ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 May；21 (5)：396 – 404
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Ras farnesyltransferase inhibition：a novel and safe approach for cancer chemotherapy

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KEY WORDS proto-oncogene protein p21 (ras)；dimethylallyltranstransferase；protein isoprenylation；Ca^{2+}-calmodulin dependent protein kinase；antineoplastic agents；signal transduction

ABSTRACT

The 21-kDa Ras proteins are well known for their regulatory role in oncogenic，mitogenic，and developmental signaling pathways. GTP activated Ras interacts directly with the Raf protein to recruit the MAP kinases and their subordinates. Attachment of Ras protein to the plasma membrane that requires farnesylation by farnesyl pyrophosphate at its C-terminus，is essential for its biological activity. Ras oncogenes are associated with a wide variety of solid tumors and leukemias for which existing chemotherapeutics have limited utility. A promising pharmacological approach of antagonizing oncogenic Ras activity is to develop inhibitors of farnesyl transferase. These inhibitors may be useful in blocking the action of Ras onco-proteins.

INTRODUCTION

The application of modern molecular genetics to the study of cancer biology has produced enormous advances to understand the principles underlying mammalian cell transformation. The evidence from the limited capacity of existing agents to cure or significantly prolong the survival of patients with disseminated tumors or certain leukemias shows that there is much need for new chemotherapeutic agents in cancer. The present knowledge on the molecular genetic defects underlying tumorigenesis and the biological complexities of cancer has afforded new opportunities for cancer drug discovery and development. To understand the difference between the proliferation of normal cells and cancer cells，one needs to consider the cell cycle of dividing cells. A dividing cell starts in G1 Phase，where the necessary enzymes are synthesized and tools up for DNA synthesis. A restriction or check-point is present before DNA replication. Growth factors provide progression signals and are necessary for the cycle to progress beyond the checkpoint. Inhibitory feed back controls hold up DNA synthesis if there is DNA damage and allows time for repair，failure in it leads to apoptosis.

A normal cell turns into a cancer cell because of alteration in its DNA. The two main categories of genetic change that lead to cancer are inactivation of tumor suppressor gene and activation of proto-oncogenes to oncogenes. Gene p53，a cellular phosphonic protein of molecular weight 53 000 has been identified as a tumor suppressor gene. If the DNA is damaged，p53 gene products accumulate and arrest DNA replication at the checkpoint. Cells in which p53 is altered by mutation or binding to viral or altered host proteins，cannot stop the abnormal DNA replication leading to cancer. Proto-oncogenes，which are converted into active oncogenes not only by certain viruses but also as a result of point mutations or chromosomal translocations，are now known to be responsible for major forms of neoplasm. Among some of the most commonly occurring mutations in cancer are alterations of the Ras genes.

THE RAS GENE

A mammalian cell contains an estimated 50 – 100 different GTP binding proteins，which function as molecular switches in biological processes[1,2]. The Ras related super families of GTP-binding proteins are small 21 kDa proteins. The mammalian three Ras genes H-Ras，K-Ras，and N-Ras are collectively known as p21ras[3].

Ras is an essential component in the transduction of extracellular signals that induce proliferation and differentiation[4,5]. It is localized to the interface of the plasma

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Received 1999-09-06 Accepted 2000-01-24

membrane. Cycling between the active GTP bound form and inactive GDP bound form, p21ras serves as a molecular switch, interfacing between receptors and intracellular effected protein[3]. In normal cell signaling, inactivation of Ras occurs through hydrolysis of bound GTP to GDP by the protein intrinsic GTPase coupled with strong stimulation by GTPase activating protein (GAP)[6-8].

Specific point mutations at positions Gly 12, Gly 13, or Gln 61 render it oncogenic resulting in uncontrolled cell growth and morphological transformation of normal cells[9-11]. Mutated Ras genes have been found in 30 % of all human carcinomas, with much higher occurrences in pancreatic adenocarcinomas (90 %), colonic human tumors (50 %), lung cancers (30 %), and 20 % – 30 % