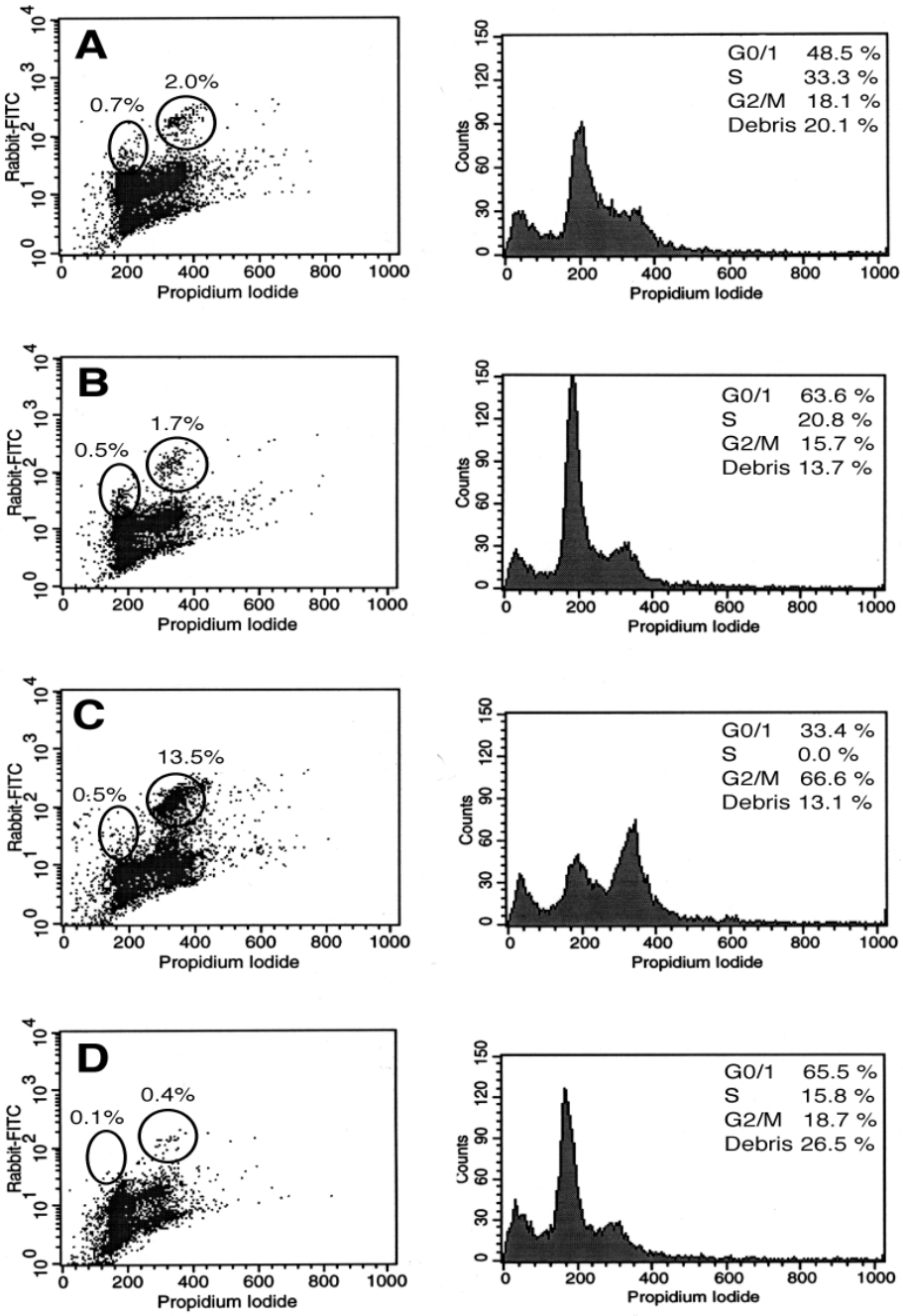

Figure 22. Effects of U0126 or FTI-277 treatment for 36 hours on the cell cycle dependent activation of MEK-1/2. NB-4 cells were incubated in the absence or the presence of 50 μM U0126 or 20 μM FTI-277. After 36 hours, double staining with propidium iodide and an antibody specific for activated PP-MEK-1/2 was performed as described in Methods. Representative cell cycle profiles (left) and FACS profiles of the double staining (right) are shown. A, untreated NB-4 cells; B, solvent-treated (DMSO) NB-4 cells; C, NB-4 cells treated with 20 μM FTI-277; D, NB-4 cells treated with 50 μM of U0126. FPT-3 treated NB-4 cells are not shown because of nearly complete apoptosis at this time point.

increased to 46.8%. PP-MEK-1/2 positive cells were reduced to 1.3% in G_2/M, but remained unchanged in G_0/G_1 (1.1 vs. 0.8 and 1.3 % for DMSO and negative controls). Treatment of NB-4 cells with FPT-3 for 36 h resulted in nearly complete apoptotic DNA fragmentation (not shown).

Incubation with FTI-277 for 18 h lead to an increase of cellular debris to 16.1%, an increase of PP-MEK-1/2-positive cells in G_2/M to 5.5% but a decrease in G_0/G_1 to 0.3% (Figure 21D). FTI-277 treatment for 36 h induced a G_2/M block with 66.6% of cells in G_2/M as well as an increase of PP-MEK-1/2-positive cells in G_2/M to 13.5%, but a decrease in G_0/G_1 to 0.5% (Figure 22C). Similarly, incubation of NB4 with FTI L-744,832 for 18 h lead to an increase of PP-MEK-1/2-positive cells in G_2/M from 5.2% to 7.5% but a slight decrease in G_0/G_1 from 2.6% to 2.1% (Table 6). Treatment with FTI L-744,832 for 36 h caused a G_2/M block with 32.5% of cells in G_2/M and a further increase of PP-MEK-1/2-positive cells in G_2/M to 8.7%, but a decrease in G_0/G_1 to 0.3% (Table 6).

To elucidate the effects of the most potent GGTIs on cell cycle progression of leukemia cells, NB-4 cells were incubated 18 h and 36 h with 20 μM of these inhibitors (Table 6). After incubation with GGTI-286, GGTI-298, GGTI-2133 and GGTI-2147, double staining with propidium iodide and anti-PP-MEK-1/2 antibodies was performed and cells were analyzed by flow cytometry. Incubation with DMSO elicited no substantial changes in NB-4 cell cycle progression as compared to non-treated cells (Table 6). After 18 h, 2.6% of DMSO-treated control cells showed strong activation of MEK-1/2 in the G_0/G_1 phase and 5.2% in the G_2/M phase of the cell cycle. These subpopulations were reduced after 36 h reflecting an accumulation of resting cells (Table 6).

After 18 h incubation with 20 μM GGTI-286, a slight increase of the sub-G_0/G_1 fraction (debris) was observed suggesting rapid induction of apoptotic DNA fragmentation (increase of debris from 6.6 to 10.8%) (Table 6). Furthermore, the G_0/G_1 fraction and PP-MEK-1/2 positive cells in G_0/G_1 increased from 43.2% to 57.2% and from 2.6% to 7.5%, respectively. In contrast, the G_2/M fraction and PP-MEK-1/2 positive cells in G_2/M were reduced to 10.3% and 2.0%. Similar results were obtained for GGTI-298, GGTI-2133 and GGTI-2147 (Table 6) suggesting that treatment with GGTIs led to a G_0/G_1 cell cycle block with accumulation of PP-MEK-1/2 positive cells in G_0/G_1.

Treatment with GGTI-286 for 36 h induced a further increase of cellular debris to 19.8%, an increase in the G_0/G_1 fraction to 61.7%, and a substantial decrease in PP-MEK-1/2 positive-cells in G_0/G_1 and G_2/M as compared to solvent control (0.5% and 0.1% vs. 1.5% and 1.4%, respectively) (Table 6). A similar increase of cellular debris and decrease

Table 6. Effect of GGTI treatment on cell cycle progression and induction of apoptosis in NB4 cells.

Inhibitor		Cells (%) Cell cycle phase G0/G1	S	G2/M	Sub-G0	Apoptosis (45h) TUNEL/AnnexinV/7AAD
Control	**18h**	46.0±1.2	37.7±0.8	16.3±1.1	8.0±0.4	2.9±0.8
PP-MEK-1/2+		3.3		4.9		
	36h	51.8±0.8	39.5±0.9	8.7±0.4	9.1±0.3	13.4±1.6
PP-MEK-1/2+		1.6		1.4		
DMSO	**18h**	43.2±1.5	38.7±1.3	18.1±1.9	6.6±1.2	0.6±0.3
PP-MEK-1/2+		2.6		5.2		
	36h	53.3±0.5	38.9±0.3	7.9±0.3	9.8±0.4	19.1±7.5
PP-MEK-1/2+		1.5		1.4		
GGTI-286	**18h**	57.2±0.6	32.4±0.8	10.3±1.1	10.8±0.4	60.3±9.9
PP-MEK-1/2+		7.5		2.0		
	36h	61.7±0.8	34.0±0.8	4.3±0.4	19.8±1.5	71.6±17.1
PP-MEK-1/2+		0.5		0.1		
GGTI-298	**18h**	56.1±0.5	33.4±0.8	10.4±1.3	8.3±0.4	62.3±9.3
PP-MEK-1/2+		4.4		1.7		
	36h	60.4±2.1	34.4±2.3	5.2±0.5	24.9±4.6	79.1±10.4
PP-MEK-1/2+		0.7		0.3		
GGTI-2133	**18h**	44.6±0.7	36.8±0.5	18.6±0.3	12.0±0.4	21.1±6.1
PP-MEK-1/2+		4.4		3.4		
	36h	49.2±0.8	47.3±0.4	3.5±1.2	17.0±0.3	28.8±3.6
PP-MEK-1/2+		0.5		0.4		
GGTI-2147	**18h**	52.6±0.5	31.5±0.6	15.8±0.2	9.1±0.1	86.8
PP-MEK-1/2+		5.9		2.8		
	36h	50.6±0.8	47.5±0.3	1.9±0.5	17.1±2.4	65.3±7.7
PP-MEK-1/2+		0.7		0.2		
FTI L-744,832	**18h**	48.7±1.2	31.9±2.7	19.4±1.6	7.3±1.5	32.6
PP-MEK-1/2+		2.1		7.5		
	36h	32.5±2.4	35.0±3.1	32.5±0.7	15.5±3.7	86.9±6.8
PP-MEK-1/2+		0.3		8.7		

Legend: NB-4 cells were incubated with or without 20 μM GGTI-286, GGTI-298, GGTI-2133, GGTI-2147 or FTI-744,832. After 18 h and 36 h, double staining with propidium iodide and an antibody specific for activated MEK-1/2 (PP-MEK-1/2^{+}) was performed and cells were analyzed by flow cytometry as described in Methods. Apoptosis of NB4 cells was determined after 45 h incubation using *in situ* TUNEL and annexin V/7-AAD assays as described in Methods. Mean values ± standard deviation are given (n=3). Note : According to ModFit, cell cycle fractions (G_0/G_1, S and G_2M phase) are shown in percent of viable cells. Sub-G_0 fractions are expressed as percent debris of total cell counts.

of PP-MEK-1/2 positive cells in G_0/G_1 and G_2/M was observed after 36 h incubation with GGTI-298, GGTI-2133 and GGTI-2147 (Table 6). However, the extent of the GGTI-induced G_0/G_1 block varied between the different GGTIs at this time point. The initial increase in PP-MEK-1/2$^+$ cells in G_0/G_1 after 18h treatment with GGTIs and the elimination of activated MEK after 36h exposure to GGTIs suggests that GGTI treatment interferes with and subsequently disrupts the Ras-to-MAPK cascade.

Treatment with U0126 for 18 h induced an increase of cellular debris to 22.4%, an increase in the G_0/G_1 fraction to 46.6%, and a slight decrease in PP-MEK-1/2 positive-cells in G_0/G_1 (0.6% vs. 0.8%) (Figure 21B and D). After 36 h incubation with U0126 the sub-G_0/G_1 debris increased to 26.5% and the PP-MEK-1/2 positive-cells were further reduced to 0.1% in G_0/G_1 and 0.4% in G_2/M (Figure 22D).

3.2.4. ***Apoptosis induction by Ras signaling inhibitors.*** Propidium iodide staining demonstrated an increase of the sub-G_0/G_1 fraction (debris) after treatment of NB-4 cells with U0126 and FPT-3 (Figures 21 and 22) suggesting apoptotic DNA fragmentation. To confirm induction of apoptosis by these inhibitors, NB-4 cells were incubated for 18 and 36 hours with FPT-3, U0126 and FTI-277. DNA strand breaks were detected by labeling with flourescein-dUTP using the TUNEL method. Externalization of membrane phosphatidylserine during early stages of apoptosis was monitored by an Annexin V-PE/7-amino-actinomycin (7-AAD) assay. Annexin V binds to negatively charged phospholipid surfaces with a higher specificity for phosphatidylserine than most other phospholipids. 7-AAD was used for the exclusion of nonviable cells. Quantification of apoptosis at the single cell level was accomplished by flow cytometry. The presence of DMSO had no effect on DNA degradation or exposure of phosphatidylserine (Figure 23B). FPT-3 treatment resulted in apoptotic DNA fragmentation (86.5% after 18h) and exposure of phosphatidylserine on the outer leaflet of the plasma membrane (87.3% after 36 h incubation) (Figure 23C). Similarly, treatment with U0126 induced DNA cleavage (47.4%) and externalization of phosphatidylserine (81.5%) (Figure 23E). In contrast, FTI-277 treatment for 18 and 36 h, which caused a G2/M block, did not induce apoptosis at these time points (Figure 23D).

GGTI-induced apoptosis was also quantified by the both TUNEL method and the Annexin V-PE/7-AAD double staining method. The presence of DMSO had no effect on DNA degradation or exposure of phosphatidylserine (Table 6). GGTI-286 treatment

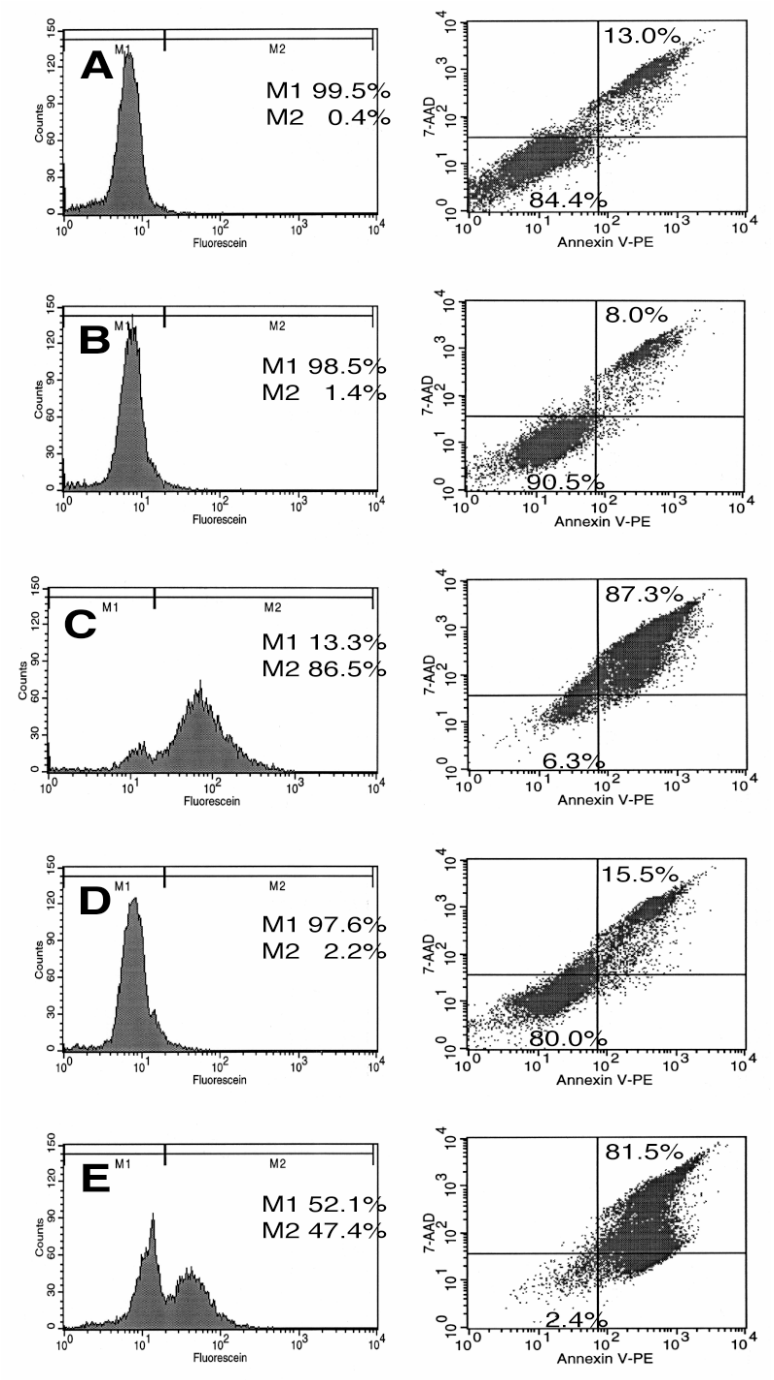

Figure. 23. Induction of apoptosis by treatment with FPT-3, FTI-277 or U0126. NB-4 cells were incubated in the presence or absence of 50 μM FPT3, 50 μM U0126 or 20 μM FTI-277. After 18 hours, cells were harvested and labeling of DNA strand breaks was performed applying the TUNEL method as described in Methods (left). M1, no DNA fragmentation, M2, DNA fragmentation. Exposure of phosphatidylserine on the outer leaflet of the plasma membrane was detected using an Annexin V-PE/7-AAD double staining method as described in Methods (right). Apoptotic exposure of phosphatidylserine is shown in the upper right square. Results are given in percentage of total cell population. A, untreated NB-4 cells; B, solvent-treated (DMSO) NB-4 cells; C, FPT-3 treated NB-4 cells; D, FTI-277 treated NB-4 cells; E, U0126 treated NB-4 cells.

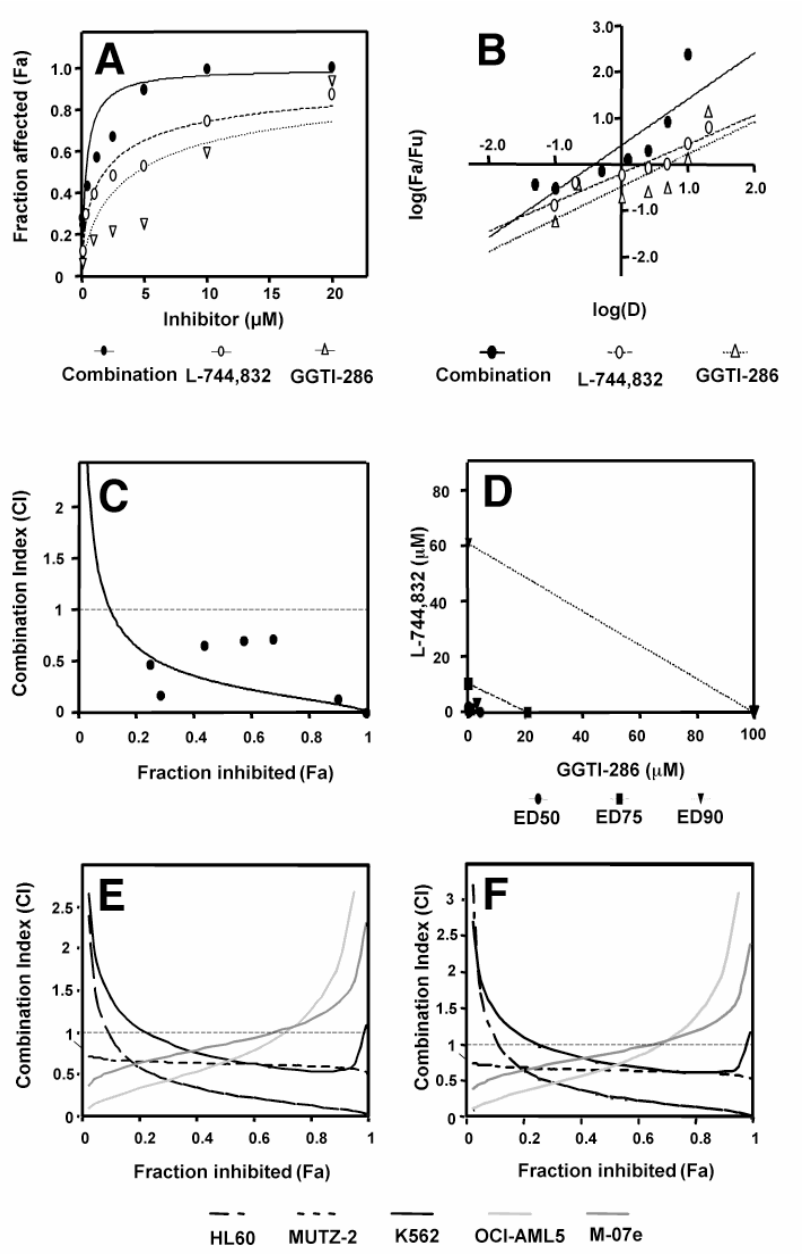

Figure 24. Treatment of myeloid leukemia cells with a combination of FTI L-744,832 and GGTI-286 leads to synergistic growth inhibition. Myeloid leukemia cell lines were incubated in liquid suspension cultures with increasing concentrations of FTI L-744,832, GGTI-286 or with a fixed 1:1 ratio of L-744,832 and GGTI-286. After four days, viability of the cells was determined by trypan blue dye exclusion assays. Aliquots of the samples were incubated in methylcellulose for an additional 7-14 days in the presence of freshly added inhibitors. Colony formation was plotted relative to solvent-treated (DMSO) control cells. A, Colony formation (= fraction affected) of HL60 cells in the presence of increasing concentrations of FTI L-744,832, GGTI-286 or a fixed 1:1 ratio of both inhibitors; B, median effect plot of data in A; C, plot of combination index (CI) versus cytotoxicity calculated from data in B under the assumption that agents are mutually exclusive; D, isobolograms for data in A; E, plot of CI calculated from data obtained for HL60, MUTZ-2, K562, OCI-AML5 and M-07e cells under the assumption that agents are mutually exclusive or non-exclusive (F). Each panel of results was accomplished at least three times.

resulted in apoptotic DNA fragmentation (60.3% after 45 h) and exposure of phosphatidylserine on the outer leaflet of the plasma membrane (71.6% after 45 h incubation) (Table 6). Similarly, treatment with GGTI-298, GGTI-2133 and GGTI-2147 induced DNA cleavage (62.3%, 21.1% and 86.8%, respectively) and externalization of phosphatidylserine (79.1%, 28.8% and 65.3%, respectively) (Table 6).

3.2.5. ***Effects of FTI/GGTI co-treatment on myeloid leukemia cell growth.*** To overcome resistance caused by alternative geranylgeranylation of K-Ras and N-Ras in the presence of FTI treatment, myeloid cell lines were exposed to a fixed ratio (1 : 1) of FTI L-744,832 and GGTI-286. Co-treatment of HL60 and other myeloid cell lines with FTI and GGTI resulted in stronger inhibition of colony growth than treatment with FTIs or GGTIs alone (Figure 24). The data were analyzed by the median effect method (Chou and Talalay, 1984) to determine whether the inhibitory effects were additive, synergistic or antagonistic. Median effect plots were generated to determine x-intercepts (IC_{50}) and slopes (*m*) and to calculate the combination indices (CI). CIs < 1 indicate synergism, CIs = 1 indicate additive effects and CIs >1 indicate antagonism. For the combination of FTI L-744,832 and GGTI-286, the CI calculated under the assumption that the drugs were mutually exclusive was <1 over much of the range examined, with mean CI values of 0.272±0.179 at the IC_{50}, 0.172±0.179 at the IC_{70} and 0.083±0.093 at the IC_{90} (n=4) (Figure 24C). The effect of combining FTI-L-744,832 with GGTI-286 was also examined in other myeloid cell lines (K562, MUTZ-2, AML-OCI5, M-07e) (Figure 24E and F). The CI values consistently dropped below 1 over much of the range examined (Figure 24E and F). These results indicate that co-treatment of myeloid cell lines with FTIs and GGTIs has synergistic cytotoxic effects.

Interestingly, co-treatment with 10 μM GGTI-286 and 10 μM FTI-L-744,832 reduced the FTI-induced G_2/M-block (at 18 h and 36 h, respectively) and increased cellular debris (sub-G_0/G_1 fraction) (Figure 26E). Co-treatment of NB-4 cells with GGTI-286 also reduced the FTI-induced accumulation of PP-MEK-1/2^+ cells in G_2/M, suggesting more efficient inhibition of Ras prenylation (Figures 25 and 26, panels D and E).

In agreement with these results, co-treatment of NB-4 cells with 10 μM FTI L-744,832 and 10 μM GGTI-286 was found to be more effective than treatment with either 20 μM FTI L-744,832 or 20 μM GGTI-286 alone in inducing translocation of phosphatidylserine (99.5% versus 93.6% and 85.6%) and DNA fragmentation (72.0% versus 32.6% and 46.2%) (Figure 27).

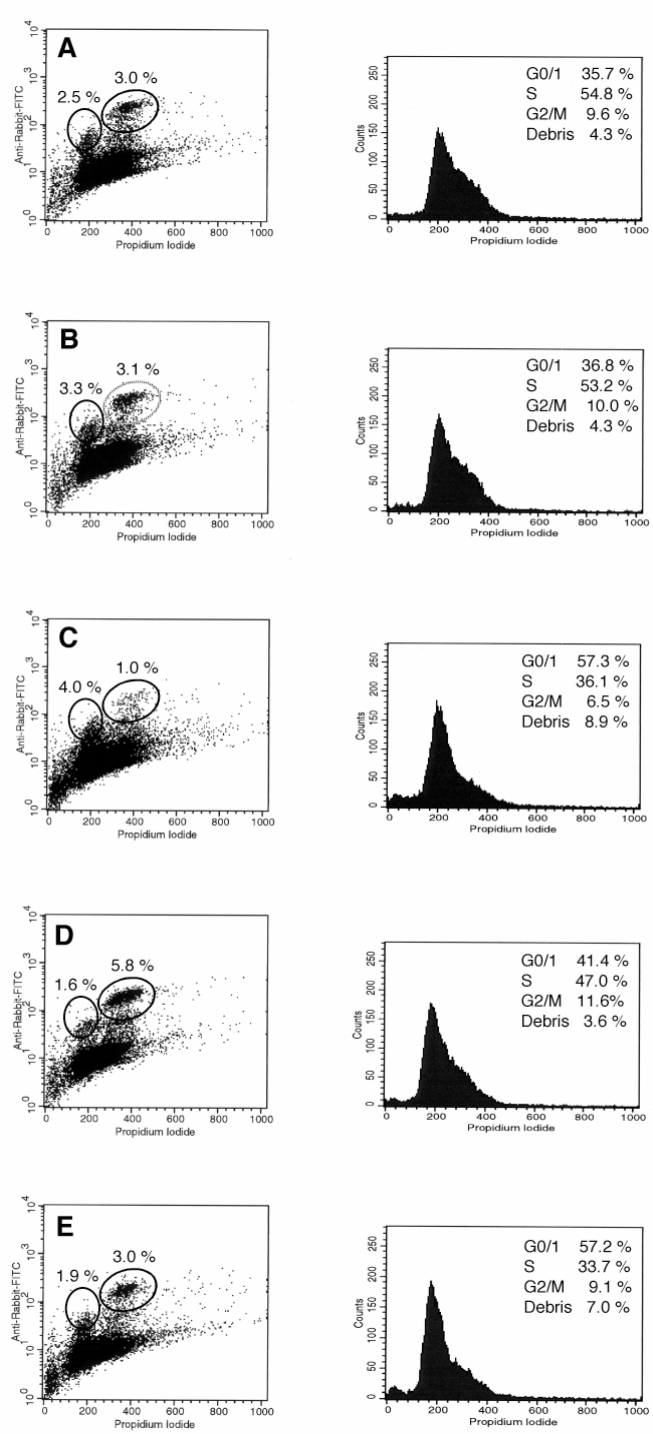

Figure 25. Effects of 18 h co-treatment with FTI L-744,832 and GGTI-286 on cell cycle progression and the cell cycle-dependent activation of MEK-1/2 in myeloid leukemia cells. NB-4 cells were incubated in the absence or the presence of 20 μM GGTI-286, FTI L-744,832 or a combination of 10 μM GGTI-286 and 10 μM L-744,832. After 18 h, double staining with propidium iodide and an antibody specific for activated PP-MEK-1/2 was performed as described in Methods. Representative cell cycle profiles (right) and FACS profiles of the double staining (left) are shown. A, untreated NB-4 cells; B, solvent-treated (DMSO) NB-4 cells; C, 20 μM GGTI-286; D, 20 μM FTI L-744,832; E, combination of 10 μM GGTI-286 and 10 μM L-744,832.

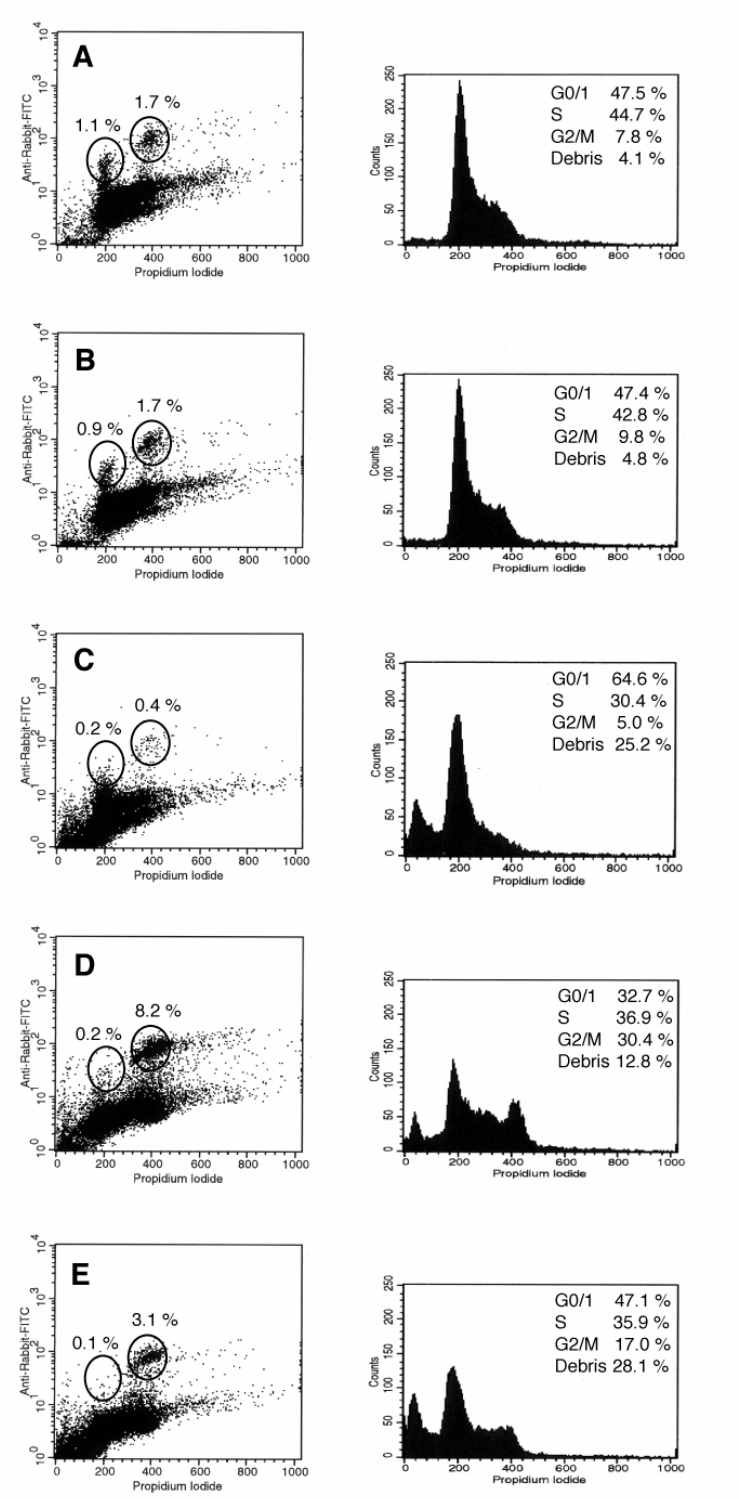

Figure. 26. Effects of 36 h co-treatment with FTI L-744,832 and GGTI-286 on cell cycle progression and the cell cycle-dependent activation of MEK-1/2 in myeloid leukemia cells. NB-4 cells were incubated in the absence or the presence of 20 μM GGTI-286, FTI L-744,832 or a combination of 10 μM GGTI-286 and 10 μM L-744,832. After 36 h, double staining with propidium iodide and an antibody specific for activated PP-MEK-1/2 was performed as described in Methods. Representative cell cycle profiles (right) and FACS profiles of the double staining (left) are shown. A, untreated NB-4 cells; B, solvent-treated (DMSO); C, 20 μM GGTI-286; D, 20 μM FTI L-744,832; E, 10 μM GGTI-286 and 10 μM L-744,832.

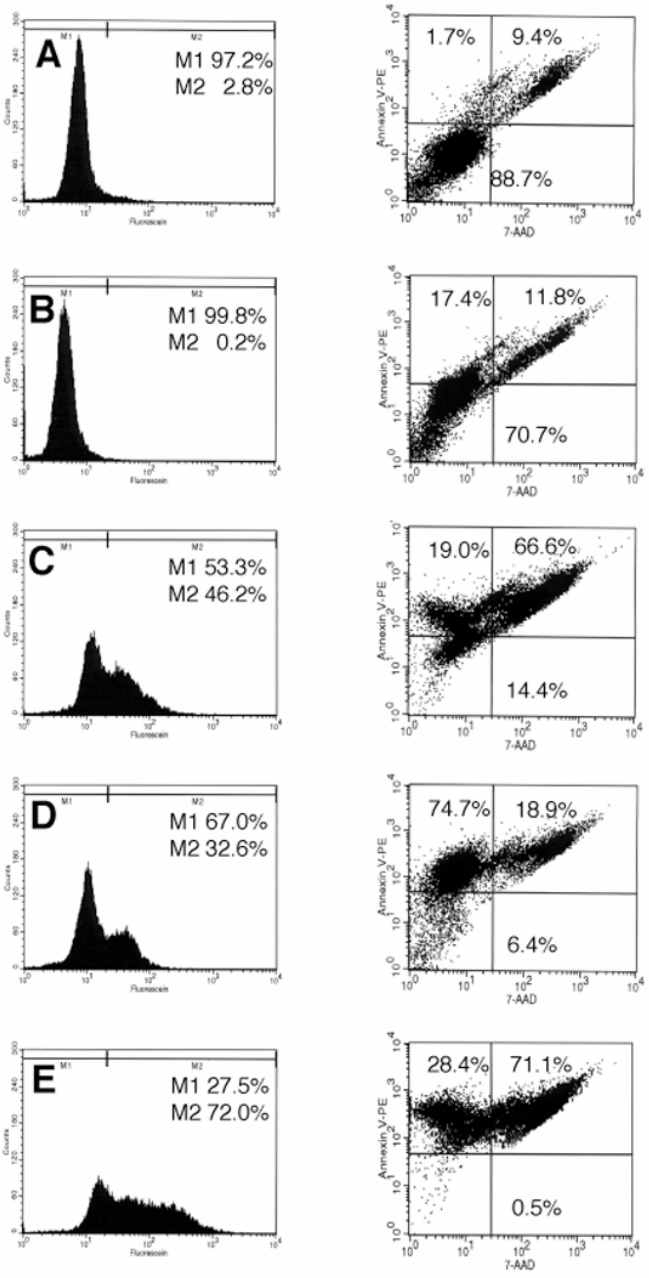

Figure 27. Co-treatment of myeloid leukemia cells with GGTI-286 and FTI L-744,832 leads to induction of apoptosis. NB-4 cells were incubated in the presence or absence of 20 μM GGTI-286, 20 μM FTI L-744,832, or a combination of 10 μM GGTI-286 and 10 μM L-744,832. After 42-45 h, cells were harvested and labeling of DNA strand breaks was performed applying the TUNEL method as described in Methods (left). M1, no DNA fragmentation, M2, DNA fragmentation. Exposure of phosphatidylserine on the outer leaflet of the plasma membrane was detected using an Annexin V-PE/7-AAD double staining method as described in Methods (right). Apoptotic exposure of phosphatidylserine is shown in the upper left (early apoptosis) and upper right square (late apoptosis). Results are given in percentage of total cell population. (A) untreated NB-4 cells; (B) solvent-treated (DMSO); (C) GGTI-286 alone; (D) FTI L-744,832 alone; (E) GGTI-286 and FTI L-744,832 co-incubation

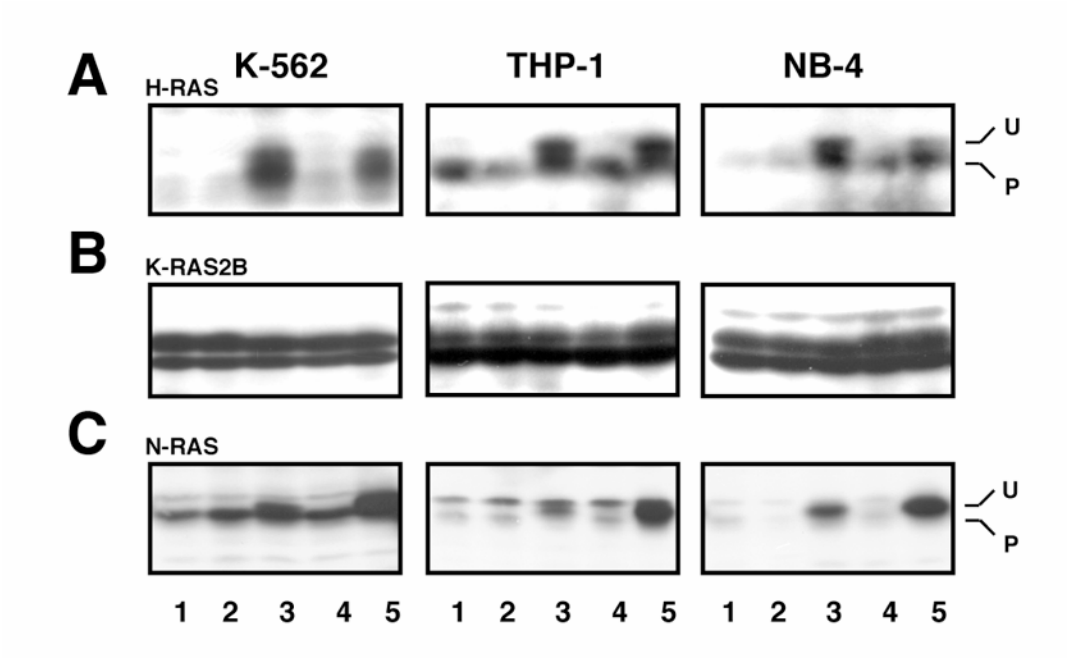

Figure 28. SDS-PAGE analyses of H-, K- and N-Ras prenylation in myeloid leukemia cell lines treated with FTI, GGTI or FTI/GGTI combination. K-562, THP-1 and NB-4 cells were incubated 24 h alone (lane 1), with DMSO solvent control (lane 2), 20μM FTI L-744,832 (lane 3), 20 μM GGTI-286 (lane 4) or a combination of 10 μM FTI L-744,832 and 10 μM GGTI-286 (lane 5). Cell lysates were probed for H-Ras (A), K-Ras2B (B) and N-Ras (C). Unprocessed (U) and processed (P) Ras proteins are indicated.

3.2.6. ***Effects of FTI L-744,832 and GGTI-286 on Ras prenylation.*** Treatment with 20 μM FTI L-744,832 resulted in an electrophoretic mobility shift corresponding to unprocessed H-Ras indicating FTI-induced inhibition of posttranslational prenylation (Figure 28A). Similar results were obtained with a combination of 10 μM FTI L-744,832 and 10 μM of GGTI-286, whereas treatment with GGTI-286 alone had no effect on H-Ras prenylation (Figure 28A). In some cases an electrophoretic mobility shift of K-Ras2B was observed in FTI, GGTI or FTI/GGTI-treated cells (not shown). However, in contrast to the mobility shifts of H-Ras and N-Ras, these changes were not consistent in all experiments. K-Ras2A was not detected using an antibody specific for this splice variant (not shown). Interestingly, a strong accumulation of unprocessed N-Ras was observed in cells treated with FTI L-744,832. Furthermore, FTI/GGTI co-treatment lead to a substantial increase in unprocessed N-Ras, indicating potent inhibition of N-Ras geranylgeranylation in the presence of FTI by GGTI-co-treatment (Figure 28C).

3.2.7. ***Effects of FTI L-744,832 and GGTI-286 on prenylation of non-Ras proteins.*** As FTIs induce multiple effects on cancer cells, it has been suggested that multiple

farnesylated proteins may be important in mediating the FTI-induced effects (Tamanoi *et al* 2001). Among these, the G-proteins Rheb, Rap2, RhoB, RhoD, Rho6-8, the kinetochore-binding proteins CENP-E and CENP-F, the tyrosine phosphatases PTPCAAX1 and PTPCAAX2 and the nuclear Lamins A and B have been characterized. In order to evaluate the processing inhibition of other FTase substrates in myeloid leukemia cells by FTI treatment, several farnesylated proteins known to undergo mobility shifts upon FTase inhibition (Adjei *et al* 2000) were analyzed. In most experiments, a mobility shift of the H-Ras-related G-proteins Rap2A and Rheb was observed (Figure 29A and 29B). Furthermore, FTI-treatment resulted in a shift of intranuclear intermediate filament protein Lamin A in K562 cells (Figure 29C). In the case of Lamin A, this mobility shift reflects inhibition of a farnesylation-dependent proteolytic processing that removes a 13-amino acid peptide from the C-terminus of Prelamin A (Sinensky *et al*

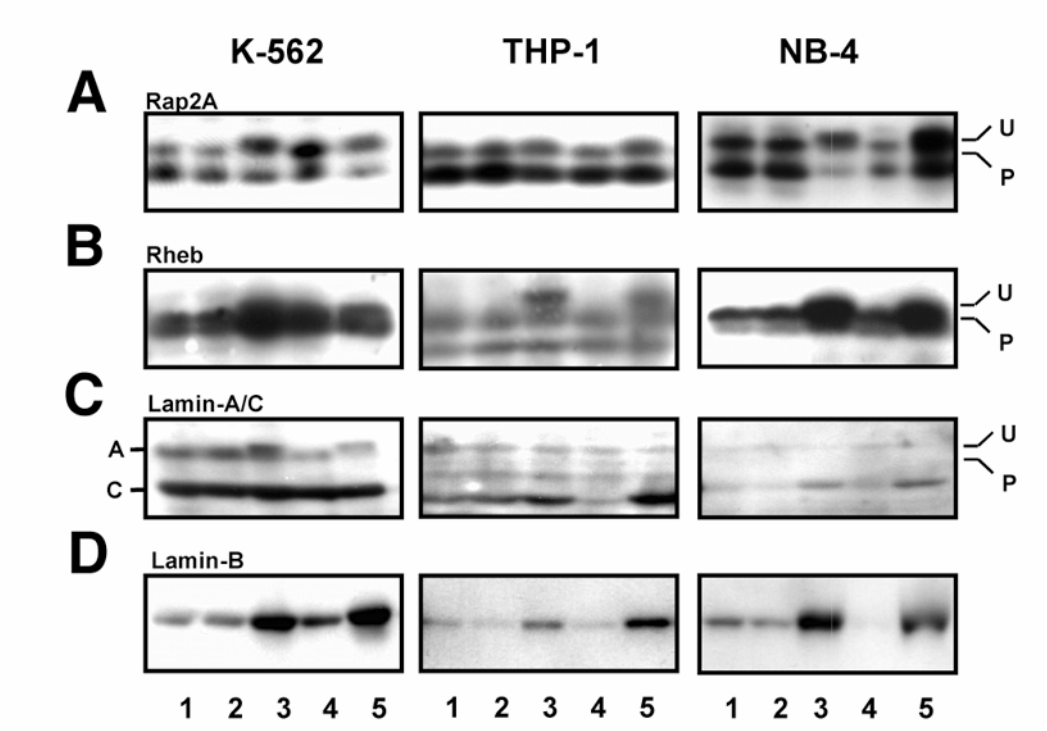

Figure 29. SDS-PAGE analyses of Rap2A, Rheb, Lamin-A/C and Lamin B in myeloid leukemia cell lines treated with FTI, GGTI or a FTI/GGTI combination. K-562, THP-1 and NB-4 cells were incubated 24 h alone (lane 1), with DMSO solvent control (lane 2), 20µM FTI L-744,832 (lane 3), 20 µM GGTI-286 (lane 4) or a combination of 10 µM FTI L-744,832 and 10 µM GGTI-286 (lane 5). Cell Lysates were probed for Rap2A (A), Rheb (B), Lamin-A/C (C), or Lamin-B (D). Unprocessed (U) and processed (P) proteins are indicated.

1994). While farnesylation of Lamin B and RhoB has previously been reported to be sensitive to FTI treatment, no mobility shifts of these two proteins were observed in myeloid cells treated with FTI L-744,832 alone or in combination with GGTIs (Figure 29D, not shown). However, FTI treatment resulted in an accumulation of Lamin B which might be due to cell-cycle-dependent or FTI-induced expression. CENP-E and CENP-F, which function as centromere-associated kinesin motors and play critical roles in mitosis, could not be detected in myeloid cell lysates by Western blotting with commercially available antibodies (not shown). The predominant effect of FTI L-744,832 on N-Ras suggests that this FTI exhibits a greater specificity against farnesylation of N-Ras proteins.

3.2.8. ***Effects of FTI L-744,832 and GGTI-286 on Ras activation.*** To identify the presence of GTP-Ras, cell lysates were incubated with the minimal Ras-binding domain (RBD) of C-Raf-1. Western blotting with antibodies against H-, K- and N-Ras was performed to detect the binding of GTP-Ras with GST-RBD. Cellular lysates of H-*Ras* (L-61)-transformed NIH-3T3 fibroblasts were used as a positive control for activated H-Ras. Mutated L61- and V-12-H-Ras bound to GST-RBD (not shown). As shown in Figure 28, high levels of activated N-Ras were found in NB-4 and THP-1 cell lysates. FTI-and FTI/GGTI-co-treatment resulted in binding of unprocessed N-Ras to GST-RBD, suggesting that unprocessed N-Ras is capable of forming inactive N-Ras-Raf complexes when GTP-loaded. This finding supports the hypothesis that unprocessed N-Ras may elicit a dominant negative effect on Ras-signaling.

3.2.9. ***Effect of FTI/GGTI co-treatment on primary AML cells.*** To assess the potential clinical relevance of our findings, the effects of FTI and GGTI treatment on primary AML cells were analyzed. Treatment with FTI L-744,832 or GGTIs alone induced apoptosis in a fraction of cells (Figure 31). In agreement with results using myeloid cell lines, GGTI-2147 was found to be more effective than GGTI-286 or GGTI-298 in inducing apoptosis and inhibiting AML cell growth (Table 7). As shown in Table 7 and Figure 31 (panel H vs panels B and G), FTI/GGTI co-treatment was found to be even more effective in inhibiting cell proliferation and inducing apoptosis than treatment with FTI or GGTI alone. Propidium iodide and anti-PP-MEK-1/2 double staining demonstrated lower proliferating rates of primary AML blasts as compared to myeloid cell lines (Tables 6 and 7). Double staining of short term cultures of primary AML blasts in RPMI containing 20% FCS revealed a large portion of cells in G_0/G_1 and very low amounts of $PP\text{-}MEK^+$ cells in the G_0/G_1 and G_2/M phases. Proliferation of primary AML cells was increased by culturing in

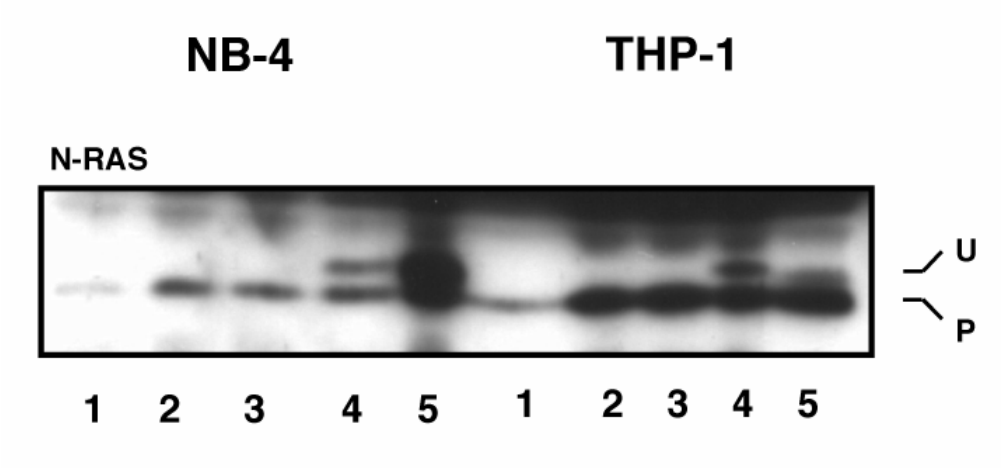

Figure 30. FTI/GGTI co-treatment of myeloid leukemia cells causes accumulation of unprocessed, activated N-Ras as demonstrated by affinity precipitation with GST-RBD. NB-4 and THP-1 cells were incubated 48 h alone (lane 1), with DMSO solvent control (lane 2), 20μM FTI L-744,832 (lane 3), 20 μM GGTI-286 (lane 4) or a combination of 10 μM FTI L-744,832 and 10 μM GGTI-286 (lane 5). Cellular lysates were subjected to affinity precipitation (AP) with GST-RBD as described in "Methods". N-Ras proteins were detected by immunoblotting with N-Ras specific antibodies. Unprocessed (U) and processed (P) N-Ras proteins are indicated.

StemSpan™ containing cytokines (SCF, IL-3, IL-6 and Flt-3 ligand) (Figure 32). GGTI-treatment primarily resulted in G_0/G_1 blocks, whereas FTI-treatment caused either G_0/G_1 or G_2/M arrests in primary AML cells, suggesting that FTI-induced G_2/M blocks were dependent on AML cell type and/or proliferation. In order to demonstrate synergism of FTI/GGTI co-treatment in primary AML cells, AML blasts from 5 AML patients were titrated with increasing concentrations of FTI L-744,832, the most effective GGTase I inhibitor (GGTI-2147) and a fixed ratio (1 : 1) of both inhibitors. After 72 h inhibitor treatment, cell viability was measured using the trypan blue exclusion assay and the MTS cell proliferation assay. The data were analyzed by the median effect method (Chou and Talalay, 1984) and median effect plots were generated to determine x-intercepts (IC_{50}) and slopes (*m*) and to calculate the combination indices (CIs). As shown in Figure 33, the CI values for the FTI/GGTI combination were consistently below 1 over much of the range examined, indicating synergism in all AML cases investigated.

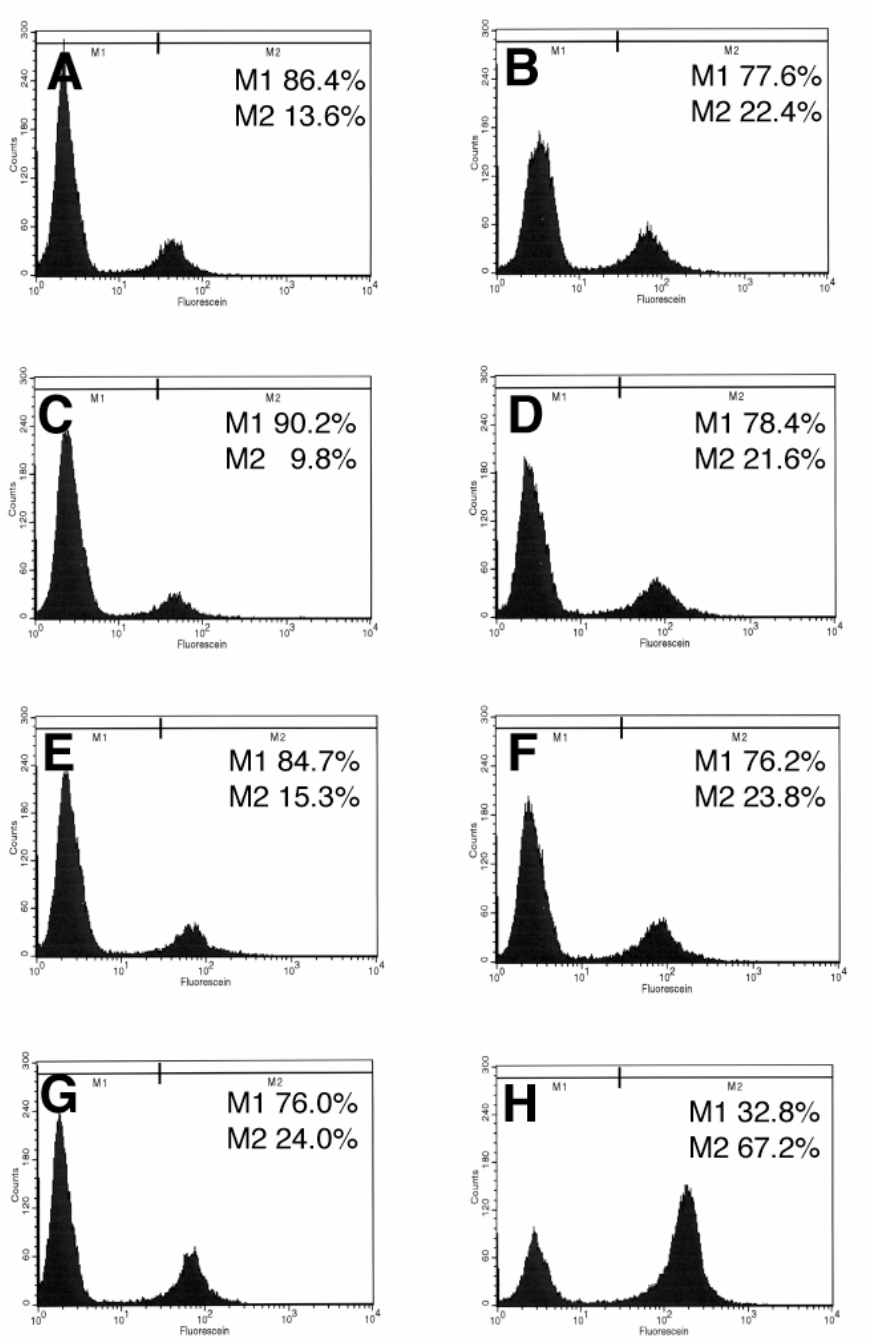

Figure 31. FTI/GGTI co-treatment leads to induction of apoptosis in primary AML cells. Primary AML cells from patient 5 were incubated in the presence or absence of 20 μM FTI L-744,832, 20 μM GGTIs, or a combination of 10 μM L-744,832 and 10 μM GGTIs. After 42-45 h, cells were harvested and DNA strand breaks were labeled by the TUNEL method as described in Methods. M1, no DNA fragmentation, M2, DNA fragmentation. Results are given in percentage of total cell population. A, solvent-treated (DMSO); B, 20 μM FTI L-744,832; C, 20 μM GGTI-286; D, 10 μM GGTI-286 and 10 μM L-744,832; E, 20 μM GGTI-298; F, 10 μM GGTI-298 and L-744,832; G, 20 μM GGTI-2147; H, 10 μM GGTI-2147 and 10 μM FTI L-744,832.

Table 7. Effect of FTI and GGTI treatment on cell cycle progression and induction of apoptosis of primary AML cells.

Patient sex/age/ leukemia/ karyotype	Cell cycle phase/ TUNEL (+/- cytokines)	Cells (%)/Increase in Apoptosis (%) DMSO	FTI L-744,832	GGTI-286	L-744,832+ GGTI-286	GGTI-298	L-744,832+ GGTI-298	GGTI-2147	L-744,832+ GGTI-2147
Patient 1	G0/G1	71.5±1.9	71.9±3.5	79.5±3.5	83.8±2.1	80.6±2.1	83.4±5.4	82.4±1.1	82.6±2.0
m/59	S	28.5±1.9	25.0±1.9	19.2±2.3	16.2±2.1	18.7±2.3	16.2±5.1	17.6±1.1	17.4±2.0
sAML/MDS	G2/M	0.1±0.1	3.1±2.2	1.1±1.5	0±0	0.7±0.3	0.4±0.4	0±0	0±0
46 XY	Sub-G0	6.6±0.4	14.3±0.4	10.1±1.3	15.3±0.9	7.7±0.4	11.7±1.1	15.5±2.7	14.8±0.8
NRAS/61									
	TUNEL + cytokines	0	7.5	5.9	11.3	0.6	9.5	12.3	24.8
	TUNEL - cytokines	0	4.8	1.4	4.0	4.8	5.3	20.2	34.7
Patient 2	G0/G1	85.5±0.3	90.2±1.6	86.3±0.5	91.5±0.5	84.1±1.6	92.9±0.9	86.3±0.9	92.9±0.5
f/71	S	11.5±0.7	8.7±1.9	10.5±0.5	7.2±0.8	12.2±1.0	6.1±1.3	9.9±0.8	5.8±0.2
AML M2, 2. relapse	G2/M	3.0±0.7	1.0±0.5	3.1±0.1	1.3±0.4	3.8±0.7	0.9±0.4	3.9±0.2	1.3±0.6
	Sub-G0	5.7±0.5	8.3±0.8	7.4±0.4	14.8±0.3	5.9±0.9	13.2±0.5	7.5±0.2	16.2±0.2
	TUNEL + cytokines	0	5.9	1.5	2.7	-0.9	9.8	0.5	11.3
	TUNEL - cytokines	0	1.4	-1.0	7.2	-1.2	5.7	7.0	16.3
Patient 3	G0/G1	72.1±0.5	77.8±0.9	n.d.	n.d.	n.d.	n.d.	78.2±1.0	84.4±2.8
m/44	S	26.8±0.6	14.5±0.6	n.d.	n.d.	n.d.	n.d.	14.5±0.6	10.4±1.6
AML M2	G2M	1.2±1.0	7.7±0.3	n.d.	n.d.	n.d.	n.d.	7.3±0.6	5.2±1.4
	Sub-G0	18.2±1.5	34.8±0.2	n.d.	n.d.	n.d.	n.d.	24.0±2.7	57.9±3.2
	TUNEL + cytokines	0	30.6	9.8	41.7	9.3	40.3	23.0	66.1
	TUNEL - cytokines	0	26.6	5.1	27.6	7.5	30.4	40.5	48.2
Patient 4	G0/G1	42.2±2.1	56.6±0.8	n.d.	n.d.	n.d.	n.d.	45.9±0.4	54.3±3.9
m/57	S	40.0±1.4	25.3±1.0	n.d.	n.d.	n.d.	n.d.	30.9±0.7	29.1±1.8
sAML/MDS	G2M	17.8±0.7	18.1±0.9	n.d.	n.d.	n.d.	n.d.	23.2±0.8	16.5±5.5
46 XY 20q-	Sub-G0	12.6±0.5	20.2±2.4	n.d.	n.d.	n.d.	n.d.	16.2±0.2	21.1±0.6
NRAS/12									
	TUNEL + cytokines	0	0.9	-3.4	6.0	-3.1	-2.6	0.5	10.7
	TUNEL - cytokines	0	14.5	1.1	4.7	2.4	5.4	9.0	26.5
Patient 5	G0/G1	71.3±0.2	59.1±4.2	73.2±0.3	67.1±0.8	72.5±0.9	79.0±1.7	78.5±1.0	82.4±3.1
m/66	S	24.4±1.0	27.7±3.6	21.4±0.8	20.0±1.2	24.2±0.3	19.5±0.3	17.3±0.3	10.4±1.6
sAML/MDS	G2M	4.2±1.2	13.2±7.2	5.5±0.6	12.8±1.3	3.4±0.8	1.5±1.7	4.2±1.2	7.2±3.2
NRAS/61	Sub-G0	9.3±0.4	21.1±0.6	6.0±0.2	13.3±0.1	7.4±0.2	22.3±0.4	14.5±0.7	32.5±2.5
	TUNEL + cytokines	0	8.8	-3.8	8.0	1.7	10.2	10.4	53.6
	TUNEL - cytokines	0	8.4	0.6	12.4	2.3	18.2	46.3	67.7

Legend to Table 7: Primary AML cells were incubated with 20 μM FTI L-744,832, 20 μM GGTIs or a combination of 10 μM L-744,832 and 10 μM GGTIs for 48 h. AML blasts from patients 1, 4 and 5 were found to harbor N-Ras mutations (CAA⇒CGA resulting in Q61R, GGT⇒GAT resulting in G12D, and CAA⇒CAC resulting in Q61H, respectively). Cell cycle progression and apoptosis were determined as described in Methods. Mean values ± standard deviation are given (n=3). According to ModFit, cell cycle fractions (G_0/G_1, S and G_2M phase) are shown in percent of viable cells. Sub-G_0 fractions are expressed as percent debris of total cell counts. For *in situ* TUNEL apoptosis assays, cells were cultured in the presence (+ cytokines) and absence (- cytokines) of cytokines (SCF, IL-3, IL-6, Flt-3 ligand). Increase in apoptosis is given as percent solvent control (DMSO).

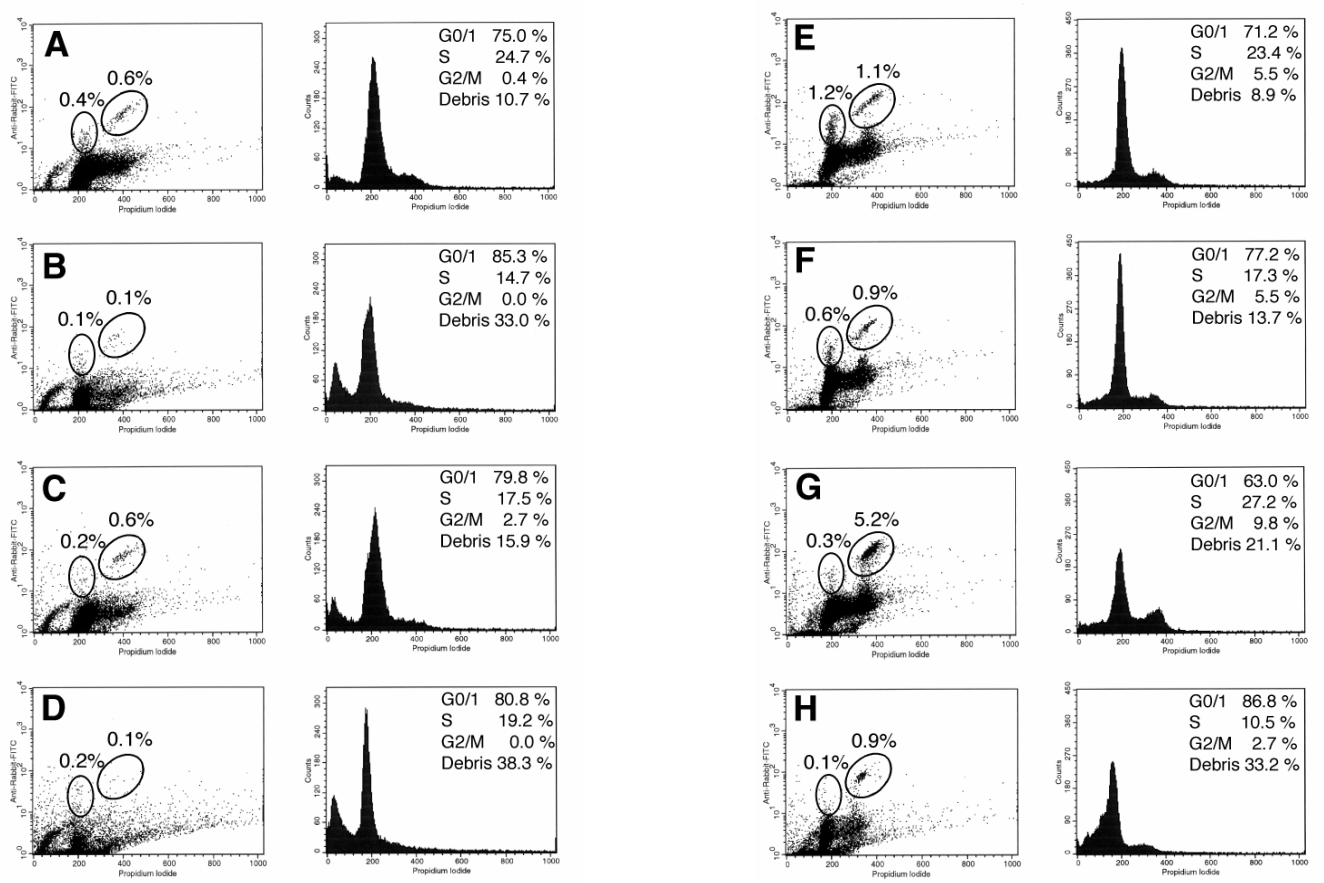

Figure 32. Effects of 48 h co-treatment with GGTI-2147 and FTI L-744,832 on cell cycle progression and the cell cycle-dependent activation of MEK-1/2 in primary AML cells. AML blasts from patient 5 were incubated in the absence or the presence of 20 μM GGTI-2147, FTI L-744,832 or a combination of 10 μM GGTI-2147 and 10 μM L-744,832. After 18 h, double staining with propidium iodide and an antibody specific for activated PP-MEK-1/2 was performed as described in Methods. Representative cell cycle profiles (right) and FACS profiles of the double staining (left) are shown. (A-D), AML cells were cultured in RPMI containing 20 % FCS; (E-H), AML cells were cultured in IMDM (Stemspan^{TM}) containing SCF, IL-3, Il-6 and Flt3-ligand. A and E, solvent-treated (DMSO) AML cells; B and F, 20 μM GGTI-2147; C and G, 20 μM FTI L-744,832; D and H, combination of 10 μM GGTI-2147 and 10 μM L-744,832.

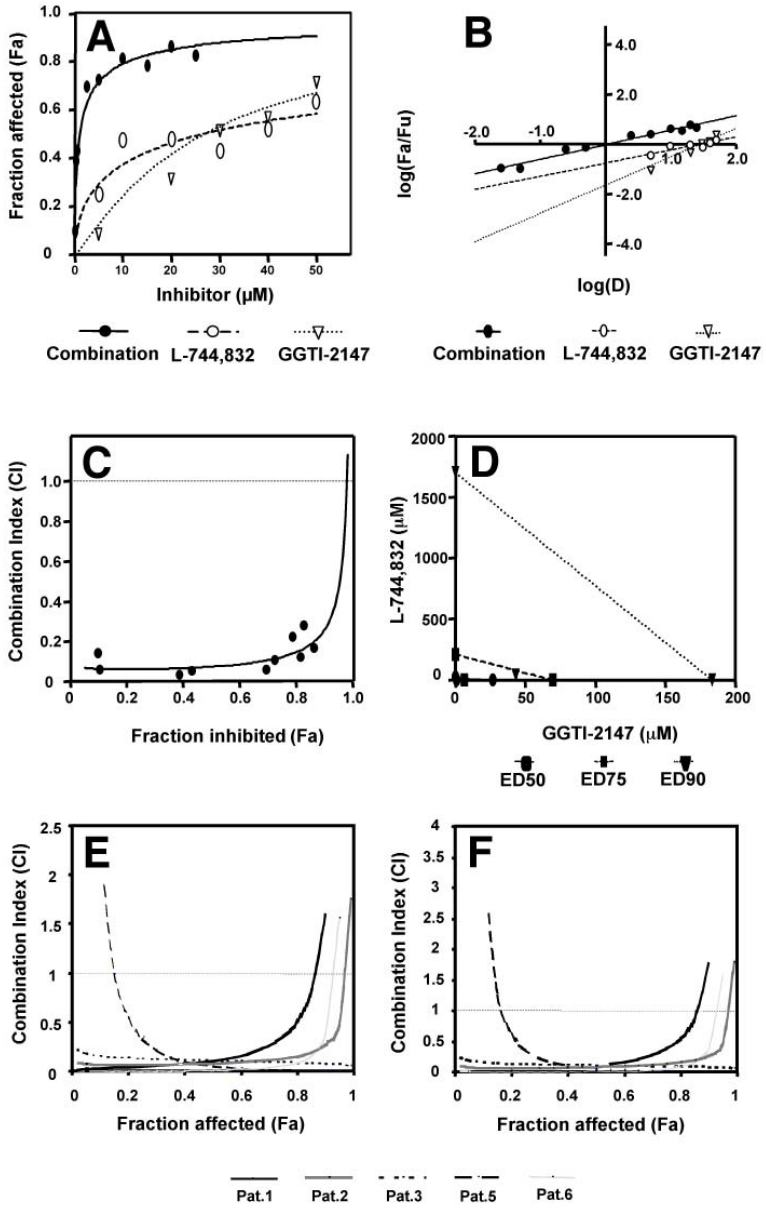

Figure 33. Treatment of primary AML cells with a combination of GGTI-2147 and FTI L-744,832 leads to synergistic growth inhibition. AML cells were incubated in liquid suspension cultures (StemSpan™) with increasing concentrations of GGTI-2147, FTI L-744,832 or with a fixed 1:1 ratio of GGTI-2147 and L-744,832. After 72 h, viability of the cells was determined by trypan blue dye exclusion and MTS proliferation assays. A, Viability (= fraction affected) of AML cells treated with GGTI-2147, FTI L-744,832 or a fixed 1:1 ratio of both inhibitors; B, median effect plot of data in A; C, plot of combination index (CI) versus cytotoxicity calculated from data in B under the assumption that agents are mutually exclusive; D, isobolograms for data in A; E, plot of CI calculated from data obtained for leukemic blasts from different AML patients (1, 2, 3, 5, 6) under the assumption that agents are mutually exclusive or non-exclusive (F). Note: CI values for patient 4 could not be calculated because FTI L-744,832 had no effect on blast viability. However, FTI/GGTI co-treatment was more effective than GGTI alone.

4. Discussion

4.1. ***Role of Ras in myeloid leukemias.*** There is accumulating evidence supporting a role of the deregulation of Ras function in the molecular pathogenesis of myeloid leukemias (Sawyers & Denny 1994; Byrne & Marshall 1998; Beaupre & Kurzrock 1999; Reuter *et al* 2000). The importance of the Ras-to-MAPK pathway is underscored by the positioning of several oncogene and tumor suppressor gene products on this pathway (e.g. activated receptor tyrosine kinases such as CSF-1, fusion tyrosine kinases such as BCR-Abl, and the tumor suppressor NF-1, a Ras-GAP). Furthermore, it has recently been demonstrated that mutant N-*Ras* induces myeloproliferative disorders resembling human chronic myelogenous leukemia (CML), acute myeloid leukemias and apoptotic syndromes similar to human myelodysplastic syndromes (MDS) in bone marrow repopulated mice (MacKencie *et al* 1999). Additionally, expression of a dominant negative form of Ras was shown to inhibit growth of Bcr-Abl-transformed K562 chronic myeloid leukemia cells by 90% (Sonoyama *et al* 2002).

Aberrant signaling through the Ras-to-MAP kinase cascade can lead to changes in several cellular processes, including proliferation, cell cycle progression, differentiation, apoptosis and migration. These are accomplished by activation of and signaling through different Ras effectors. Ras signaling through Raf proteins has been demonstrated to be involved in proliferation and differentiation functions in many cell systems (Marshall 1995). Activated PI-3K recruits the serine/threonine kinase Akt to the membrane where Akt is activated and subsequently inactivates several targets along pro-apoptotic pathways (Carpenter & Cantley 1996; Gire *et al* 2000). Additionally, changes in cytoskeletal organization by RalGDS, another Ras effector, affect adhesion and migration properties of cells (Ohta *et al* 1999). It was recently reported that activation of the RalGDS pathway increases tumor invasiveness and metastasis (Ward *et al* 2001).

Expression of matrix metalloproteinases (MMPs) has also been shown to be upregulated by Ras (Yanagihara *et al* 1995, Himelstein *et al* 1997; Hernandez-Alcoceba *et al* 2000). MMPs are a family of zinc-dependent endopeptidases which have been implicated in extracellular matrix turnover and bone remodeling events such as angiogenesis, bone resorption and tumor invasiveness (Woessner 1991; Kleiner & Stetler-Stevenson 1993; Matrisian 1992; Barille *et al* 1997). Activity of MMPs is controlled at the level of gene expression and by interaction with specific tissue inhibitors of MMPs, known as TIMPs. Both active and inactive MMPs interact with their specific TIMPs, so the balance of MMPs to TIMPs seems to be important for the control of MMP activation.

Recently, inhibition of geranylgeranylation has been reported to suppress secretion of MMP-9 and cell migration in the acute myeloid monocytic leukemia cell line THP-1 (Wong *et al* 2001). The Ras-to-(MAPK) pathway also upregulates transcription of the adhesion molecule CD44, which has been demonstrated to contribute to cell migration (Hoffman *et al* 1993). Additionally, other members of the Ras-like family of G-proteins mediate cellular adhesion and migration (eg. Rho, Rac and Rap).

4.2. ***Activation of Ras signaling in myeloid leukemias.*** It has previously been reported that approximately 50% (37 out of 73) of AML cases showed a constitutive activation of ERKs (Towatari *et al* 1997; Iida *et al* 1999; Kim *et al* 1999). Consistently, the data summarized in Table 2 demonstrates an activation of the ERK cascade in 9/14 AML and 2/5 CML cell lines. Furthermore, 4 of 14 AML cell lines (28.6%) and AML blasts from 4 of 6 patients harbored activating mutations of N-*Ras* and K-*Ras* (Figure 11). Interestingly, in MV4-11 two allelic K-*Ras* mutations were detected (codons 12 and 18). Previous studies have shown that mutations of "non-hot spot" codons 15, 16, 18 and 31 in K-*Ras* frequently occur in addition to the classical codon 12, 13 and 61 mutations (Lin *et al* 1998; Lin *et al* 2000). The identification of K-*Ras* and "non-hot spot" mutations demonstrates that the frequency of *Ras* mutations in myeloid leukemia may be underestimated in the published literature as most studies did not analyze all three *Ras* genes and "non-hot spot" codons such as 15, 16, 18, 31.

The observation that ERK-1/2 activation in myeloid leukemia cell lines does not always correlate with the presence of activating *Ras* mutations or BCR-Abl is also in agreement with recent reports (Towatari *et al* 1997; Iida *et al* 1999; Kim *et al* 1999). It has been suggested that the constitutive activation of ERK-1/2 in AML cells results from ERK-1/2 hyperexpression and downregulation of PAC1, which is a member of the MAPK phosphatase family (Kim *et al* 1999).

The immunocytochemical staining shown above (Figure 16) reveals that approximately 5% of all cells showed strong ERK-1/2 and MEK-1/2 activation. Two patterns of staining were observed: cytoplasmatic and nuclear staining. These observations are in agreement with reports that ERKs and MEKs are phosphorylated and activated after growth factor stimulation in the cytoplasm and then translocated into the nucleus (Fukuda *et al* 1997; Brunet *et al* 1999; Tolwinski *et al* 1999). Nuclear translocation of ERK-1/2 is a key signaling event for activation of nuclear processes such as transcription (Fukuda *et al* 1997; Brunet *et al* 1999). In contrast to stable nuclear presence of PP-ERK for several

hours, a nuclear export signal (NES) between residues 32 and 42 of MEK-1/2 leads to rapid removal of MEKs from the nucleus (Fukuda *et al* 1997; Tolwinski *et al* 1999).

4.3. ***Ras and the cell cycle.*** Cell cycle-dependent activation of the ERK cascade in myeloid leukemia cell lines is demonstrated in Figure 17. While most cells were strongly positive for MEK-1, two subpopulations of cells were strongly positive for PP-MEK-1/2. These two subpopulations correspond to the G_0/G_1 and the G_2/M phases of the cell cycle. Several studies report that ERK activation is required in the G_1 phase and the M phase of the cell cycle of fibroblasts (Dobrowolski *et al* 1994; Liu *et al* 1995; Winston *et al* 1996; Shapiro *et al* 1998; Chiri *et al* 1998; Zecevic *et al* 1998; Brunet *et al* 1999). ERK-1/2 expression is increased in mid-G_1 phase and ERK phosphorylation and activation occurs during G_1 phase (Liu *et al* 1995; Winston *et al* 1996). After phosphorylation and activation in the cytoplasm, PP-ERK translocates to the nucleus during late/mid-G1 phase (Liu *et al* 1995; Winston *et al* 1996). Nuclear ERK-regulated events required for progression into S phase include expression of immediate early genes such as Fos and Egr-1, and transcriptional upregulation of cyclin D1. Phosphorylation of Rb-1 by the cyclin D1/CDK4 complex inactivates the Rb-1 induced block, thus allowing cell cycle progression through G_1 to S phase. The observation of cyclin-D1 over-expression in myeloma implicates Ras influence in control of the malignant cell cycle (Ronchetti *et al* 1999; Arber *et al* 1996).

ERKs and MEKs are also activated early in prophase of mitosis before nuclear envelope breakdown (Sun *et al* 1995; Chiri *et al* 1998; Zecevic *et al* 1998; Shapiro *et al* 1999). In prophase, activated ERKs associate at kinetochores and within the chromosomal periphery of condensed chromosomes (Sun *et al* 1995; Shapiro *et al* 1999). Both activated ERKs and MEKs localize to spindle poles between prophase and anaphase, and to the midbody during cytokinesis (Sun *et al* 1995). The kinetochore motor protein CENP-E and topoisomerase IIa are potential mitotic substrates and suggest possible targets for the ERK pathway in the modulation of chromatin reorganization events during mitosis and in other phases of the cell cycle (Sun *et al* 1995; Shapiro *et al* 1999).

4.4. ***Effects of Ras-signaling inhibitors in myeloid leukemia.*** Although leukemogenesis is a multi-step process, the positioning of several oncogenes and tumor suppressor genes on the Ras-to-MAPK pathway strongly suggests that inhibition of oncogenic Ras function and signaling is a very promising pharmacological strategy. Therefore, a panel of 10

inhibitors of Ras-to-MAPK signaling was evaluated for growth inhibition of myeloid leukemia cell lines. This panel included inhibitors of the posttranslational modification of Ras and specific inhibitors of MEK (reviewed in Gibbs & Oliff 1997; Omer & Kohl 1997; Reuter *et al* 2000). Several FTase inhibitors were not very effective in the inhibition of leukemia cell growth (e.g. B581, FPT-2, Cys-4-Abs-Met).

Treatment with the CAAX-based FTI-277 or L-744,832 resulted in significant reduction of cell viability and colony formation in all myeloid leukemia cell lines tested (Table 3; Figures 19 and 20). Unfortunately, these inhibitors also displayed a strong toxicity towards purified human CD34+ cells, suggesting that myelotoxicity may be a side effect of treatment with some FTase inhibitors. While similar toxicities have not been reported in recent animal studies with these inhibitors (Sun *et al* 1998; Lantry *et al* 2000), mild myelosuppression, neutropenia and anemia have been observed in phase I clinical trials of another FTase inhibitor, R115777 (Miquel *et al* 1997; Zujewski *et al* 2000). Treatment of myeloid cell lines with FTI-277 or L-744,832 resulted in a G_2/M block and a subsequent increase in the number of PP-MEK-1/2 positive cells (Figure 22; Table 6). Similarly, FTI-277 treatment of lung adenocarcinoma A-549 cells has been reported to cause enrichment in the G_2/M phase of the cell cycle (Emanuel *et al* 2000). In primary AML cells, FTI treatment induced G_0/G_1 or G_2/M blocks. These observations are in agreement with previous results which demonstrated G_0/G_1 or G_2/M blocks after treatment with CAAX-based FTIs depending on cell type (Miquel *et al* 1997; Vogt *et al* 1997; Ashar *et al* 2000; Ashar *et al* 2001; Morgan *et al* 2001; Tamanoi *et al* 2001). Induction of $p21^{WAF1/CIP1}$ is one pathway through which FTIs cause a G_1 arrest. The FTI-induced G_2/M arrest is a consequence of an alteration of the microtubule-centromere interaction during mitosis by blocking bipolar spindle formation and chromosome alignment (Crespo *et al* 2001). It has been suggested that FTI-induced accumulation of cells in prometaphase is due to inhibition of farnesylation of the centromeric protein CENP-E (Ashar *et al* 2000). However, as CENP-E is not essential for spindle pole separation, others have suggested that dynein- or Eg5-interacting proteins may be more relevant biologic FTI targets, and are responsible for the prometaphase arrest (Crespo *et al* 2001).

Additionally, L-739,749 and L-744,832, both CAAX-based FTase inhibitors, have recently been shown to inhibit spontaneous JMML granulocyte-macrophage colony growth at a dose range of 1-10 μM (Hung & Chuang 1998; Mahgoub *et al* 1999). L-744,832 inhibited H-Ras prenylation and colony growth of NF-1 deficient hematopoietic cells in response to GM-CSF, but did not reduce constitutively activated MAPK activity in these

cells. Furthermore, a myeloproliferative disorder in NF-1 deficient (NF-/-) mice did not respond to L-744,832 treatment (Mahgoub *et al* 1999). Its was speculated that the lack of efficacy in this model was due to the resistance of N-Ras and K-Ras processing to inhibition by this FTase inhibitor (Mahgoub *et al* 1999).

The FPP-based farnesyl transferase inhibitor FPT-3 caused significant inhibition of the colony formation of 10/19 myeloid cell lines at concentrations which did not significantly affect colony growth of purified human CD34+ cells (Table 3; Figure 19). Incubation of NB-4 cells with FPT-3 resulted in rapid induction of apoptotic DNA fragmentation and exposure of phosphatidylserine (Figure 23). Recently, FPT-3 has also been found to induce apoptosis in ovarian cancer cells by upregulation of Bax and Bcl-xs expression and activation of caspase family proteases (Hung & Chuang 1998a,b). FTIs have been demonstrated to induce apoptosis in a wide variety of cancer cell lines by release of cytochrome c from mitochondria into the cytosol (Tamanoi *et al* 2001). Cytochrome c forms a complex with Apaf-1 and pro-caspase-9 that results in activation of caspase-9 and caspase-3. Caspase-3 is a key regulator that triggers a variety of apoptotic changes, including nuclear condensation and chromosomal DNA fragmentation (Tamanoi *et al* 2001). Additionally, inhibition of phosphoinositide 3-OH kinase/AKT2-mediated cell survival and adhesion pathway was recently shown as a critical target for FTI-induced apoptosis (Jiang *et al* 2000). Interestingly, it has recently been demonstrated that the common alpha subunit of FTase and GGTase I is cleaved by caspase 3 during apoptosis, suggesting that inactivation of prenyltransferases by caspases contributes to progression of apoptosis (Kim *et al* 2001).

Significant growth inhibition (>70%) was also observed upon treatment of myeloid leukemia cells with GGTI-286 (9/19), GGTI-298 (14/19) and GGTI-2147 (16/19), whereas GGTI-287, GGTI-297 and GGTI-2133 were less effective (Table 4). Treatment of NB-4 cells with GGTIs resulted in an increase of cells in G_0/G_1, induction of apoptosis and a decrease in cell-cycle-dependent MEK activation (Table 6). The GGTI-induced G_1-block has recently been shown to be due to upregulation of transcription of the CDK inhibitor $p21^{WAF1/CIP1}$ (Vogt *et al* 1997; Adnane *et al* 1998). One mechanism by which GGTIs upregulate $p21^{WAF1/CIP1}$ is via inhibition of RhoA geranylgeranylation. Rho proteins facilitate progression from G_1 to S phase in growth-stimulated cells by promoting degradation of the CDK inhibitor $p27^{Kip1}$ and by downregulating the $p21^{WAF1/CIP1}$ promoter (Hirai *et al* 1997; Adnane *et al* 1998).

As shown in Table 5, treatment with the MEK inhibitors PD098059 and U0126 significantly inhibited viability, growth and colony formation of most cell lines tested (10/19 and 19/19, respectively). Surprisingly, the MEK inhibitors, which display broad inhibitory effects, caused only minor toxicity in purified human CD34+ cells (Figure 19). The stronger effect of U0126 is most likely due to a significantly higher affinity of U0126 to all forms of MEK (44- to 357-fold) as compared to PD098059 (Reuter *et al* 2000). In contrast to PD098059, U0126 has recently been reported to reverse Ki-Ras-mediated transformation by blocking MAPK and p70 S6 kinase pathways (Fukazawa & Uehara 2000).

In agreement with previous observations (Miquel *et al* 1997), a correlation between susceptibility towards these inhibitors and the Ras status (e.g. mutation or activation) was not always observed in the study presented here. For example, NB4 cells displayed a strong activation of the Ras-to-MAPK cascade and were very sensitive to most inhibitors, whereas Mono-Mac-1 cells were resistant to most inhibitors in spite of a strong MAPK activation.

The different cellular responses invoked by the FPP-based inhibitor FPT-3 and the CAAX-box based inhibitors FTI-277 and FTI L-744,832 are particularly interesting as these compounds all target Ras farnesylation. This suggests the possibility that specific types of FTIs may have different mechanisms of action.

It has been speculated that alternative geranylgeranylation of K-Ras and N-Ras in the presence of FTIs might represent a possible mechanism of FTI-resistance (Sun *et al* 1998; Mahgoub *et al* 1999). As the majority of *Ras* mutations in acute myeloid leukemias occur in K- and N-*Ras*, this mechanism of FTI-resistance may have therapeutic consequences. This hypothesis was tested by investigating the effects of GGTIs alone and in combination with FTI on myeloid leukemia cell growth, cell cycle progression, induction of apoptosis, Ras processing and signaling.

Co-treatment of myeloid cells with FTI and GGTI resulted in synergistic cytotoxic effects which correlated with an increased accumulation of unprocessed N-Ras (Figures 24, 27 and 28). The observation that FTI-induced inhibition of H-Ras processing is not augmented by co-addition of GGTIs underscores the fact that H-Ras, in contrast to K- and N-Ras, is solely farnesylated (Lerner *et al* 1997; Rowell *et al* 1997; Whyte *et al* 1997; Zhang *et al* 1997). The resistance of K-Ras processing to inhibition by FTIs may be due to the increased binding affinity of K-Ras to farnesyl transferase (James *et al* 1996; Zhang *et al* 1997). Inhibition of oncogenic K-Ras4B processing required concentrations of FTI-277

100-fold higher than those needed for H-Ras inhibition (Lerner *et al* 1995b). Ras-Raf binding assays demonstrated that unprocessed N-Ras binds to the minimal Ras-binding domain of Raf (GST-RBD) (Figure 30). The accumulation and Raf-binding of unprocessed N-Ras in cells treated with FTIs alone or in combination with GGTIs suggests dominant negative effects of this biologically inactive version of N-Ras. Accumulation of inactive Ras-Raf complexes in the cytosolic fraction and inhibition of Ras-induced constitutive activation of MAPK has been observed in FTI-277-treated cells overexpressing oncogenic H-Ras (Lerner *et al* 1995a).

The results demonstrating synergistic toxicity of FTI/GGTI co-treatment in myeloid leukemia cells are in agreement with recent reports describing similar synergistic efficiency of a FTI/GGTI combination in adrenocortical and human colon cancer cells containing mutant K-Ras (Mazet *et al* 1999; Di Paolo *et al* 2001). In a nude mouse xenograft model, both FTI and GGTI are required to inhibit prenylation of oncogenic K-Ras, but each alone is sufficient to suppress human tumor growth (Sun *et al* 1998; Sun *et al* 1999). Furthermore, FTI/GGTI cotreatment and treatment with a dual prenylation inhibitor (DPI) with both FTI and GGTI activity, resulted in higher levels of apoptosis in K-Ras transformed cells relative to FTI and GGTI alone (Lobell *et al* 2001). While the GGTIs described by Sun et al. and used in the study presented here were non-toxic in mice, the chemically distinct GGTIs and DPIs used by Lobell et al. revealed strong toxicity in mice which may be caused by an activity unrelated to GGTase-I inhibitory activity (Lobell *et al* 2001).

As there are over twenty known farnesyltransferase substrates (Table 8), and as it has been suggested that Ras may not be the therapeutically relevant loci of FTI treatment (Adjei *et al* 2000a; Prendergast 2001; Tamanoi *et al* 2001), the effects of FTIs and GGTIs on processing of other farnesyl transferase substrates were investigated. The results presented in Figure 29 demonstrate that FTI treatment inhibited processing of the G-proteins Rap2A and Rheb and of the intranuclear intermediate filament Lamin A in some myeloid leukemia cells. Rap2A and Rheb are two other members of Ras family proteins that are farnesylated (Tamanoi *et al* 2001). While the biologic function of these G-proteins is currently under investigation, it has recently been shown that failure to farnesylate Rheb proteins contributes to enrichment of G_0/G_1 phase cells in *S. pombe* (Yang *et al* 2001).

Although inhibition of H-Ras and N-Ras prenylation was always observed after FTI or FTI/GGTI treatment, FTI/GGTI-induced inhibition of the prenylation of K-Ras2B,

Table 8. Substrates of Farnesyltransferase (modified from Tamanoi *et al* 2001).

Protein	CAAX Motif	Function	Reference
H-Ras	CLVS	Proliferation, Differentiation, Apoptosis inhibition	Farnsworth *et al* 1994
K-Ras	CVVM	Proliferation, Differentiation, Apoptosis inhibition	
N-Ras	CVIM	Proliferation, Differentiation, Apoptosis inhibition	
Rheb	CSVM	Cell cycle (G1/S)?	Tamanoi *et al* 2001
Rap2A	CNIQ	G-Protein with unknown function	
TC10	CLIT	Transformation, Cytoskeleton	
RhoB	CKVL	Endocytosis, Apoptosis, Transcription	Lebowitz *et al* 1998
RhoD	CVVT	Cytoskeleton, Endocytosis	Tamanoi *et al* 2001
Rho6/Rnd1	CSIM	Adhesion, Cytoskeleton	
Rho7/Rnd2	CNLM	Cytoskeleton	
RhoE/Rho8	CTVM	Cytoskeleton, Adhesion	
Lamin A	CSIM	Nuclear membrane structural protein	Farnsworth *et al* 1989
Lamin B	CAIM		
CENP-E	CKTQ	Cell cycle (G2/M), Centromere binding	Ashar *et al* 2000
CENP-F	CKVQ		
HDJ-2	CQTS	Chaperone protein	Adjei *et al* 2000
PxF	CLIM	Peroxisomal protein with unknown function	James *et al* 1992
Transducin α	CGLF	Visual protein	Lai *et al* 1990
γ	CVIS		
Rhodopsin Kinase	CVLS	Visual protein	Inglese *et al* 1992
cGMP-PDE α	CCIQ	Visual signal transduction	Anant *et al* 1992
cGMP-PDE β	CCIL		
Phosphorylase Kinase α	CAMQ	Thrombocyte function	Heilmeyer *et al* 1992
β	CLVS		
InsP3 5-Phos-phatase I	CVVQ	Inositol signal transduction	Tamanoi *et al* 2001
IV	CSVS		
PTPCAAX1/ hPRL1	CCIQ	Tyrosine phosphatase	Tamanoi *et al* 2001
PTPCAAX2/ hPRL2	CCVQ		
hPRL3	CCVM		

Abbreviations : InsP3 phosphatase, inositol-polyphosphate phosphatase; PDE, phosphodiesterase; PRL-1, -2, -3,; cGMP, cyclic guanosine monophosphate; CAAX, C = cysteine, A = aliphatic amino acid, X = serine, glutamine, or methionine.

Rap2A, Rheb and Lamin A seemed to be less frequent and cell type-dependent. Co-treatment with FTI and GGTI did not result in an increased accumulation of unprocessed Rap2A and Rheb, suggesting that these G proteins do not undergo alternative prenylation.

Work of several laboratories previously demonstrated that Lamin B and Prelamin/Lamin A are farnesylated proteins (Farnsworth *et al* 1989; Beck *et al* 1990) and that FTI treatment causes inhibition of Lamin A and B farnesylation (Sepp-Lorenzino *et al* 1995; Adjei *et al* 2000; Karp *et al* 2001; Kelland *et al* 2001). While the immunoblotting studies described above failed to demonstrate a mobility shift of Lamin B, elevated protein levels of Lamin B were observed in cells treated with FTI alone and in combination with GGTI. Furthermore, co-treatment with FTI and GGTI had no synergistic effect on Lamin A, B and C prenylation (Figure 29).

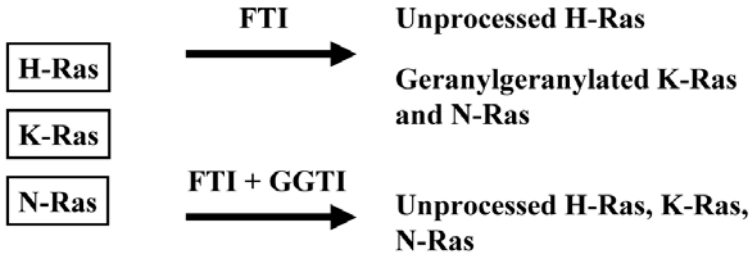

Figure 34. Addition of GGTI is one strategy to overcome alternative prenylation of K- and N-Ras in FTI-treated cells.

RhoB, an endosomal Rho protein that functions in receptor trafficking and which has been shown to be both farnesylated and geranylgeranylated, has been proposed as a key target of FTIs (Prendergast 2001). These studies have examined epitope-tagged RhoB that is expressed after transfection. It has been suggested that FTI treatment shifts the prenylation status of RhoB to an exclusively geranylgeranylated form which causes dominant effects to induce apoptotic and antineoplastic responses (Du *et al* 1999; Du & Prendergast 1999; Liu *et al* 2000; Prendergast 2001). However, induction of apoptosis by overexpression of either farnesylated or geranylgeranylated versions of RhoB in Panc-1 cells suggests that both RhoB-F and RhoB-GG function similarly and argues against the idea that RhoB-GG has a role different from that of RhoB-F (Chen *et al* 2000).

It has been suggested that Ras may not be the therapeutically relevant loci (Prendergast 2000). Determining whether leukemic growth inhibition occurs because of an inhibition of Ras-specific cellular events such as MAPK induced cell cycle progression or because of alteration of other cellular targets such as RhoB, an endosomal Rho protein that

functions in receptor trafficking, is currently under investigation. The findings reported here support a potential role of inhibitors of Ras signaling in the future treatment of myeloid leukemias.

Phase I and II clinical trials investigating FTIs have reported that FTIs are tolerated well in humans, with myelosuppression and neurotoxicity as the main dose limiting factors (Adjei *et al* 2000; Reuter *et al* 2000; Zujewski *et al* 2000; Britten *et al* 2001; Crul *et al* 2001; Eskens *et al* 2001; Karp *et al* 2001; Karp 2001; Punt *et al* 2001). The relatively disappointing results of FTI monotherapy may be a result of resistance of K- and N-Ras to FTI therapy. However, a recent study demonstrates synergistic induction of apoptosis upon co-treatment of a Ras signaling inhibitor (e.g. MEK inhibitor PD184352) and antisense oligonucleotides directed against Bcl-2 (Milella *et al* 2002). As it has recently been reported that lovastatin inhibits Ras prenylation (Wang *et al* 2000), the statins are another potentially interesting class of compounds to combine with FTI in the hopes of better combating leukemias.

In conclusion, the results presented here demonstrate that disruption of Ras signaling may be an effective strategy to treat myeloid leukemias and suggest that synergistic cytotoxic effects of FTI/GGTI combination is – at least in part – due to increased inhibition of N-Ras prenylation. Regardless of the locus of action, the results presented here demonstrate profound *in vitro* inhibitory effects of FTIs and GGTIs on the growth of myeloid leukemia cells irrespective of the presence of *Ras* mutations, expression of BCR-Abl or MAPK activation. The accumulation of unprocessed N-Ras after FTI/GGTI co-treatment of myeloid leukemia cells, suggests that combining FTIs and GGTIs in the treatment of myeloid leukemias may lead to higher efficacy by overcoming the partial resistance of K- and N-Ras to FTI monotherapy.

5. References

Abu-Duhier FM, Goodeve AC, Wilson GA, Care RS, Peake IR, Reilly JT. Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. *Br J Haematol.* 2001;**113**:983-938.

Abe A, Emi N, Tanimoto M, Terasaki H, Marunouchi T, Saito H. Fusion of the platelet-derived growth factor beta to a novel gene CEV14 in acute myelogenous leukemia after clonal evolution. *Blood.* 1997;**90**:4271-4277.

Adachi M, Fukuda M, Nishida E. Two co-existing mechanisms for nuclear import of MAP kinase: passive diffusion of a monomer and active transport of a dimer. *EMBO J.* 1999;**19**:5347-5358.

Adachi T & Alam R. The mechanism of IL-5 signal transduction. *Am J Physiol.* 1998;**275**:C623-633.

Adamson P, Marshall CJ, Hall A, Tilbrook PA. Post-translational modifications of p21rho proteins. *J Biol Chem.* 1992;**267**:20033-20038.

Adjei AA, Davis JN, Erlichman C, Svingen PA, Kaufmann SH. Comparison of potential markers of farnesyltransferase inhibition. *Clin Cancer Res.* 2000;**6**:2318-2325.

Adjei AA, Erlichman C, Davis JN, Cutler DL, Sloan JA, Marks RS, Hanson LJ, Svingen PA, Atherton P, Bishop WR, Kirschmeier P, Kaufmann SH. A phase I trial of the farnesyl transferase inhibitor SCH66336: evidence for biological and clinical activity. *Cancer Res.* 2000;**60**:1871-1877.

Adnane J, Bizouarn FA, Qian Y, Hamilton AD, Sebti SM. p21(WAF1/CIP1) is upregulated by the geranylgeranyltransferase I inhibitor GGTI-298 through a transforming growth factor beta- and Sp1-responsive element: involvement of the small GTPase rhoA. *Mol Cell Biol.* 1998;**18**:6962-6970.

Aharonson Z, Gana-Weisz M, Varsano T, Haklai R, Marciano D, Kloog Y. Stringent structural requirements for anti-Ras activity of S-prenyl analogues. *Biochim Biophys Acta.* 1998;**1406**:40-50.

Akopyan TN, Couedel Y, Orlowski M, Fournie-Zaluski MC, Roques BP. Proteolytic processing of farnesylated peptides: assay and partial purification from pig brain membranes of an endopeptidase which has the characteristics of E.C. 3.4.24.15. *Biochem Biophys Res Comm.* 1994;**198**:787-794.

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AD. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J Biol Chem.* 1995;**270**:27489-27494.

Anant JS, Ong OC, Xie HY, Clarke S, O'Brien PJ, Fung BK. In vivo differential prenylation of retinal cyclic GMP phosphodiesterase catalytic subunits. *J Biol Chem.* 1992;**267**:687-690.

Arber N, Sutter T, Miyake M, Kahn SM, Venkatraj VS, Sobrino A, Warburton D, Holt PR, Weinstein IB. Increased expression of cyclin D1 and the RB tumor suppressor gene in c-K-*ras* transformed rat enterocytes. *Oncogene*. 1996;**12**:1903-1908.

Ashar HR, James L, Gray K, Carr D, Black S, Armstrong L, Bishop WR, Kirschmeier P. Farnesyl transferase inhibitors block the farnesylation of CENP-E and CENP-F and alter the association of CENP-E with the microtubules. *J Biol Chem.* 2000;**275**:30451-30457.

Ashar HR, James L, Gray K, Carr D, McGuirk M, Maxwell E, Black S, Armstrong L, Doll RJ, Taveras AG, Bishop WR, Kirschmeier P. The farnesyl transferase inhibitor SCH 66336 induces a G(2) --> M or G(1) pause in sensitive human tumor cell lines. *Exp Cell Res.* 2001;**262**:17-27.

Barbacid M. ras genes. *Annu Rev Biochem.* 1987;**56**:779-827.

Bardwell L & Thorner J. A conserved motif at the amino termini of MEKs might mediate high affinity interaction with the cognate MAPKs. *Trends Biol Sci.* 1996;**21**:373-374.

Barille S, Akhoundi C, Collette M, Mellerin M-P, Rapp M-J, Harousseau J-L, Bataille R and Amiot M. Metalloproteinases in multiple myeloma: production of matrix metalloproteinase-9 (MMP-9), activation of proMMP-2, and induction of MMP-1 by myeloma cells. *Blood*. 1997; **90**:1649-1655.

Barrington RE, Subler MA, Rands E, Omer CA, Miller PJ, Hundley JE, Koester SK, Troyer DA, Bearss DJ, Conner MW, Gibbs JB, Hamilton K, Koblan KS, Mosser SD, O'Neill TJ, Schaber MD, Senderak ET, Windle JJ, Oliff A, Kohl NE. A farnesyltransferase inhibitor induces tumor regression in transgenic mice harboring multiple oncogenic mutations by mediating alterations in both cell cycle control and apoptosis. *Mol Cell Biol.* 1998;**18**:85-92.

Beaupre DM & Kurzrock R. RAS and leukemia: from basic mechanisms to gene – directed therapy. *J Clin Oncol.* 1999;**17**:1071-1079.

Beck LA, Hosick TJ, Sinensky M. Isoprenylation is required for the processing of the lamin A precursor. *J Cell Biol.* 1990;**110**:1489-1499.

Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Young SG. Targeted inactivation of the isoprenylcysteine carboxyl methyltransferase gene causes mislocalization of K-Ras in mammalian cells. *J Biol Chem.* 2000;**275**:17605-17610.

Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Gomes AQ, Seabra MC, Young SG. Isoprenylcysteine carboxyl methyltransferase deficiency in mice. *J Biol Chem.* 2001;**276**:5841-5848.

Bergo MO, Ambroziak P, Gregory, George. Otto JC, Kim, Nagase, Casey PJ, Balmain, Young SG. Absence of the CAAX endoprotease Rce1: effects on cell growth and transformation. *Mol Cell Biol.* 2002;**22**:171-181.

Bernhard EJ, McKenna WG, Hamilton AD, Sebti SM, Qian Y, Wu JM, Muschel RJ. Inhibiting Ras prenylation increases the radiosensivity of human tumor cell lines with activating mutations of ras oncogenes. *Cancer Res.* 1998;**58**:1754-1761.

Bezieau S, Devilder MC, Avet-Loiseau H, Mellerin MP, Puthier D, Pennarun E, Rapp MJ, Harousseau JL, Moisan JP, Bataille R. High incidence on N and K-Ras activating mutations in multiple myeloma and primary plasma cell leukemia at diagnosis. *Hum Mutat.* 2001;**18**:212-224.

Birnbaum RA, O´Marcaigh A, Wardak Z. NF1 and GM-CSF interact in myeloid leukemogenesis. *Mol Cell.* 2000;**5**:189-195.

Bishop WR, Bond R, Petrin J, Wang L, Patton R, Doll R, Njoroge G, Catino J, Schwartz J, Windsor W, Syto R, Schwartz J, Carr D, James L, Kirschmeier P. Novel tricyclic inhibitors of farnesyl protein transferase. *J Biol Chem.* 1995;**270**:30611-30618.

Bollag G, Clapp DW, Shih S, Adler F, Zhang YY, Thompson P, Lange BJ, Freedman MH, McCormick F, Jacks T, Shannon K. Loss of NF-1 results in activation of the Ras signaling pathway and leads to aberrant growth in hematopoietic cells. *Nature Genet.* 1996;**12**:144-148.

Bonfini L, Migliaccio E, Pelicci G, Lanfrancone L, Pelicci PG. Not all Shc`s roads lead to Ras. *Trends Biol Sci.* 1996;**21**:257-261.

Booden MA, Baker TL, Solski PA, Der CJ, Punke SG, Buss JE. A non-farnesylated Ha-Ras protein can be palmitoylated and trigger potent differentiation and transformation. *J Biol Chem.* 1999;**274**:1423-1431.

Boulton TG & Cobb MH. Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regul.* 1991;**2**:357-371.

Bos JL, Verlaan-de-Vries M, van-der-Eb AJ, Janssen JW, Delwel R, Lowenberg B, Colly LP. Mutations in N-Ras predominate in acute myeloid leukemia. *Blood.* 1987;**69**:1237-1241.

Bos JL. All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *EMBO J.* 1998;**17**:6776-6782.

Bos JL. *RAS* oncogenes in human cancer: a review. *Cancer Res.* 1989;**49**:4682-4689.

Boyartchuk VL, Ashby MN, Rine J. Modulation of Ras and a-factor function by carboxyl-terminal proteolysis. *Science.* 1997;**275**:1796-1800.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;**72**:248-254.

Bredel M, Pollack IF, Freund JM, Hamilton AD, Sebti SM. Inhibition of Ras and related G-Proteins as a therapeutic strategy for blocking malignant glioma growth. *Neurosurgery.* 1998;**43**:124-131.

Britten CD, Rowinsky EK, Soignet S, Patnaik A, Yao SL, Deutsch P, Lee Y, Lobell RB, Mazina KE, McCreery H, Pezzuli S, Spriggs D. A phase I and pharmacological study of the farnesyl protein transferase inhibitor L-778,123 in patients with solid malignancies. *Clin Cancer Res.* 2001;**7**:3894-3903.

Browett PJ & Norton JD. Analysis of *RAS* gene mutations and methylation state in human leukemias. *Oncogene.* 1989;**4**:1029-1036.

Browett PJ, Yaxley JC, Norton JD. Activation of Harvey ras oncogene by mutation at codon 12 is very rare in hematopoietic malignancies. *Leukemia.* 1989;**3**:86-88.

Brown MS, Goldstein JL, Paris KJ, Burnier JP, Marsters JCJr. Tetrapeptide inhibitors of protein farnesyltransferase: amino-terminal substitution in phenylalanine-containing tetrapeptides restores farnesylation. *Proc Natl Acad Sci USA.* 1992;**89**:8313-8316.

Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouysségur J. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO J.* 1999;**18**:664-674.

Buday L. Membrane-targeting of signalling molecules by SH2/SH3 domain-containing adaptor proteins. *Biochimica et Biophysica Acta.* 1999;**1422**:187-204.

Buttner C, Henz BM, Welker P, Sepp NT, Grabbe J. Identification of activating c-kit mutations in adult-, but not in childhood-onset indolent mastocytosis: a possible explanation for divergent clinical behavior. *J Invest Dermatol.* 1998;**111**:1227-1231.

Byrne JL & Marshall CJ. The molecular pathophysiology of myeloid leukaemias: Ras revisited. *Brit J Haematol.* 1998;**100**:256-264.

Camp LA, Verkruyse LA, Afendis SJ, Slaughter CA, Hofmann SL. Molecular cloning and expressing of palmitoyl-protein thioesterase. *J Biol Chem.* 1994;**269**:23212-23219.

Carpenter CL & Cantley LC. Phosphoinositide kinases. Curr Opin Cell Biol. 1996;8:153-158.

Casey S & Dautry F. Inactivation of the murine N-ras gene by gene targeting. *Oncogene.* 1992;**12**:2525-2528.

Casey PJ & Seabra MC. Protein prenyltransferases. *J Biol Chem.* 1996;**271**:5289-5292.

Catling AD, Schaeffer H-J, Reuter CWM, Reddy GR, Weber MJ. A proline-rich sequence unique to MEK1 and MEK2 is required for Raf binding and regulates MEK function. *Mol Cell Biol.* 1995;**15**:5214-5225.

Cazzaniga G, Tosi S, Aloisi A, Giudici G, Daniotti M, Pioltelli P, Kearney L, Biondi A. The tyrosine kinase abl-related gene ARG is fused to ETV6 in an AML-M4Eo patient with a t(1;12)(q25;p13): molecular cloning of both reciprocal transcripts. *Blood.* 1999;**94**:4370-4373.

Chen TH, Sarnecki C, Blenis J. Nuclear localization and regulation of ERK- and RSK-encoded protein kinases. *Mol Cell Biol.* 1992;**12**:915-927.

Chen Y. Selective inhibition of ras-transformed cell growth by a novel fatty acid-based chloromethyl ketone designed to target Ras endoprotease. *Ann NY Acad Sci.* 1999;**886**:103-108.

Chen Z, Sun J, Pradines A, Favre G, Adnane J, Sebti SM. Both farnesylated and geranylgeranylated RhoB inhibit malignant transformation and suppress human tumor growth in nude mice. *J Biol Chem.* 2000;**275**:17974-17978.

Chiri S, Nadai CD, Ciapa B. Evidence for MAP kinase activation during mitotic division. *J Cell Sci.* 1998;**111**:2519-2527.

Chou TC & Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul.* 1984;**22**:27-55.

Clark GJ & Der CJ. Ras proto-oncogene activation in human malignancy, in: *Cellular cancer markers.* Eds Garrett CT & Sell S, Humana Press Totowa, New Jersey. 1995;17-52.

Corradini P, Ladetto M, Voena C, Palumbo A, Inghirami G, Knowles DM, Boccadoro M, Pileri A. Mutational activation of N- and K-*RAS* oncogenes in plasma cell dyscrasias. *Blood.* 1993;**81**:2708-2713.

Cox AD, Brtva TR, Lowe DG, Der CJ. R-Ras induces malignant, but not morphologic, transformation of NIH3T3 cells. *Oncogene.* 1994;**9**:3281-3288.

Cox AD & Der CJ. Farnesyltransferase inhibitors and cancer treatment: targeting simply Ras? *Biochim Biophys Acta.* 1997;**1333**:F51-71.

Cox AD, Garcia AM, Westwick JK, Kowalczyk JJ, Lewis MD, Brenner DA, Der CJ. The CAAX peptidomimetic compound B581 specifically blocks farnesylated, but not geranylgeranylated or myristylated, oncogenic ras signaling and transformation. *J Biol Chem.* 1994;**269**:19203-19206.

Cox LY, Motz J, Troll W, Garte SJ. Antipain-induced suppression of oncogene expression in H-ras-transformed NIH3T3 cells. *Cancer Res.* 1991;**51**:4810-4814.

Crespo NC, Ohkanda J, Yen TJ, Hamilton AD, Sebti SM. The farnesyltransferase inhibitor, FTI-2153, blocks bipolar spindle formation and chromosome alignment and causes prometaphase accumulation during mitosis of human lung cancer cells. *J Biol Chem.* 2001;**276**:16161-16167.

Crews CM, Alessandrini A, Erikson RL. The primary structure of MEK, a protein that phosphorylates the ERK gene product. *Science.* 1992;**258**:478-480.

Crul M, de Klerk GJ, Beijnen JH, Schellens JH. Ras biochemistry and farnesyl transferase inhibitors: a literature survey. *Anticancer Drugs.* 2001;**12**:163-184.

Dalton MB, Fantle KS, Bechtold HA, DeMaio L, Evans RM, Krystosek A, Sinensky M. The farnesyl protein transferase inhibitor BZA-5B blocks farnesylation of nuclear lamins and p21ras but does not affect their function or localization. *Cancer Res.* 1995;**55**:3295-3304.

D'Andrea RJ & Gonda TJ. A model for assembly and activation of the GM-CSF, IL-3 and IL-5 receptors: insights from activated mutants of the common beta subunit. *Exp Hematol.* 2000;**28**:231-243.

Daum G, Eisenmann-Tappe I, Fries HW, Troppmair J, Rapp U. The ins and outs of Raf kinases. *Trends Biol Sci.* 1994;**19**:474-480.

De Rooij J & Bos JL. Minimal Ras-binding domain of Raf1 can be used as an activation specific probe for Ras. *Oncogene.* 1997;**14**:623-625.

DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC, Lowy DR. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. *Cell.* 1992;**69**:265-273.

Del Villar K, Urano J, Guo L, Tamanoi F. A mutant form of human protein farnesyltransferase exhibits increased resistance to farnesyltransferase inhibitors. *J Biol Chem.* 1999;**274**:27010-27017.

Demiroglu A, Steer E, Heath C, Taylor K, Bentley M, Allen S, Koduru P, Brody J, Hawson G, Rodwell R, Doody M-L, Carnicero F, Reiter A, Goldman J, Melo J, Cross N. The t(8;22) in chronic myeloid leukemia fuses BCR to FGFR1: transforming activity and specific inhibition of FGFR1 fusion proteins. *Blood.* 2001;**98**:3778-3783.

DeSilva DR, Jones EA, Favata MF, Jaffee BD, Magolda RL, Trzaskos JM, Scherle PA. Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent anergy. *J Immunol.* 1998;**160**: 4175-4181.

DeSolms SJ, Giuliani EA, Graham SL, Koblan KS, Kohl NE, Mosser SD, Oliff AI, Pompliano DL, Rands E, Scholz TH, Wiscount CM, Gibbs JB, Smith RL. N-Arylalkyl pseudopeptide inhibitors of farnesyltransferase. *J Med Chem.* 1998;**41**:2651-2656.

Devedjiev Y, Dauter Z, Kuznetsov SR, Jones TL, Derewenda ZS. Crystal structure of the human acyl protein thioesterase I from a single X-ray data set to 1.5 A. *Structure Fold Des.* 2000;**8**:1137-1146.

Di Paolo, A., Danesi, R., Caputo, S., Macchia, M., Lastella, M., Boggi, U., Mosca, F., Marchetti, A., and Del Tacca, M. Inhibition of protein farnesylation enhances the chemotherapeutic efficacy of the novel geranylgeranyltransferase inhibitor BAL9611 in human colon cancer cells. *Br J Cancer.* 2001;**84**: 1535-1543.

Dobrowolski S, Harter M, Stacey DW. Cellular Ras activity is required for passage through multiple points of the G0-G1 phase in BALB-c3T3 cells. *Mol Cell Biol.* 1994;**14**:5441-5449.

Dosil M, Wang S, Lemischka IR. Mitogenic signaling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. *Mol Cell Biol.* 1993;**13**:6572-6585.

Drexler HG, Matsuo AY, MacLeod AF. Continuous hematopoietic cell lines as model systems for leukemia-lymphoma research. *Leuk Res.* 2000;**24**:881-911.

Du W, Lebowitz PF, Prendergast GC. Cell growth inhibition by farnesyltransferase inhibitors is mediated by gain of geranylgeranylated RhoB. *Mol Cell Biol.* 1999;**19**:1831-1840.

Du W & Prendergast GC. Geranylgeranylated RhoB mediates suppression of human tumor cell growth by farnesyltransferase inhibitors. *Cancer Res.* 1999;**59**:5492-5496.

Dudler T & Gelb MH. Palmitoylation of Ha-Ras facilitates membrane binding, activation of downstream effectors and meiotic maturation in *Xenopus* oocytes. *J Biol Chem.* 1996;**271**:11541-11547.

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci USA.* 1995;**92**:7686-7689.

Duncan JA & Gilman AG. A cytoplasmic acyl-protein thioesterase that removes palmitate from G protein alpha subunits and p21(RAS). *J Biol Chem.* 1998;**273**:15830-15837.

Eguchi M, Eguchi-Ishimae M, Tojo A, Morishita K, Suzuki K, Sato Y, Kudoh S, Tanaka K, Setoyama M, Nagamura F, Asano S, Kamada N. Fusion of ETV6 to neurotrophin-3 receptor TRKC in acute myeloid leukemia with t(12;15)(p13;q25). *Blood.* 1999;**93**:1355-1363.

Elion EA. Routing MAP kinase cascades. *Science.* 1998;**281**:1625-1626.

Elmberger PG, Lozano MD, Weisenburger DD, Sanger W, Chan WC. Transcripts of the npm-alk fusion gene in anaplastic large cell lymphoma, Hodgkin´s disease, and reactive lymphoid lesions. *Blood.* 1995;**86**:3517-3521.

Emanuel, PD, Snyder RC, Wiley T, Gopurala B, Castleberry RP. Inhibition of juvenile myelomonocytic leukemia cell growth in vitro by farnesyltransferase inhibitors. *Blood.* 2000;**95**:639-645.

English JM & Cobb MH. Pharmacological inhibitors of MAPK pathways. *TRENDS in Pharmacological Sciences.* 2002;**23**:40-45.

Eskens FA, Awada A, Cutler DL, de Jonge MJ, Luyten GP, Faber MN, Statkevich P, Sparreboom A, Verweij J, Hanauske AR, Piccart M. Phase I and pharmacokinetic study of the oral farnesyl transferase inhibitor SCH 66336 given twice daily to patients with advanced solid tumors. *J Clin Oncol.* 2001;**19**:1167-1175.

Faderl S, Talpaz M, Estrow Z, O`Brien S, Kurzrock R, Kantarjian HM. The biology of chronic myeloid leukemia. *N Engl J Med.* 1999;**341**:164-172.

Fanger GR, Gerwins P, Widmann C, Jarpe MB, Johnson GL. MEKKs, GCKs, MLKs, PAKs, TAKs, and Tpls: upstream regulators of the c-Jun amino-terminal kinases? *Curr Opin Genet Dev.* 1997;**7**:67-74.

Farnsworth CC, Wolda SL, Gelb MH, Glomset JA. Human lamin B contains a farnesylated cysteine residue. *J Biol Chem.* 1989;**264**:20422-20429.

Farnsworth CC, Seabra MC, Ericsson LH, Gelb MH, Glomset JA. Rab geranylgeranyl transferase catalyzes the geranylgeranylation of adjacent cysteines in the small GTPases Rab1A, Rab3A, and Rab5A. *Proc Natl Acad Sci U S A.* 1994;**91**:11963-11967.

Farr CJ, Saiki RK, Erlich HA, McCormick F, Marshall CJ. Analysis of Ras gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucteotide probes. *Proc Natl Acad Sci USA.* 1988;**85**:1629-1633.

Favata MF, Horiuchi KJ, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem.* 1998;**273**:18623-18632.

Feig LA, Urano T, Cantor S. Evidence for a Ras/Ral signaling cascade. *Trends Biochem Sci.* 1996;**21**:438-441.

Fukazawa H & Uehara Y. U0126 reverses Ki-ras-mediated transformation by blocking both mitogen-activated protein kinase and p70 S6 kinase pathways. *Cancer Res.* 2000;**60**:2104-2107.

Fukuda M, Gotoh Y, Nishida E. Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmatic transport of MAP kinase. *EMBO J.* 1997;**16**:1901-1908.

Gana-Weisz M, Haklai R, Marciano D, Egozi Y, Ben-Baruch G, Kloog Y. The Ras antagonist S-farnesylthiosalicylic acid induces inhibition of MAPK activation. *Biochem Biophys Res Comm.* 1997;**239**:900-904.

Garrington TP & Johnson GL. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol.* 1999;**11**:211-218.

Gelb MH, Tamanoi F, Yokoyama K, Ghomashchi F, Esson K, Gould MN. The inhibition of protein prenyltransferases by oxygenated metabolites of limonene and perillyl alcohol. *Cancer Lett.* 1995;**91**:169-175.

Gelb MH. Protein prenylation, et cetera: Signal transduction in two dimensions. *Science.* 1997;**275**:1750-1751.

Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med.* 1993;**328**:1313-1316.

Gibbs JB & Oliff A. The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. *Annu Rev Pharmacol Toxicol.* 1997;**37**:143-166.

Gibbs JB, Oliff A, Kohl NE. Farnesyltransferase inhibitors: Ras research yields a potential cancer therapeutic. *Cell.* 1994;**77**:175-178.

Gibbs JB & Oliff A. Pharmaceutical research in molecular oncology. *Cell.* 1994;**79**:193-198.

Gibbs JB, Pompliano DL, Mosser SD, Rands E, Lingham RB, Singh SB, Scolnick EM, Kohl NE, Oliff A. Selective inhibition of farnesyl-protein transferase blocks ras processing in vivo. *J Biol Chem.* 1993;**268**:7617-7620.

Gibbs JB. Ras C-terminal processing enzymes: new drug targets? *Cell.* 1991;**65**:1-4.

Gibbs JB, Sigal IS, Poe M, Scolnick EM. Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. *Proc Natl Acad Sci USA*. 1984;**81**:5704-5708.

Gilliland DG & Griffin JD. Role of FLT3 in leukemia. *Curr Opin Hematol*. 2002;**9**:274-281.

Gire V, Marshall C, Wynford-Thomas D. PI-3-kinase is an essential anti-apoptotic effector in the proliferative response of primary human epithelial cells to mutant RAS. *Oncogene*. 2000;**19**:2269-2276.

Glomset JA & Farnsworth CC. Role of protein modification reactions in programming interactions between Ras-related GTPases and cell membranes. *Ann Rev Cell Biol.* 1994;**10**:181-205.

Golub T, Barker G, Lovett M, Gilliland D. Fusion of PDGF receptor β to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. *Cell.* 1994;**77**:307-316.

Golub TR, Goga A, Barker GF, Afar DE, McLaughlin J, Bohlander SK, Rowley JD, Witte ON, Gilliland DG. Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. *Mol Cell Biol.* 1996;**16**:4107-4116.

Gonzalez FA, Seth A, Raden DL, Bowman DS, Fay FS, Davis RJ. Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. *J Cell Biol.* 1993;**122**:1089-1101.

Graham SM, Cox AD, Drivas G, Rush MG, D`Eustachio P, Der CJ. Abberant function of the Ras-related protein TC21/R-Ras2 triggers malignant transformation. *Mol Cell Biol.* 1994;**14**:4108-4115.

Guthridge MA, Stomski FC, Thomas D, Wookcock JM, BagleyCJ, Berndt MC, Lopez AF. Mechanism of activation of the GM-CSF, IL-3, and IL-5 family of receptors. *Stem cells.* 1998;**16**:301-313.

Haklai R, Weisz MG, Elad G, Paz A, Marciano D, Egozi Y, Ben-Baruch G, Kloog Y. Dislodgment and accelerated degradation of Ras. *Biochemistry.* 1998;**37**:1306-1314.

Hallek M, Leif Bergsagel P, Anderson KC. Multiple myeloma: increasing evidence for a multistep transformation process. *Blood.* 1998;**91**:3-21.

Hall-Jackson CA, Eyers PA, Cohen P, Goedert M, Boyle FT, Hewitt N, Plant H, Hedge P. Paradoxical activation of Raf by a novel Raf inhibitor. *Chem Biol.* 1999;**6**:559-568.

Hall-Jackson CA, Goedert M, Hedge P, Cohen P. Effect of SB 203580 on the activity of c-Raf in vitro and in vivo. *Oncogene*. 1999;**18**:2047-2054.

Hancock J, Magee A, Childs J, Marshall C. All ras proteins are polyisoprenylated but only some are palmitoylated. *Cell.* 1989;**57**:1167-1177.

Hara M, Akasaka K, Akinaga S, Okabe M, Nakano H, Gomez R, Wood D, Uh M, Tamanoi F. Identification of Ras farnesyltransferase inhibitors by microbial screening. *Proc Natl Acad Sci USA.* 1993;**90**:2281-2285.

Heilmeyer LM Jr, Serwe M, Weber C, Metzger J, Hoffmann-Posorske E, Meyer HE. Farnesylcysteine, a constituent of the alpha and beta subunits of rabbit skeletal muscle phosphorylase kinase: localization by conversion to S-ethylcysteine and by tandem mass spectrometry. *Proc Natl Acad Sci U S A.* 1992;**89**:9554-9558.

Heimbrook D *et al.* Identification of potent, selective kinase inhibitors of Raf. *Proc. Amer. Assoc. Cancer Res.* 1998;**39**:3793.

Heimbrook DC & Oliff A. Therapeutic intervention and signaling. *Curr Opin Cell Biol.* 1998;**10**:284-288.

Hernandez-Alcoceba R, del Peso R and Lacal JC. The Ras family of GTPases in cancer cell invasion. *Cell Mol Life Sci.* 2000;**57**:65-76.

Herrmann C, Block C, Geisen C, Haas K, Weber C, Winde G, Moroy T, Muller O. Sulindac sulfide inhibits Ras signaling. *Oncogene.* 1998;**17**:1769-1776.

Himelstein BP, Lee EJ, Sato H, Seiki M, Muschel RJ. Transcriptional activation of the matrix metalloproteinase-9 gene in an H-ras and v-myc transformed rat embryo cell line. *Oncogene*. 1997;**14**:1995-1998.

Hirai A, Nakamura S, Noguchi Y, Yasuda T, Kitagawa M, Tatsuno I, Oeda T, Tahara K, Terano T, Narumiya S, Kohn LD, Saito Y. Geranylgeranylated rho small GTPase(s) are essential for the degradation of p27Kip1 and facilitate the progression from G1 to S phase in growth-stimulated rat FRTL-5 cells. *J Biol Chem.* 1997;**272**:13-16.

Hirsch-Ginsberg C, LeMaistre AC, Kantarjian H, Talpaz M, Cork A, Freireich EJ, Trujillo JM, Lee MS, Stass SA. Ras mutations are rare events in Philadelphia chromosome-negative/bcr gene rearrangement-negative chronic myelogenous leukemia, but are prevalent in chronic myelomonocytic leukemia. *Blood.* 1990;**76**:1214-1219.

Hofman M, Rudy W, Gunthert U Zimmer SG, Zawadzki V, Zoller M, Lichtner RB, Herrlich P, Ponta H. A link between ras and metastatic behaviour of tumor cells: ras induces CD44 promoter activity and leads to low-level expression of metastasis-specific variants of CD44 in CREF cells. *Cancer Res.* 1993;**53**:1516-1521.

Hollander I, Frommer E, Mallon R. Human ras-converting enzyme (hRCE1) endoproteolytic activity on K-ras-derived peptides. *Anal Biochem.* 2000;**286**:129-137.

Hulleman E, Bijvelt JJ, Verkleij AJ, Verrips CT, Boonstra J. Nuclear translocation of mitogen-activated protein kinase p42MAPK during ongoing cell cycle. *J Cell Physiol.* 1999;**180**:325-333.

Hung WC & Chuang LY. Involvement of caspase family proteases in FPT inhibitor III-induced apoptosis in human ovarian cancer cells. *Int J Oncol.* 1998a;**12**:1339-1342.

Hung WC & Chuang LY. The farnesyltransferase inhibitor, FPT inhibitor III upregulates Bax and Bcl-xs expression and induces apoptosis in human ovarian cancer cells. *Int J Oncol.* 1998b;**12**:137-140.

Hunter T. Oncoprotein networks. *Cell.* 1997;**88**:333-346.

Iida M, Towatari M, Nakao A, Iida H, Kiyoi H, Nakano Y, Tanimoto M, Saito H, Naoe T. Lack of constitutive activation of MAP kinase pathway in human acute myeloid leukemia cells with N-Ras mutation. *Leukemia*. 1999;**13**:585-589.

Iijima Y, Ito T, Oikawa T, Eguchi M, Euchi-Ishimae M, Kamada N, Kishi K, Asano S, Sakaki Y, Sato Y. A new ETV/TEL partner gene, ARG (ABL-related gene or ABL2), identified in an AML-M3 cell line with a t(1;12)(q25;p13) translocation. *Blood.* 2000;**95**:2126-2131.

Inglese J, Glickman JF, Lorenz W, Caron MG, Lefkowitz RJ. Isoprenylation of a protein kinase. Requirement of farnesylation/alpha-carboxyl methylation for full enzymatic activity of rhodopsin kinase. *J Biol Chem.* 1992;**267**:1422-1425.

Ito T, Kawata S, Tamura S, Igura T, Nagase T, Miyagawa JI, Yamazaki E, Ishiguro H, Matasuzawa Y. Suppression of human pancreatic cancer growth in BALB/c nude mice by manumycin, a farnesyl:protein transferase inhibitor. *Jpn J Cancer Res.* 1996;**87**:113-116.

Itoh O, Kuroiwa S, Atsumi S, Umezawa K, Takeuchi T, Mar M. Induction by guanosine analogue oxanosine of reversion toward the normal phenotype of K-ras transformed rat kidney cells. *Cancer Res.* 1989;**49**:996-1000.

Jaaro H, Rudinfeld H, Hanoch T, Seger R. Nuclear translocation of mitogen-activated protein kinase kinase (MEK1) in response to mitogenic stimulation. *Proc Natl Acad Sci USA*. 1997;**94**:3742-3747.

James G, Goldstein JL, Brown MS. Resistance of K-RasB^{V12} proteins to farnesyltransferase inhibitors in Rat1 cells. *Proc Natl Acad Sci USA.* 1996;**93**:4454-4458.

James GL, Goldstein JL, Pathak RK, Anderson RG, Brown MS. PxF, a prenylated protein of peroxisomes. *J Biol Chem.* 1994;**269**:14182-14190.

James GL, Brown MS, Cobb MH, Goldstein JL. Benzodiazepine peptidomimetic BZA-5B interrupts the MAP kinase activation pathway in H-Ras-transformed Rat-1 cells, but not in untransformed cells. *J Biol Chem.* 1994;**269**:27705-27714.

James GL, Goldstein JL, Brown MS, Rawson TE, Somers TC, McDowell RS, Crowley CW, Lucas BK, Levinson AD, Marsters JC Jr. Benzodiazepine

peptidomimetics: potent inhibitors of Ras farnesylation in animal cells. *Science.* 1993;**260**:1937-1942.

James GL, Goldstein JL, Brown MS. Polylysine and CVIM sequences of K-RasB dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic *in vitro*. *J Biol Chem.* 1995;**270**:6221-6226.

Jansen B, Schlagbauer-Wadl H, Kahr H, Heere-Ress E, Mayer BX, Eichler H, Pehamberger H, Gana-Weisz M, Ben-David E, Kloog Y, Wolff K. Novel Ras antagonist blocks human melanoma growth. *Proc Natl Acad Sci USA.* 1999;**96**:14019-14024.

Janssen J, Steenvoorden A, Lyons J, Anger B, Bohlke JU, Bos JL, Seliger H, Bartram CR. Ras gene mutations in acute and chronic myelocytic leukemias, chronic myeloproliferative disorders, and myelodysplastic syndromes. *Proc Natl Acad Sci USA.* 1987;**84**,9228-9232.

Jayasuriya H, Ball RG, Zink DL, Smith JL, Goetz MA, Jenkins RG, Nallin-Omstead M, Silverman KC, Bills GF, Lingham RB, et al. Barcelonic acid A, a new farnesyl-protein transferase inhibitor from Phoma species. *J Natural Prod.* 1995;**58**:986-991.

Jiang K, Coppola D, Crespo NC, Nicosia SV, Hamilton AD, Sebti SM, Cheng JQ. The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. *Mol Cell Biol.* 2000;**20**:139-148.

Johnson L, Greenbaum D, Cichowski K, Mercer K, Murphy E, Schmitt E, Bronson RT, Umanoff H, Edelmann W, Kucherlapati R, Jacks T. K-ras is an essential gene in the mouse with partial functional overlap with N-ras. *Genes Dev.* 1997;**11**:2468-2481.

Jousset C, Carron C, Boureux A, Quang CT, Oury C, Dusanter-Fourt I, Charon M, Levin J, Bernard O, Ghysdael J. A domain of TEL conserved in a subset of ETS proteins defines a specific oligomerization interface essential to the mitogenic properties of the TEL-PDGFRβ oncoprotein. *EMBO J.* 1997;**16**:69-82.

Kadam S & McAlpine JB. Dorrigocins: novel antifungal antibiotics that change the morphology of ras-transformed NIH/3T3 cells to that of normal cells. III. Biological properties and mechanism of action. *J Antibiot Toyko.* 1994;**47**:875-880.

Kainuma O, Asano T, Hasegawa M, Kenmochi T, Nakagohri T, Tokoro Y, Isono K. Inhibition of growth and invasive activity of human pancreatic cancer cells by a farnesyltransferase inhibitor, manumycin. *Pancreas.* 1997;**15**: 379-383.

Kalakonda N, Rothwell DG, Scarffe JH, Norton JD. Detection of N-Ras codon 61 mutations in subpopulations of tumor cells in multiple myeloma at presentation. *Blood.* 2001;**98**:1555-1560.

Kalra R, Paderanga DC, Olson K, Shannon KM. Genetic analysis is consistent with the hypothesis that NF-1 limits myeloid cell growth through p21ras. *Blood.* 1994;**84**:3435-3439.

Kang MS, Stemerick DM, Zwolshen JH, Harry BS, Sunkara PS, Harrison BL. Farnesyl-derived inhibitors of Ras farnesyl transferase. *Biochem Biophys Res Comm.* 1995;**217**:245-249.

Karp JE, Lancet JE, Kaufmann SH, End DW, Wright JJ, Bol K, Horak I, Tidwell ML, Liesveld J, Kottke TJ, Ange D, Buddharaju L, Gojo I, Highsmith WE, Belly RT, Hohl RJ, Rybak ME, Thibault A, Rosenblatt J. Clinical and biologic activity of the farnesyltransferase inhibitor R115777 in adults with refractory and relapsed acute leukemias: a phase 1 clinical-laboratory correlative trial. *Blood.* 2001;**97**:3361-3369.

Karp JE. Farnesyl protein transferase inhibitors as targeted therapies for hematologic malignancies. *Semin Hematol.* 2001;**38**:16-23.

Kashani-Sabet M, FunatoT, Florenes VA, Fodstad O, Scanlon KJ. Suppression of the neoplastic phentotype *in vivo* by an anti-ras ribozyme. *Cancer Res.* 1994;**54**:900-902.

Kato K, Cox AD, Hisaka MM, Graham SM, Buss JE, Der CJ. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. *Proc Natl Acad Sci USA.* 1992;**89**:6403-6407.

Katz ME & McCormick F. Signal transduction from multiple Ras effectors. *Curr Opin Genet Dev.* 1997;**7**:75-79.

Kelland LR, Smith V, Valenti M, Patterson L, Clarke PA, Detre S, End D, Howes AJ, Dowsett M, Workman P, Johnston SR. Preclinical antitumor activity and pharmacodynamic studies with the farnesyl protein transferase inhibitor R115777 in human breast cancer. *Clin Cancer Res.* 2001;**7**:3544-3550.

Khokhlatchev AV, Canagarajah B, Wilsbacher J, Robinson M, Atkinson M, Goldsmith E, Cobb MH. Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. *Cell.* 1998;**93**:605-615.

Khosravi-Far R, Solski PA, Clark GJ, Kinch MS, Der CJ. Activation of Rac1, RhoA and mitogen-activated protein kinases is required for Ras transformation. *Mol Cell Biol.* 1995;**15**: 6443-6453.

Khosravi-Far R, Campbell S, Rossman KL, Der CJ. Increasingcomplexity of *ras* signal transduction: involvement of *rho* family proteins. *Adv Cancer Res.* 1998;**72**:57-107.

Khosravi-Far R, White MA, Westwick JK, Solski PA, Chrzanowska-Wodnicka M, Van Aelst L, Wigler MH, Der CJ. Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. *Mol Cell Biol.* 1996;**16**:3923-3933.

Kim KW, Chung HH, Chung CW, Kim IK, Miura M, Wang S, Zhu H, Moon KD, Rha GB, Park JH, Jo DG, Woo HN, Song YH, Kim BJ, Yuan J, Jung YK. Inactivation of farnesyltransferase and geranylgeranyltransferase I by caspase-3: cleavage of the common alpha subunit during apoptosis. *Oncogene.* 2001;**20**:358-366.

Kim SC, Hahn JS, Min YH, Yoo NC, Ko YW, Lee WJ. Constitutive activation of extracellular signal-regulated kinase in human acute leukemias: combined role of

activation of MEK, hyperexpression of extracellular signal-regulated kinase, and downregulation of a phosphatase, PAC1. *Blood.* 1999;**93**:3893-3899.

Kitayama H, Kanakura Y, Furitsu T, Tsujimura T, Oritani K, Ikeda H, Sugahara H, Mitsui H, Kanayama Y, Kitamura Y, et al. Constitutively activating mutations of c-Kit receptor tyrosine kinase confer factor-independent growth and tumorigenicity of factor-dependent hematopoietic cell lines. *Blood.* 1995;**85**:790-798.

Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, Asou N, Kuriyama K, Jinnai I, Shimazaki C, Akiyama H, Saito K, Oh H, Motoji T, Omoto E, Saito H, Ohno R, Ueda R. Prognostic implication of FLT3 and N-Ras gene mutations in acute myeloid leukemia. *Blood.* 1999;**93**:3074-3080.

Kleiner D & Stetler-Stevenson W. Structural biochemistry and activation of matrix metalloproteinases. *Curr Opin Cell Biol.* 1993;**5**:891.

Koblan KS, Culberson JC, DeSolms SJ, Giuliani EA, Mosser SD, Omer CA, Pitzenberger SM, Bogusky MJ. NMR studies of novel inhibitors bound to farnesyl-protein transferase. *Protein Sci.* 1995;**4**:681-688.

Koera K, Nakamura K, Nakao K, Miyoshi J, Toyoshima K, Hatta T, Otani H, Aiba A, Katsuki M. K-ras is essential for the development of the mouse embryo. *Oncogene.* 1997;**15**:1151-1159.

Kohl NE, Mosser SD, DeSolms SJ, Giuliani EA, Pompliano DL, Graham SL, Smith RL, Scolnick EM, Oliff A, Gibbs JB. Selective inhibition of ras-dependent transformation by a farnesyltransferase inhibitor. *Science.* 1993;**260**:1934-1937.

Kohl NE, Omer CA, Conner MW, Anthony NJ, Davide JP, deSolms SJ, Giuliani EA, Gomez RP, Graham SL, Hamilton K, et al. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. *Nature Med.* 1995;**1**:792-797.

Kohl NE, Wilson FR, Mosser SD, Giuliani E, deSolms SJ, Conner MW, Anthony NJ, Holtz WJ, Gomez RP, Lee TJ, Smith RL, Graham SL, Hartman GD, Gibbs JB, Oliff A. Protein farnesyltransferase inhibitors block the growth of ras-dependent tumors in nude mice. *Proc Natl Acad Sci USA.* 1994;**91**:9141-9145.

Kumar CC, Prorock-Rogers C, Kelly J, Dong Z, Lin JJ, Armstrong L, Kung HF, Weber MJ, Afonso A. SCH51344 inhibits ras transformation by a novel mechanism. *Cancer Res.* 1995;**55**:5106-5117.

Kurzrock R, Gutterman J, Talpaz M. The molecular genetics of Philadelphia chromo-some-positive leukemias. *N Engl J Med.* 1988;**319**:990-998.

Lackey K, Cory M, Davis R, Frye SV, Harris PA, Hunter RN, Jung DK, McDonald OB, McNutt RW, Peel MR, Rutkowske RD, Veal JM, Wood ER. The discovery of potent cRaf1 kinase inhibitors. *Bioorg Med Chem Lett.* 2000;**10**:223-226.

Lai RK, Perez-Sala D, Canada FJ, Rando RR. The gamma subunit of transducin is farnesylated. *Proc Natl Acad Sci U S A.* 1990;**87**:7673-7677.

Lantry LE, Zhang Z, Yao R, Crist KA, Wang Y, Ohkanda J, Hamilton AD, Sebti SM, Lubet RA, You M. Effect of farnesyltransferase inhibitor FTI-276 on established lung adenomas from A/J mice induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Carcinogenesis.* 2000;**21**:113-116.

Largaespada DA, Brannan CI, Jenkins NA, Copeland NG. NF-1 deficiency causes Ras-mediated granulocyte/macrophage colony stimulating factor hypersensitivity and chronic myeloid leukemia. *Nat Genet.* 1996;**12**:137-143.

Lebowitz PF, Davide JP, Prendergast GC. Evidence that farnesyltransferase inhibitors suppress Ras transformation by interfering with Rho activity. *Mol Cell Biol.* 1995;**15**:6613-6622.

Lebowitz PF & Prendergast GC. Non-Ras targets of farnesyltransferase inhibitors: focus on Rho. *Oncogene.* 1998;**17**:1439-1445.

Lebowitz PF, Sakamuro D, Prendergast GC. Farnesyl transferase inhibitors induce apoptosis of Ras-transformed cells denied substratum attachment. *Cancer Res.* 1997;**57**:708-713.

Lee S, Park S, Oh JW, Yang C. Natural inhibitors for protein prenyltransferase. *Planta Med.* 1998;**64**:303-308.

Leftheris K, Kline T, Vite GD, Cho YH, Bhide RS, Patel DV, Patel MM, Schmidt RJ, Weller HN, Andahazy ML, Carboni JM, Gullo-Brown JL, Lee FY, Ricca C, Rose WC, Yan N, Barbacid M, Hunt JT, Meyers CA, Seizinger BR, Zahler R, Manne V. Development of potent inhibitors of Ras farnesyltransferase possessing cellular and *in vivo* activity. *J Med Chem.* 1996;**39**:224-236.

Lenormand P, Brondello J-M, Brunet A, Pouysségur J. Growth factor-induced p42/p44 MAPK nuclear translocation and retention requires both MAPK activation and neosynthesis of nuclear anchoring proteins. *J Cell Biol.* 1998;**142**:625-633.

Lenormand P, Sardet G, Pagès G, L`Allemain G, Brunet A, Pouysségur J. Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J Cell Biol.* 1993;**122**:1079-1089.

Lerner EC, Qian Y, Blaskovich MA, Fossum RD, Vogt A, Sun J, Cox AD, Der CJ, Hamilton AD, Sebti SM. Ras CAAX peptidomimetic FTI-277 selectively blocks oncogenic Ras signaling by inducing cytoplasmic accumulation of inactive Ras-Raf complexes. *J Biol Chem.* 1995a;**270**:26802-26806.

Lerner EC, Qian Y, Hamilton AD, Sebti SM. Disruption of oncogenic K-Ras4B processing and signalling by a potent geranylgeranyltransferase I inhibitor. *J Biol Chem.* 1995b;**270**:26770-26773.

Lerner EC, Zhang TT, Knowles DB, Qian Y, Hamilton AD, Sebti SD. Inhibition of the prenylation of K-*RAS*, but not H- or N-*RAS* is highly resistant to CAAX peptidomimetics and requires both a farnesyltransferase and a geranylgeranyltransferase I inhibitor in human tumor cell lines. *Oncogene.* 1997;**15**:1283-1288.

Lin SR, Hsu CH, Tsai JH, Wang JY, Hsieh TJ, Wu CH. Decreased GTPase activity of K-Ras deriving from human functional adrenocortical tumours. *Br J Cancer.* 2000;**82**:1035-1040.

Lin SR, Tsai JH, Yang YC, Lee SC. Mutations of K-Ras oncogene in human adrenal tumors in Taiwan. *Br J Cancer.* 1998;**77**:1060-1065.

Liu Ax, Du W, Liu JP, Jessell TM, Prendergast GC. RhoB alteration is necessary for apoptotic and antineoplastic responses to farnesyltransferase inhibitors. *Mol Cell Biol.* 2000;**20**:6105-6113.

Liu J-J, Chao J-R, Jiang M-C, Ng S-Y, Yen JJ-Y, Jang-Yen H-F. Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH3T3 cells. *Mol Cell Biol.* 1995;**15**:3654-3663.

Liu L, Dudler T, Gelb MH. Purification of a protein palmitoyltransferase that acts on H-Ras protein and on a C-terminal N-Ras peptide. *J Biol Chem.* 1996;**271**:23269-23276.

Liu M, Bryant MS, Chen J, Yaremko B, Lipari P, Malkowski M, Ferrari E, Nielsen L, Prioli N, Dell J, Sinha D, Syed J, Korfmacher WA, Nomeir AA, Lin CC, Wang L, Taveras AG, Doll RJ, Njoroge FG, Mallams AK, Remiszewski S, Catino JJ, Girijavallabhan VM, Bishop WR, et al. Antitumor activity of SCH66336, an orally bioavailable tricyclic inhibitor of farnesyl protein transferase, in human tumor xenograft models and Wap-ras transgenic mice. *Cancer Res.* 1998;**58**:4947-4956.

Liu M, Bryant MS, Chen J, Lee S, Yaremko B, Li Z, Dell J, Lipari P, Malkowski M, Prioli N, Rossman RR, Korfmacher WA, Nomeir AA, Lin CC, Mallams AK, Doll RJ, Catino JJ, Girijavallabhan VM, Kirschmeier P, Bishop WR. Effects of SCH59228, an orally bioavialable farnesyl protein transferase inhibitor, on the growth of oncogene-transformed fibroblasts and a human colon carcinoma xenograft in nude mice. *Cancer Chemother Pharmacol.* 1999;**43**:50-58.

Liu Q, Schwaller J, Kutok J, Cain D, Aster JC, Williams IR, Gilliland DG. Signal transduction and transforming properties of the TEL-TRKC fusions associated with t(12;15)(p13;q25) in congenital fibrosarcoma and acute myelogenous leukemia. *EMBO J.* 2000;**19**:1827-1838.

Lloyd AC, Obermueller F, Staddon S, Barth CF, McMahon M, Land H. Cooperating oncogenes converge to regulate cyclin/cdk complexes. *Genes Dev.* 1997;**11**:663-677.

Lobell RB, Omer CA, Abrams MT, Bhimnathwala HG, Brucker MJ, Buser CA, Davide JP, deSolms SJ, Dinsmore CJ, Ellis-Hutchings MS, Kral AM, Liu D, Lumma WC, Machotka SV, Rands E, Williams TM, Graham SL, Hartman GD, Oliff AI, Heimbrook DC, Kohl NE. Evaluation of farnesyl:protein transferase and geranylgeranyl:protein transferase inhibitor combinations in preclinical models. *Cancer Res.* 2001;**61(24)**:8758-8768.

Lowy DR & Willumsen BM. Rational cancer therapy. *Nature Med.* 1995;**1**:747-748.

Lubbert M, Mertelmann R, Herrmann F. Detection of allele-specific expression of N-Ras oncogenes in human leukaemia cells. *Br J Haematol.* 1992;**81**:370-373.

MacKencie KL, Dolnikov A, Millington M, Shounan Y, Symonds G. Mutant N-ras induces myeloproliferative disorders and apoptosis in bone marrow repopulated mice. *Blood.* 1999;**93**:2043-2056.

Mahgoub N, Taylor BR, Gratiot M, Kohl NE, Gibbs JB, Jacks T, Shannon KM. In vitro and in vivo effects of a farnesyltransferase inhibitor on NF-1-deficient hematopoietic cells. *Blood.* 1999;**94**:2469-2476.

Mallams AK, Rossman RR, Doll RJ, Girijavallabhan VM, Ganguly AK, Petrin J, Wang L, Patton R, Bishop WR, Carr DM, Kirschmeier P, Catino JJ, Bryant MS, Chen KJ, Korfmacher WA, Nardo C, Wang S, Nomeir AA, Lin CC, Li Z, Chen J, Lee S, Dell J, Lipari P, Liu M, et al. Inhibitors of farnesyl protein transferase. 4-Amido, 4-Carbamoyl, and 4-Carboxamido Derivatives of 1-(8-Chloro-6,11-dihydro-5H-benzo[5,6] cyclohepta[1,2b]pyridin-11-yl)piperazine and 1-(3-Bromo-8-chloro-6,11-dihydro-5H-benzo[5,6]cyclohepta[1,2b]pyridin-11-yl)piperazine. *J Med Chem.* 1998;**41**:877-893.

Mangues R, Corral T, Kohl NE, Symmans WF, Lu S, Malumbres M, Gibbs JB, Oliff A, Pellicer A. Antitumor effect of a farnesyl protein transferase inhibitor in mammary and lymphoid tumors overexpressing N-*RAS* in transgenic mice. *Cancer Res.* 1998;**58**:1253-1259.

Manne V, Yan N, Carboni JM, Tuomari AV, Ricca CS, Brown JG, Andahazy ML, Schmidt RJ, Patel D, Zahler R, et al. Bisubstrate inhibitors of farnesyltransferase: a novel class of specific inhibitors of ras-transformed cells. *Oncogene.* 1995;**10**:1763-1779.

Marciano D, Ben-Baruch G, Marom M, Egozi Y, Haklai R, Kloog Y. Farnesyl derivatives of rigid carboxylic acids - inhibitors of Ras-dependent cell growth. *J Med Chem.* 1995;**38**:1267-1272.

Margolis B, Li N, Koch A, Mohammadi M, Hurwitz DR, Zilberstein A, Ullrich A, Pawson T, Schlessinger J. The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-gamma. *EMBO J.* 1990;**9**:4375-4380.

Marom M, Haklai R, Ben-Baruch G, Marciano D, Egozi Y, Kloog Y. Selective inhibition of Ras-dependent cell growth by farnesylthiosalisylic acid. *J Biol Chem.* 1995;**270**:22263-22270.

Marshall CJ. Raf gets it together. *Nature.* 1996a;**383**:127-128.

Marshall CJ. Ras effectors. *Curr Opin Cell Biol.* 1996b;**8**:197-204.

Marshall CJ. Specificity of receptor tyrosine kinase signalling: transient versus sustained extracellular signal-regulated kinase activation. *Cell.* 1995;**80**:179-185.

Mazet JL, Padieu M, Osman H, Maume G, Mailliet P, Dereu N, Hamilton AD, Lavelle F, Sebti SM, Maume BF. Combination of the novel farnesyltransferase inhibitor RPR130401 and the geranylgeranyltransferase-1 inhibitor GGTI-298 disrupts MAP kinase activation and G(1)-S transition in Ki-Ras-overexpressing transformed adrenocortical cells. *FEBS Lett.* 1999;**460**: 235-240.

McGuire TF, Qian Y, Vogt A, Hamilton AD, Sebti SM. Platelet-derived growth factor recptor tyrosine phosphorylation requires protein geranylgeranylation but not farnesylation. *J Biol Chem.* 1996;**271**:27402-27407.

McNamara DJ, Dobrusin E, Leonard DM, Shuler KR, Kaltenbronn JS, Quin J 3rd, Bur S, Thomas CE, Doherty AM, Scholten JD, Zimmerman KK, Gibbs BS, Gowan RC, Latash MP, Leopold WR, Przybranowski SA, Sebolt-Leopold JS. C-terminal modifications of histidyl-N-benzylglycinamides to give improved inhibition of Ras farnesyltransferase, cellular activity, and anticancer activity in mice. *J Med Chem.* 1997;**40**:3319-3322.

Milella M, Estrov Z, Kornblau SM, Carter BZ, Konopleva M, Tari A, Schober WD, Harris D, Leysath CE, Lopez-Berestein G, Huang Z, Andreeff M. Synergistic induction of apoptosis by simultaneous disruption of the Bcl-2 and MEK/MAPK pathways in acute myelogenous leukemia. *Blood* 2002;**99**:3461-3464.

Milligan G, Parenti M, Magee AI. The dynamic role of palmitoylation in signal transduction. *Trends Biochem Sci.* 1995;**20**:181-187.

Miquel K, Pradines A, Sun J, Qian Y, Hamilton AD, Sebti SM, Favre G. GGTI-298 induces G0-G1 block and apoptosis whereas FTI-277 causes G2-M enrichment in A549 cells. *Cancer Res.* 1997;**57**:1846-1850.

Miyauchi J, Asada M, Sasaki M, Tsunematsu Y, Kojima S, Mizutani S. Mutations of N-ras gene in juvenile chronic myelogenous leukemia. *Blood.* 1994;**83**:2248-2254.

Moasser MM, Sepp-Lorenzino L, Kohl NE, Oliff A, Balog A, Su DS. Farnesyl transferase inhibitors cause enhanced mitotic sensitivity to taxol and epothilones. *Proc Natl Acad Sci USA.* 1998;**95**:1369-1374.

Moores SL, Schaber MD, Mosser SD, Rands E, O'Hara MB, Garsky VM, Marshall MS, Pompliano DL, Gibbs JB. Sequence dependence of protein isoprenylation. *J Biol Chem.* 1991;**266**:14603-14610.

Moran MF, Koch CA, Anderson D, Ellis C, England L, Martin GS, Pawson T. Src homology region 2 domains direct protein-protein interactions in signal transduction. *Proc Natl Acad Sci U S A.* 1990;**87**:8622-8626.

Morgan MA, Dolp O, Reuter CW. Cell-cycle-dependent activation of mitogen-activated protein kinase kinase (MEK-1/2) in myeloid leukemia cell lines and induction of growth inhibition and apoptosis by inhibitors of RAS signaling. *Blood.* 2001;**97**:1823-1834.

Mortazavi Y, Tooze JA, Gordon-Smith EC, Rutherford TR. N-RAS gene mutation in patients with aplastic anemia and aplastic anemia/ paroxysmal nocturnal hemoglobinuria during evolution to clonal disease. *Blood.* 2000;**95**:646-50.

Mukhopadhyah T, Tainsky M, Cavender AC, Roth JA. Specific inhibition of K-ras expression and tumorigenicity of lung cancer cells by antisense RNA. *Cancer Res.* 1991;**51**:1744-1748.

Mumby SM. Reversible palmitoylation of signaling proteins. *Curr Opin Cell Biol.* 1997;**9**:148-154.

Nagase T, Kawata S, Tamura S, Matsuda Y, Inui Y, Yamasaki E, Ishiguro H, Ito T, Miyagawa J, Mitsui H, Yamamoto K, Kinoshita M, Matsuzawa Y. Manumycin and gliotoxin derivative KT7595 block Ras farnesylation and cell growth but do not disturb lamin farnesylation and localization in human tumour cells. *Br J Cancer.* 1997;**76**:1001-1010.

Nagasu T, Yoshimatsu K, Rowell C, Lewis MD, Garcia AM. Inhibition of human tumor xenograft growth by treatment with the farnesyl transferase inhibitor B956. *Cancer Res.* 1995;**55**:5310-5314.

Nagata H, Worobec AS, Oh CK, Chowdhury BA, Tannenbaum S, Suzuki Y, Metcalfe DD. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci USA.* 1995;**92**:10560-10564.

Nakata Y, Kimura A, Katoh O, Kawaishi K, Hyodo H, Abe K, Kuramoto A, Satow Y. c-kit point mutation of extracellular domain in patients with myeloproliferative disorders. *Br J Haematol.* 1995;**91**:661-663.

Neri A, Knowes DM, Greco A, McCormick F, Dalla-Favera R. Analysis of Ras oncogene mutations in human lymphoid malignancies. *Proc Natl Acad Sci USA.* 1988;**85**:9268-9272.

Neri A, Murphy JP, Cro L, Ferrero D, Tarella C, Baldini L, Dalla-Favera R. Ras oncogene mutation in multiple myeloma. *J Exp Med.* 1989;**170**:1715-1725.

Niemeyer CM, Arico M, Basso G, Biondi A, Cantu Rajnoldi A, Creutzig U, Haas O, Harbott J, Hasle H, Kerndrup G, Locatelli F, Mann G, Stollmann-Gibbels B, van't Veer-Korthof ET, van Wering E, Zimmermann M. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. *Blood.* 1997;**89**:3534-3543.

Njoroge FG, Taveras AG, Kelly J, Remiszewski S, Mallams AK, Wolin R, Afonso A, Cooper AB, Rane DF, Liu YT, Wong J, Vibulbhan B, Pinto P, Deskus J, Alvarez CS, del Rosario J, Connolly M, Wang J, Desai J, Rossman RR, Bishop WR, Patton R, Wang L, Kirschmeier P, Ganguly AK, et al. (+)-4-[2-[4-(8-Chloro-3,10-dibromo-6,11-dihydro-5H-benzo[5,6]cycloheptal[1,2b]-pyridin-11(R)-yl)-1-piperidinyl]-2-oxo-ethyl]-1-piperidinecarboxamide (SCH66336): A very potent farnesyl protein transferase inhibitor as a novel antitumor agent. *J Med Chem.* 1998a;**41**:4890-4902.

Njoroge FG, Vibulbhan B, Pinto P, Bishop WR, Bryant MS, Nomeir AA, Lin C, Liu M, Doll RJ, Girijavallabhan V, Ganguly AK. Potent, selective, and orally bioavailable tricyclic pyridyl acetamide N-oxide inhibitors of farnesyl protein transferase with enhanced *in vivo* antitumor activity. *J Med Chem.* 1998b;**41**:1561-1567.

Norgaard P, Law, B, Joseph H, Page DL, Shyr Y, Mays D, Pietenpol JA, Kohl NE, Oliff A, Coffey RJ Jr, Poulsen HS, Moses HL. Treatment with farnesyl-protein transferase inhibitor induces regression of mammary tumors in transforming growth factor

(TGF) alpha and TGF alpha/neu transgenic mice by inhibition of mitogenic activity and induction of apoptosis. *Clin Cancer Res.* 1999;**5**:35-42.

Ohta Y, Suzuki N, Nakamura S, Hartwig JH, Stossel TP. The small GTPase RalA targets filamin to induce filopodia. *Proc Natl Acad Sci U S A.* 1999;**96**:2122-2128.

Omer CA & Kohl NE. CA1A2X-competitive inhibitors of farnesyltransferase as anti-cancer agents. *Trends Pharmacol Sci.* 1997;**18**:437-444.

Omer CA, Chen Z, Diehl RE, Conner MW, Chen HY, Trumbauer ME, Gopal-Truter S, Seeburger G, Bhimnathwala H, Abrams MT, Davide JP, Ellis MS, Gibbs JB, Greenberg I, Koblan KS, Kral AM, Liu D, Lobell RB, Miller PJ, Mosser SD, O'Neill TJ, Rands E, Schaber MD, Senderak ET, Oliff A, Kohl NE. Mouse mammary tumor virus-Ki-rasB transgenic mice develop mammary carcinomas that can be growth-inhibited by a farnesyl:protein transferase inhibitor. *Cancer Res.* 2000;**60**:2680-2688.

Otto JC, Kim E, Young SG, Casey PJ. Cloning and characterization of a mammalian prenyl protein-specific protease. *J Biol Chem.* 1999**;274**:8379-8382.

Padua RA, Carter G, Hughes D, Gow J, Farr C, Oscier D, McCormick F, Jacobs A. Ras mutations in myelodysplasia detected by amplification, oligonucteotide hybridization and transformation. *Leukemia.* 1988;**2**:503-510.

Padua RA, Guinn BA, Al-Sabah AI Smith M, Taylor C, Pettersson T, Ridge S, Carter G, White D, Oscier D, Chevret S, West R. Ras, FMS and p53 mutations and poor clinical outcome in myelodysplasias: a 10-year follow-up. *Leukemia.* 1998;**12**:887-892.

Papadopoulos P, Raidge SA, Boucher CA, Stocking C, LM Wiedemann. The novel activation of abl by fusion to an ets-related gene, tel. *Cancer Res.* 1995;**55**:34-38.

Park H-W, Boduluri SR, Moomaw JF, Casey PJ, Beese LS. Crystal structure of protein farnesyltransferase at 2.25 Angstrom resolution. *Science.* 1997;**275**:1800-1804.

Parker J & Mufti GJ. Ras and myelodysplasia: lessons from the last decade. *Semin Hematol.* 1996;**33**:206-224.

Patel DV, Gordon EM, Schmidt RJ, Weller HN, Young MG, Zahler R, Barbacid M, Carboni JM, Gullo-Brown JL, Hunihan L, et al. Phosphinyl acid-based bisubstrate analog inhibitors of ras farnesyl protein transferase. *J Med Chem.* 1995;**38**:435-442.

Patel DV, Young MG, Robinson SP, Hunihan L, Dean BJ, Gordon EM. Hydroxamic acid-based bisubstrate analog inhibitors of Ras farnesyl protein transferase. *J Med Chem.* 1996;**39**:4197-4210.

Patriotis C, Makris A, Chernoff J, Tsichlis PN. Tpl-2 acts in concert with Ras and Raf-1 to activate mitogen-activated protein kinase. *Proc Natl Acad Sci USA.* 1994;**91**:9755-9759.

Pawson T & Saxton TM. Signaling networks-Do all roads lead to the same genes. *Cell.* 1999;**97**:675-678.

Pellicena P, Scholten JD, Zimmerman K, Creswell M, Huang CC, Miller WT. Involvement of the alpha subunit of farnesyl-protein transferase in substrate recognition. *Biochemistry.* 1996;**35**:13494-13500.

Pells S, Divjak M, Romanowski P, Impey H, Hawkins NJ, Clarke AR, Hooper ML, Williamson DJ. Developmentally-regulated expression of murine K-ras isoforms. *Oncogene.* 1997;**15**:1781-1786.

Perez-Sala D, Gilbert BA, Rando RR, Canada FJ. Analogs of farnesylcysteine induce apoptosis in HL-60 cells. *FEBS Lett.* 1998;**426**:319-324.

Porter AC & Vaillancourt RR. Tyrosine kinase receptor-activated signal transduction pathways which lead to oncogenesis. *Oncogene.* 1998;**17**:13434-13452.

Posado J, Yew N, Ahn NG, Vande-Woude GF, Cooper JA. Mos stimulates MAP kinase in Xenopus oocytes and activates MAP kinase kinase *in vitro*. *Mol Cell Biol.* 1993;**13**:2546-2553.

Prendergast GC, Davide JP, DeSolms JS, Giuliani EA, Graham SL, Gibbs JB, Oliff A, Kohl NE. Farnesyltransferase inhibition causes morphological reversion of ras-transformed cells by a complex mechanism that involves regulation of the actin cytoskeleton. *Mol Cell Biol.* 1994;**14**:4193-4202.

Prendergast GC, Davide JP, Lebowitz PF, Wechsler-Reya R, Kohl NE. Resistance of a variant ras-transformed cell line to phenotypic reversion by farnesyl transferase inhibitors. *Cancer Res.* 1996;**56**:2626-2632.

Prendergast GC. Farnesyltransferase inhibitors: antineoplastic mechanism and clinical prospects. *Curr Opin Cell Biol.* 2000;**12**:166-173.

Pumiglia KM & Decker SJ. Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. *Proc Natl Acad Sci USA.* 1997;**94**:448-452.

Punt CJ, van Maanen L, Bol CJ, Seifert WF, Wagener DJ. Phase I and pharmacokinetic study of the orally administered farnesyl transferase inhibitor R115777 in patients with advanced solid tumors. *Anticancer Drugs.* 2001;**12**:193-197.

Qian Y, Blaskovich MA, Saleem M, Seong CM, Wathen SP, Hamilton AD, Sebti SM. Design and structural requirements of potent peptidomimetic inhibitors of p21ras farnesyl-transferase. *J Biol Chem.* 1994;**269**:12410-12413.

Qian Y, Vogt A, Vasudevan A, Sebti SM, Hamilton AD. Selective inhibition of type-I geranylgeranyltransferase in vitro and in whole cells by CAAL peptidomimetics. *Bioorg Med Chem.* 1998;**6**:293-299.

Qiu RG, Chen J, McCormick F, Symons M. A role for Rho in Ras transformation. *Proc Natl Acad Sci USA.* 1995;**92**:11781-11785.

Quilliam LA, Castro AF, Rogers-Graham KS, Martin CB, Der CJ, Bi C. M-Ras/R-Ras3, a transforming ras protein regulated by SOS1, GRF1, and p120 Ras GTPase-

activating protein, interacts with the putative Ras effector AF6. *J Biol Chem.* 1999;**274**:23850-23857.

Rebollo A & Martinez CA. Ras proteins: recent advances and new functions. *Blood.* 1999;**94**:2971-2980.

Reiss Y, Goldstein JL, Seabra MC, Casey PJ, Brown MS. Inhibition of purified $p21^{ras}$ farnesyl protein transferase by cys-AAX tetrapeptides. *Cell.* 1990;**62**:81-88.

Reiss Y, Stradley SJ, Gierasch LM, Brown MS, Goldstein JL. Sequence requirement for peptide recognition by rat brain p21ras protein farnesyltransferase. *Proc Natl Acad Sci USA.* 1991;**88**:732-736.

Reuter C, Auf der Landwehr U I, Schleyer E, Zuhlsdorf M, Ameling C, Rolf C, Wormann B, Buchner T, Hiddemann W. Modulation of intracellular metabolism of cytosine arabinoside in acute myeloid leukemia by granulocyte-macrophage colony stimulating factor. *Leukemia.* 1994;**8**:217-225.

Reuter CWM, Catling AD, Jelinek T, Weber MJ. Biochemical analysis of MEK activation in NIH3T3 fibroblasts. *J Biol Chem.* 1995a;**270**:7644-7655.

Reuter CWM, Catling AD, Weber MJ. Immune complex kinase assays for mitogen-activated protein kinase and MEK. *Methods Enzymol.* 1995b;**205**:245-256.

Reuter CWM, Morgan MA, Bergmann L. Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematological malignancies. *Blood.* 2000;**96**:1655-1669.

Reuther GW & Der CJ. The Ras branch of small GTPases: Ras family members don't fall far from the tree. *Curr Opin Cell Biol.* 2000;**12**:157-165.

Robinson MJ & Cobb MH. Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol.* 1997;**9**:180-186.

Rohrschneider LR, Bourette RP, Lioubin MN, Algate PA, Myles GM, Carlberg K. Growth and differentiation signals regultated by the M-CSF receptor. *Mol Reprod Dev.* 1997; **46**:96-103.

Romano JD & Michaelis S. Topological and mutational analysis of *Saccharomyces cerevisiae* Ste14p, founding member of the isoprenylcysteine carboxyl methyltransferase family. *Molecular Biology of the Cell.* 2001;**12**:1957-1971.

Ross EM. Palmitoylation in G-protein signaling pathways. *Curr Biol.* 1995;**5**:107-109.

Ross TS, Bernard OA, Berger R, Gilliland DG. Fusion of Huntingtin interacting protein 1 to platelet-derived growth factor beta receptor (PDGFbetaR) in chronic myelomonocytic leukemia with t(5;7)(q33;q11.2). *Blood.* 1998;**91**:4419-4426.

Ross TS & Gilliland DG. Transforming properties of the Huntingtin interacting protein 1/ platelet-derived growth factor beta receptor fusion protein. *J Biol Chem.* 1999;**274**:22328-22336.

Rowell CA, Kowalczyk JJ, Lewis M.D, Garcia AM. Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras *in vivo*. *J Biol Chem.* 1997;**272**:14093-14097.

Saison-Behmoaras T, Tocque B, Rey I, Chassignol M, Thuong NT, Helene C. Short modified antisense oligonucleotides directed against Ha-*RAS* point mutation induce selective cleavage of the mRNA and inhibit T24 cells proliferation. *EMBO J.* 1991;**10**:1111-1118.

Sameron A, Ahmad TB, Carlile GW, Pappin D, Narsimhan RP, Ley SC. Activation of MEK-1 and SEK-2 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase. *EMBO J.* 1996;**15**:817-826.

Sawyers CL & Denny CT. Chronic myelomonocytic leukemia: Tel-a-kinase what ets all about. *Cell.* 1994;**77**:171-173.

Schaeffer HJ & Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol.* 1999;**19**:2435-2444.

Schaich M, Ritter M, Illmer T, Lisske P, Thiede C, Schakel U, Mohr B, Ehninger G, Neubauer A. Mutations in ras proto-oncogenes are associated with lower mdr1 gene expression in adult acute myeloid leukaemia. *Br J Haematol.* 2001;**112**:300-307.

Schlessinger J. How receptor tyrosine kinases activate Ras. *Trends Biol Sci.* 1993;**18**:273-275.

Schulte TW, Blagosklonny MV, Romanova L, Mushinski JF, Monia BP, Johnston JF, Nguyen P, Trepel J, Neckers LM. Destabilization of Raf-1 by geldanamycin leads to disruption of the Raf-1-MEK-mitogen–activated protein kinase signalling pathway. *Mol Cell Biol.* 1996;**16**:5839-5845.

Sekizawa R, Iinuma H, Naganawa H, Hamada M, Takeuchi T, Yamaizumi J, Umezawa K. Isolation of novel saquayamycins as inhibitors of farnesyl-protein transferase. *J Antibiot Tokyo.* 1996;**49**:487-490.

Senn HP, Tran-Thang C, Wodnar-Filipowicz A, Jiricny J, Fopp M, Gratwohl A, Signer E, Weber W, Moroni C. Mutational analysis of the N-*RAS* proto-oncogene in active and remission phase acute leukemias. *Int J Cancer.* 1988;**41**:59-64.

Sepp-Lorenzino L, Ma Z, Rands E, Kohl NE, Gibbs JB, Oliff A, Rosen N. A peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and -independent growth of human tumor cell lines. *Cancer Res.* 1995;**55**:5302-5309.

Sepp-Lorenzino L & Rosen N. A farnesyl-protein transferase inhibitor induces p21 expression and G1 block in p53 wild type tumor cells. *J Biol Chem.* 1998;**273**:20243-20251.

Shannon KM, O'Connell P, Martin GA, Paderanga D, Olson K, Dinndorf P, McCormick F. Loss of the normal NF-1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders. *N Engl J Med.* 1994;**330**:597-601.

Shapiro PS, Vaisberg E, Hunt AJ, Tolwinski NS, Whalen AM, McIntosh JR, Ahn NG. Activation of the MKK/ERK pathway during somatic cell mitosis: direct interactions of active ERK with kinetochores and regulation of the mitotic 3T3/2 phosphoantigen. *J Cell Biol.* 1998;**142**:1533-1545.

Shapiro PS, Whalen AM, Tolwinski NS, Wilsbacher J, Froelich-Ammon SJ, Garcia M, Osheroff N, Ahn NG. Extracellular signal-regulated kinase activates topoisomerase IIa through a mechanism independent of phosphorylation. *Mol Cell Biol.* 1999;**19**:3551-3560.

Shindo-Okado N, Makabe O, Nagahara H, Nishimura S. Permanent conversion of mouse and human cells transformed by activated ras or raf genes to apparently normal cells by treatment with the antibiotic azatyrosine. *Mol Carcinog.* 1989;**2**:159-167.

Shirasawa S, Furuse M, Yokoyama N, Sasazuki T. Altered growth of human colon cancer cell lines disrupted at activated Ki-Ras. *Science.* 1993;**260**:85-88.

Side L, Taylor B, Cayouette M, Conner E, Thompson P, Luce M, Shannon K. Homozygous inactivation of the NF-1 gene in bone marrow cells from children with neurofibromatosis type 1 and malignant myeloid disorders. *N Engl J Med.* 1997;**336**:1713-1720.

Silverman KC, Cascales C, Genilloud O, Sigmund JM, Gartner SE, Koch GE, Gagliardi MM, Heimbuch BK, Nallin-Omstead M, Sanchez M, Diez MT, Martin I, Garrity GM, Hirsch CF, Gibbs JB,Singh SB, Lingham RB. Actinoplanic acids A and B as novel inhibitors of farnesyl-protein transferase. *Appl Microbiol Biotechnol.* 1995;**43**:610-616.

Silverman KC, Jayasuriya H, Cascales C, Vilella D, Bills GF, Jenkins RG, Singh SB, Lingham RB. Oreganic acid, a potent inhibitor of Ras farnesyl-protein transferase. *Biochem Biophys Res Comm.* 1997;**232**:478-481.

Sinensky M, Fantle K, Dalton M. An antibody which specifically recognizes prelamin A but not mature lamin A: application to detection of blocks in farnesylation-dependent protein processing. *Cancer Res.* 1994;**54**:3229-3232.

Sinensky M. Recent advances in the study of prenylated proteins. *Biochim Biophys Acta.* 2000;**1484**:93-106.

Sonoyama J, Matsumura I, Ezoe S, Satoh Y, Zhang X, Kataoka Y, Takai E, Mizuki M, Machii T, Wakao H, Kanakura Y. Functional cooperation among Ras, STAT5, and phosphatidylinositol 3-kinase is required for full oncogenic activities of BCR/ABL in K562 cells. *J Biol Chem.* 2002;**277**:8076-8082.

Sprang SR. G protein mechanisms: Insights from structural analysis. *Ann Rev Biochem.* 1997;**66**:639-678.

Stebbins CE, Russo AA, Schneider C, Rosen N, Hartl FU, Pavletich NP. Crystal structure of an HSP90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent. *Cell.* 1997;**89**:239-250.

Stiller CA, Chessells JM, Fitchett M. Neurofibromatosis and childhood leukemia/lymphoma: a population based UKCCSG study. *Brit J Cancer.* 1994;**70**:969-972.

Stirewalt DL, Kopecky KJ, Meshinchi S, Appelbaum FR, Slovak ML, Willman CL, Radich JP. FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood.* 2001;**97**:3589-3595.

Strumberg D *et al.* Phase I and pharmacokinetic study of the Raf kinase inhibitor Bay 43-9006 in patients with locally advanced or metastatic cancer. *Proc. Amer. Assoc. Cancer Res.* 2001;**42**:2921.

Sturgill TW & Ray LB. Muscle proteins related to microtubule associated protein-2 are substrates for an insulin-stimulatable kinase. *Biochem Biophys Res Commun.* 1986;**134**:565-571.

Sturm S, Gil RR, Chai HB, Ngassapa OD, Santisuk T, Reutrakul V, Howe A, Moss M, Besterman JM, Yang SL, Farthing JE, Tait RM, Lewis JA, O'Neill MJ, Farnsworth NR, Cordell GA, Pezzuto JM, Kinghorn AD. Lupane derivatives from *Lophopetalum wallichi* with farnesyl protein transferase inhibitory activity. *J Nat Prod.* 1996;**59**:658-663.

Sun J, Blaskovich MA, Knowles D, Qian Y, Ohkanda J, Bailey R D, Hamilton A D, Sebti SM. Antitumor efficacy of a novel class of non-thiol-containing peptidomimetic inhibitors of farnesyltransferase and geranylgeranyltransferase I: combination therapy with the cytotoxic agents cisplatin, Taxol, and gemcitabine. *Cancer Res.* 1999 ;**59**: 4919-4926.

Sun J, Qian Y, Hamilton AD, Sebti SM. Both farnesyltransferase and geranylgeranyltransferase I inhibitors are required for inhibition of oncogenic K-Ras prenylation but each alone is sufficient to suppress human tumor growth in nude mouse xenografts. *Oncogene.* 1998;**16**:1467-1473.

Sun J, Qian Y, Hamilton AD, Sebti SM. Ras CAAX peptidomimetic FTI-276 selectively blocks tumor growth in nude mice of a human lung carcinoma with K-Ras mutation and p53 deletion. *Cancer Res.* 1995;**55**:4243-4247.

Sweet RW, Yokoyama S, Kamata T, Fermisco JR, Rosenberg M, Gross M. The product of ras is a GTPase and the T24 oncogenic mutant is deficient in this activity. *Nature.* 1984;**311**:273-275.

Tamanoi F, Gau C-L, Jiang C, Edamatsu H, Kato-Stankiewicz J. Protein farnesylation in mammalian cells: effects of farnesyltransferase inhibitors on cancer cells. *Cell Mol Life Sci.* 2001;**58**:1636-1649.

Tamanoi F. Inhibitors of Ras farnesyltransferases. *Trends Biol Sci.* 1993;**18**:349-353.

Tanaka K, Takechi M, Asaoku H, Dohy H, Kamada N. A high frequency of N-Ras oncogene mutations in multiple myeloma. *Int J Hematol.* 1992;**56**:119-127.

Taylor JT & Shalloway D. Cell cycle-dependent activation of Ras. *Curr Biol.* 1996;**6**:1621-1627.

Taylor SJ, Resnick RJ, Shalloway D. Nonradioactive determination of Ras-GTP levels using activated ras interaction assay. *Methods Enzymol.* 2001;**333**:333-342.

Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U, Wermke M, Bornhauser M, Ritter M, Neubauer A, Ehninger G, Illmer T. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood*. 2002;**99**:4326-4335.

Tobal K, Pagliuca A, Bhatt B, Bailey N, Layton DM, Mufti GJ. Mutation of the human FMS gene (M-CSF receptor) in myelodysplastic syndromes and acute myeloid leukemia. *Leukemia.* 1990;**4**:486-489.

Toksoz D, Farr CJ, Marshall CJ. Ras genes and acute myeloid leukemia. *Br J Haematol.* 1989;**71**:1-6.

Tolwinski NS, Shapiro PS, Goueli S, Ahn NG. Nuclear localization of mitogen-activated protein kinase kinase 1 (MKK1) is promoted by serum stimulation and G2/M progression. *J Biol Chem.* 1999;**274**:6168-6174.

Towatari M, Iida H, Tanimoto M, Iwata H, Hamaguchi M, Saito H. Constitutive activation of mitogen-activated protein kinase pathway in acute leukemia cells. *Leukemia.* 1997;**11**:479-484.

Treisman R. Regulation of transcription by MAP kinase cascades. *Curr Opin Cell Biol.* 1996;**8**:205-215.

Trueblood CE, Boyartchuk VL, Rine J. Substrate specificity determinants in the farnesyltransferase ß-subunit. *Proc Natl Acad Sci USA.* 1997;**94**:10774-10779.

Trueblood CE, Ohya Y, Rine J. Genetic evidence for in vivo cross-specificity of the CAAX-box protein prenyltransferases farnesyltransferase and geranylgeranyltransferase I in Saccharomyces cerevisiae. *Mol Cell Biol.* 1993;**13**:4260-4275.

Tsuda M, Muraoka Y, Takeuchi T, Sekizawa R, Umezawa K. Stereospecific synthesis of a novel farnesyl protein transferase inhibitor, valinoctin and its analogues. *J Antibiot Toyko.* 1996;**49**:1031-1035.

Umanoff, Edelmann W, Pellicer A, Kucherlapati R. The murine N-ras gene is not essential for growth and development. *Proc Natl Acad Sci USA.* 1995;**92**:1709-1713.

Van Aelst L, White M, Wigler M.H. Ras partners. *Cold Spring Harbor Symp Quant Biol.* 1994;**59**:181-186.

Van der Pyl D, Cans P, Debernard JJ, Herman F, Lelievre Y, Tahraoui L, Vuilhorgne M, Leboul J. RPR113228, a novel farnesyl-protein transferase inhibitor produced by *Chrysosporium lobatum*. *J Antibiot Tokyo.* 1995;**48**:736-737.

Vasudevan A, Qian Y, Vogt A, Blaskovich MA, Ohkanda J, Sebti SM, Hamilton AD. Potent, highly selective, and non-thiol inhibitors of protein geranylgeranyltransferase-I. *J Med Chem.* 1999;**42**:1333-1340.

Verhuel HM, Panigrahy D, Yuan J, D'Amato RJ. Combination oral antiangiogenic therapy with thalidomide and sulindac inhibits tumour growth in rabbits. *Br J Cancer.* 1999;**79**: 114-118.

Vogelstein B, Civin CI, Preisinger AC, Krischer JP, Steuber P, Ravindranath Y, Weinstein H, Elfferich P, Bos J. Ras gene mutations in childhood acute myeloid leukemia: a pediatric oncology group study. *Genes Chrom Canc.* 1990;**2**:159-162.

Vogt A, Qian Y, McGuire TF, Hamilton AD, Sebti SM. Protein geranylgeranylation, not farnesylation, is required for the G1 to S phase transition in mouse fibroblasts. *Oncogene.* 1996;**13**:1991-1999.

Vogt A, Sun J, Qian Y, Hamilton AD, Sebti SM. The geranylgeranyltransferase-I inhibitor GGTI-298 arrests human tumor cells in G0/G1 and induces p21(WAF1/CIP1/SDI1) in a p53-independent manner. *J Biol Chem.* 1997;**272**:27224-27229.

Volker C, Miller RA, McCleary WR, Rao A, Poenie M, Backer JM, Stock JB. Effects of farnesylcysteine analogs on protein carboxyl methylation and signal transduction. *J Biol Chem.* 1991;**266**:21515-21522.

Voss J, Posern G, Hannemann JR, Wiedemann LM, Turhan AG, Poirel H, Bernard OA, Adermann K, Kardinal C, Feller SM. The leukaemic oncoproteins BCR-Abl and Tel-Abl (ETV6/Abl) have altered substrate preferences and activate similar intracellular signaling pathways. *Oncogene.* 2000;**19**:1684-1690.

Waggott W, Lo YM, Bastard C, Gatter KC, Leroux D, Mason DY, Boultwood J, Wainscoat JS. Detection of NPM-ALK DNA rearrangement in CD30 positive anaplastic large cell lymphoma. *Br J Haematol.* 1995;**89**:905-907.

Walsh AB, Dhanasekaran M, Bar-Sagi D, Kumar CC. SCH51344-induced reversal of Ras-transformation is accompanied by the specific inhibition of the Ras and Rac-dependent cell morphology pathway. *Oncogene.* 1997;**15**:2553-2560.

Ward Y, Wang W, Woodhouse E, Linnoila I, Liotta L, Kelly K. Signal pathways which promote invasion and metastasis: critical and distinct contributions of extracellular signal-regulated kinase and Ral-specific guanine exchange factor pathways. *Mol Cell Biol.* 2001;**21**:5958-5969.

Whyte DB, Kirschmeier P, Hockenberry TN, Nunez-Oliva I, James L, Catino JJ, Bishop WR, Pai JK. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. *J Biol Chem.* 1997;**272**:14459-14464.

Williams DH, Wilkinson SE, Purton T, Lamont A, Flotow H, Murray EJ. Ro 09-2210 exhibits potent anti-proliferative effects on activated T-cells by selectively blocking MKK activity. *Biochemistry.* 1998;**37**:9579-9585.

Winston JT, Coats SR, Wang Y-Z, Pledger WJ. Regulation of the cell cycle machinery by oncogenic ras. *Oncogene.* 1996;**12**:127-134.

Wittinghofer A. Signal transduction via Ras. *Biol Chem.* 1998;**379**:933-937.

Woessner JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB.* 1991;**5**:2145.

Wong B, Lumma WC, Smith AM, Sisko JT, Wright SD and Cai TQ. Statins suppress THP-1 cell migration and secretion of matrix metalloproteinase 9 by inhibiting geranylgeranylation. *J Leukoc Biol.* 2001;**69**:959-962.

Wu J, Harrison JK, Dent P, Lynch KR, Weber MJ, Sturgill TW. Identification and characterization of a new mammalian mitogen-activated protein kinase kinase, MKK2. *Mol Cell Biol.* 1993;**8**:4539-4548.

Xing J, Ginty DD, Greenberg ME. Coupling of the Ras-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science.* 1996;**273**:959-963.

Xu G, O'Connell P, Viskochil D, Cawthon R, Robertson M, Culver M, Dunn D, Stevens J, Gesteland R, White R, et al. The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell.* 1990;**62**:599-608.

Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Saito K, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Saito H, Ueda R, Ohno R, Naoe T. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood.* 2001;**97**:2434-2439.

Yanagihara K, Nii M, Tsumuraya M, Numoto M, Seito T, Seyama T. A radiation-induced murine ovarian granulosa cell tumor line: introduction of v-ras gene potentiates a high metastatic ability. *Jpn J Cancer Res.* 1995;**86**:347-356.

Yang W, Tabancay AP, Urano J, Tamanoi F. Failure to farnesylate Rheb protein contributes to the enrichment of G0/G1 phase cells in the Schizosaccharomyces pombe farnesyltransferase mutant. *Mol Microbiol.* 2001;**41**:1339-1347.

Yokoyama K, Goodwin GW, Ghomashchi F, Glomaset JA, Gelb MH. A protein geranylgeranyltransferase from bovine brain: implications for protein prenylation specificity. *Proc Natl Acad Sci USA.* 1991;**88**:5302-5306.

Yonemoto M, Satoh T, Arakawa H, Suzuki-Takahashi I, Monden Y, Kodera T, Tanaka K, Aoyama T, Iwasawa Y, Kamei T, Nishimura S, Tomimoto K. J-104,871, a novel farnesyltransferase inhibitor, blocks Ras farnesylation *in vivo* in a farnesyl pyrophosphate-competitive manner. *Mol Pharmacol.* 1998;**54**:1-7.

Zecevic M, Catling AD, Eblen ST, Renzi L, Hittle JC, Yen TJ, Gorbsky GJ, Weber MJ. Active MAP kinase in mitosis: localization at kinetochores and association with the motor protein CENP-E. *J Cell Biol.* 1998;**142**:1547-1558.

Zhang FL & Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. *Annu Rev Biochem.* 1996;**65**:241-269.

Zhang FL, Kirschmeier P, Carr D, James L, Bond RW, Wang L, Patton R, Windsor WT, Syto R, Zhang R, Bishop WR. Characterization of Ha-ras, N-ras, Ki-Ras4A, and Ki-

Ras4B as in vitro substrates for farnesyl protein transferase and geranylgeranyl protein transferase type I. *J Biol Chem.* 1997;**272**:10232-10239.

Zheng C-F & Guan K-L. Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. *J Biol Chem.* 1993;**268**:11435-11439.

Zou X & Calame K. Signaling pathways activated by oncogenic forms of Abl tyrosine kinase. *J Biol Chem.* 1999;**274**:18141-18144.

Zujewski J, Horak ID, Bol CJ Woestenborghs R, Bowden C, End DW, Piotrovsky VK, Chiao J, Belly RT, Todd A, Kopp WC, Kohler DR, Chow C, Noone M, Hakim FT, Larkin G, Gress RE, Nussenblatt RB, Kremer AB, Cowan KH. Phase I and pharmacokinetic study of farnesyl protein transferase inhibitor R115777 in advanced cancer. *J Clin Oncol.* 2000;**18**:927-941.

6. Abbreviations

A	Adenine
7-AAD	7-Amino-actinomycin D
Ala	Alanine
AML	Acute myelogenous leukemia
Asp	Aspartic acid
ATF-1	Transcription factor that binds to the cyclic-AMP response element
ATP	Adenosine 5´-triphosphate
BSA	Bovine serum albumin
C	Cytosine
CAAX-box	peptide recognition motif for prenylation (C, cysteine; A, aliphatic amino acid; and X, any amino acid)
CML	Chronic myelogenous leukemia
cpm	counts per minute
CREB	Transcription factor that binds to the cyclic-AMP response element
C-terminal	Carboxy-terminal
Cys	Cysteine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide 5´-triphosphates
DTT	Dithiothreitol
EDTA	Ethylene diamine-tetra-acetic acid
ERK	Extracellular regulated kinase (or MAPK)
FCS	Fetal calf serum
FPP	Farnesylpyrophosphate
FTase	Farnesyltransferase
FTI	Farnesyltransferase inhibitor
G	Guanine
g	Gravitational force
GGTase	Geranylgeranyltransferase
GGTI	Geranylgeranyltransferase inhibitor
Glu	Glutamic acid
Gly	Glycine
G-protein	GTP-binding protein
GST-RBD	Ras-binding domain of c-Raf-1 fused to glutathione
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphate phosphatase
GTP-Ras	GTP-bound Ras
h	Hour
H-Ras	Harvey-Ras
IC_{50}	concentration at which 50 % of cells are affected
IgG	Immunoglobulin G
IL	Interleukin
IP	Immunoprecipitation
K-Ras	Kirsten-Ras
L-61-H-Ras	H-Ras activated by mutation to harbor leucine at amino acid position 61
LB Medium	Luria-Bertani medium
Leu	Leucine
M	Molarity
MAPK	Mitogen activated protein kinase (or ERK)

MBP	Myelin basic protein
MEK	MAPK/ERK kinase
min	Minute
N-Ras	Neuronal-Ras
N-terminal	Amino-terminal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PP-ERK-1/2	Diphospho-ERK-1/2
PP-MEK-1/2	Diphospho-MEK-1/2
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T	Thymidine
TAE	Tris-acetate/EDTA electrophoresis buffer
TBE	Tris-borate/EDTA electrophoresis buffer
TE	Tris/EDTA
TEMED	N,N,N´,N´-tetramethylethylenediamine
UV	Ultraviolet
V-12-H-Ras	H-Ras activated by mutation to harbor valine at amino acid position 12

7. Abstract

Ras proteins are prototypical G-proteins that have been shown to play a key role in signal transduction, proliferation and malignant transformation. Ras proteins are produced as cytoplasmatic precursor proteins and require several post-translational modifications to acquire full biological activity. *Ras* mutations are frequently observed in myelodysplastic syndromes (MDS), acute myeloid leukemias (AML), juvenile myelomonocytic myeloid leukemia (JMML) and in multiple myelomas. The importance of deregulation of Ras signaling in the molecular pathogenesis of myeloid leukemias is further underscored by the positioning of several oncogene and tumor suppressor gene products on this pathway. Based on the wealth of data reporting the effectiveness of Ras signaling inhibitors against human carcinomas harboring activated Ras, coupled with the implications of Ras in the pathophysiology of myeloid leukemias, the role of activated Ras signaling and the effects of these inhibitors on leukemia cell growth were investigated.

Activation of the Ras-to-MAPK cascade in 14 AML and 5 CML cell lines was examined and correlated with the effects of a panel of 10 Ras signaling inhibitors on cell viability, colony formation, cell cycle progression and induction of apoptosis. Activation of MEK, MAPK and the transcription factors CREB-1, ATF-1 and c-Myc was observed in the majority of the cell lines (9/14 AML and 2/5 CML cell lines). Activating *Ras* mutations were found in 4 of the 14 AML cell lines (28.6%), including one "non-hot spot" K-*Ras* mutation in codon 18. While activation of the MAPK cascade did not always correlate with the presence of activating *Ras* mutations or BCR-Abl, activation was found to be linked to the G_0/G_1 and G_2/M phases of the cell cycle. In contrast to most inhibitors (e.g. B581, Cys-4-Abs-Met, FPT-2, FTI-276, and FTS), significant growth inhibition was only observed for FTI L-744,832 (16/16), FTI-277 (19/19), FPT-3 (10/19), and the MEK inhibitors U0126 (19/19) and PD098059 (8/19). Treatment of NB-4 cells with FTI-277 or FTI L-744,832 primarily resulted in a G_2/M block, whereas FPT-3 and U0126 treatment lead to induction of apoptosis.

Since alternative geranylgeranylation of K-Ras and N-Ras in the presence of farnesyl transferase inhibitors (FTIs) may represent an important mechanism of FTI-resistance, six geranylgeranyl transferase-I inhibitors (GGTIs) were screened alone and in combination with FTI for growth inhibition of myeloid leukemia cell lines and primary AML blasts from patients. Significant growth inhibition (>70%) in cell lines was observed for GGTI-286 (9/19), GGTI-298 (14/19) and GGTI-2147 (16/19), whereas GGTI-287, GGTI-297 and GGTI-2133 were less effective. GGTI treatment of NB-4 cells resulted in accumulation of cells in G_0/G_1 and induction of apoptosis. In all cases, FTI/GGTI co-treatment led to

synergistic cytotoxic effects in both myeloid cell lines (5/5) and primary AML cells (6/6). This synergy coincided with increased apoptosis. FTI /GGTI co-treatment seemed to have little additional effect on processing of H-Ras, Rap2A, Rheb, and Lamins A-C. However, accumulation of unprocessed N-Ras was induced in all cell lines tested and led to increased inactive N-Ras-Raf complexes. The results presented here suggest that molecular targeting of both FTase and GGTase I may lead to higher efficacy in the treatment of myeloid leukemias by overcoming the partial resistance of N-Ras and possibly K-Ras to FTI monotherapy.

8. Zusammenfassung

Ras-Proteine sind klassische G-Proteine, welche eine Schlüsselrolle in der Signaltransduktion, Proliferation und malignen Transformation einnehmen. Sie werden als zytoplasmatische Vorläuferproteine produziert und benötigen einige posttranslationale Modifikationen, um volle biologische Aktivität zu entfalten. Ras-Mutationen werden unter anderem gehäuft in Myelodysplastischen Syndromen (MDS), akuten myeloischen Leukämien (AML), juvenilen myelomonzytären Leukämien (JMML) und in Multiplen Myelomen beobachtet. Die Bedeutung der Deregulierung der Ras-Signaltransduktion für die molekulare Pathogenese von myeloischen Leukämien wird durch die Positionierung einiger Onkogene und Tumorsuppressorgene auf diesem Signalweg unterstrichen. Aufgrund der vielen Berichte bezüglich der Effektivität von Inhibitoren der Ras-Signaltransduktion gegenüber menschlichen Tumoren, die aktiviertes Ras besitzen, und aufgrund der Bedeutung von Ras in der Pathophysiologie von myeloischen Leukämien, wurde in dieser Arbeit die Rolle einer aktivierten Ras-Signaltransduktion und der Effekt dieser Inhibitoren auf das Wachstum von myeloischen Leukämiezellen analysiert.

Die Aktivierung der Ras-MAPK-Kaskade wurde in 14 AML- und in 5 CML-Zellinien untersucht und mit den Effekten von 10 Inhibitoren der Ras-Signaltransduktion auf Zellviabilität, Kolonieformation, Zellzyklusprogression und Apoptoseinduktion korreliert. Eine Aktivierung von MEK, MAPK und den Transskriptionsfaktoren CREB-1, ATF-1 und c-Myc wurde in der Mehrheit der Zellinien beobachtet (9/14 AML und 2/5 CML). Aktivierende Ras-Mutationen wurden in 4 dieser 14 AML-Zellinien (28,6%) festgestellt, inklusive einer „non-hot spot“-Mutation von K-Ras in Kodon 18. Während die Aktivierung der MAPK-Kaskade nicht immer mit der Anwesenheit von Ras-Mutationen korrelierte, war die Aktivierung dieser Kaskade mit der G0/G1- und G2/M-Phase des Zellzyklus verknüpft. Im Gegensatz zu den meisten Inhibitoren (B581, Cys-4-Abs-Met, FPT-2, FTI-276 und FTS) wurde eine signifikante Inhibition des Leukämiezellwachstums nur für die Farnesyltransferase-Inhibitoren (FTI) L-744,832 (16/16), FTI-277 (19/19), FPT-3 (10/19) und die MEK-Inhibitoren U0126 (19/19) und PD098059 (8/19) beobachtet. Inkubation von NB-4-Zellen mit FTI-277 oder FTI L-744,832 führte hauptsächlich zu einem G2/M-Block, während die Behandlung der Zellen mit FPT-3 und U0126 Apoptose induzierte.

Da die alternative Geranylgeranylierung von K-Ras und N-Ras in Anwesenheit von FTI ein wichtiger Mechanismus der FTI-Resistenz sein könnte, wurden sechs Geranylgeranyltransferase-I-Inhibitoren (GGTI) bezüglich der Wachstumsinhibition von myeloischen Leukämiezellinien und primären AML-Zellen untersucht. Eine signifikante

Wachstumsinhibition wurde für GGTI-286 (9/19 Zellinien), GGTI-298 (14/19) und GGTI-2147 (16/19) beobachtet, während GGTI-287, GGTI-297 und GGTI-2133 kaum Wachstumshemmung induzierten. Die Inkubation von NB-4-Zellen mit GGTI führte zur Akkumulation der Zellen in der G0/G1 Phase und zur Apoptoseinduktion. FTI/GGTI Coinkubation führte sowohl in allen myeloischen Zellinien als auch in allen untersuchten primären AML-Zellen zu synergistischen zytotoxischen Effekten. Diese Synergie ging mit einer gesteigerten Apoptose einher. Die Kombination von FTI und GGTI hatte kaum zusätzliche Effekte auf die Prozessierung von H-Ras, Rap2A, Rheb und den Laminen A-C, führte aber zur zytoplasmatischen Akkumulation von nicht-prozessiertem N-Ras und von inaktiven N-Ras-Raf-Komplexen. Die hier präsentierten Daten lassen vermuten, daß FTase und GGTase I molekulare Zielstrukturen für eine Leukämietherapie sein könnten. Die Kombination von FTI und GGTI könnte durch Überwindung der partiellen Resistenz von K-Ras und N-Ras zu einem besseren Ansprechen in der Behandlung myeloischer Leukämien führen.

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Publications

Morgan MA and Hecht SM (1994). Iron (II) bleomycin-mediated degradation of a DNA-RNA heteroduplex. *Biochemistry*; **33**: 10286-10293.

Morgan MA, Kazakov SA and Hecht SM (1995). Phosphoryl migration during the chemical synthesis of RNA. *Nucleic Acids Research*; **23(19)**: 3949-3953.

Reuter CWM, **Morgan MA** and Bergmann L (2000). Targeting the Ras signaling pathway: A rational, mechanism-based treatment for hematological malignancies? *Blood*; **96**: 1655-1669.

Morgan MA, Dolp O and Reuter CWM (2001). Cell-cycle-dependent activation of mitogen-activated protein kinase kinase (MEK-1/2) in myeloid leukemia cell lines and induction of growth inhibition and apoptosis by inhibitors of RAS signaling. *Blood*; **97**: 1823-1834.

Morgan MA, Wegner J, Aydilek E, Ganser A and Reuter CWM (2002). Synergistic cytotoxic effects in myeloid leukemia cells upon co-treatment with farnesyltransferase and geranylgeranyl transferase-I inhibitors. Submitted to *Leukemia*.

Book Article

Reuter CWM, **Morgan MA**, Bergmann L (2001). Effect of mutationally activated Ras on the Ras to MAP kinase signaling pathway and growth inhibition of myeloid leukemia cells by inhibitors of the MAP kinase cascade. In: *Acute Leukemias VIII: Prognostic factors and treatment strategies.* (p. 100-108) Eds. Büchner T, Hiddemann W, Wörmann B, Schellong G, Ritter J, Creutzig U. Springer Verlag, Berlin, Heidelberg, New York, Tokyo.

Abstracts

Morgan MA, Dolp O, Bergmann L, Reuter CWM (1999). Targeting the Ras signaling pathway in myeloid leukemia cells: a potential rational approach for treatment. *Blood* **94** (Supplement 1); Abstract 277, p. 64a.

Reuter CWM, **Morgan MA**, Dolp O, Bergmann L (1999). Potential effectiveness of inhibitors of Ras signaling in the treatment of multiple myeloma. *Blood* **94** (Supplement 1); Abstract 1263, p. 282a.

Morgan MA, Bergmann L, Reuter C (1999). Potential role of inhibitors of Ras signaling in the treatment of myeloid leukemias. *Onkologie* **22** (Supplement 1) ; Abstract 335, p. 92.

Morgan MA, Bergmann L, Reuter CWM (2000). Activation of Ras and effect of inhibitors targeting Ras signaling in myeloid leukemia. *Int. J. Hematology* **72** (Supplement 1); Abstract 16011, p. 28.

Morgan MA, Dolp O, Reuter CWM (2000). Cell-cycle dependent activation of the Ras-to-MAPK pathway in myeloid leukemia and effect of inhibitors of Ras signaling on leukemia cell growth. *Onkologie* **23** (Sonderheft 7); Abstract 0074, p. 22.

Morgan MA, Dolp O, Ganser A and Reuter CWM (2001). Cell-cycle dependent activation of MAP Kinase Kinase (MEK) in myeloid leukemia cells. *The Hematology Journal* **1** (Supplement 1); Abstract 315, p. 90.

Reuter CWM, **Morgan MA**, Wegner J, Dolp O and Ganser A (2001). Induction of growth inhibition and apoptosis by inhibitors of the Ras-to-MAPK pathway in myeloid leukemia cell lines. *The Hematology Journal* **1** (Supplement 1); Abstract 354, p. 102.

Morgan MA, Wegner J, Ganser A and Reuter CWM (2001). Effects of FTase and GGTase inhibitors in multiple myeloma cells. *Blood* **98**; Abstract 4957, p. 302B.

Reuter CWM, **Morgan MA**, Wegner J and Ganser A (2001). Induction of growth inhibition and apotosis by FTase and GGTase inhibitors in myeloid leukemia cell lines. *Blood* **98**; Abstract 432, p. 103A.

Morgan MA, Wegner J, Ganser A and Reuter CWM (2002). Induction of growth inhibition, apoptosis and accumulation of unprocessed N-Ras by co-treatment of myeloid leukemia cells with farnesyltransferase and geranylgeranyltransferase-I inhibitors.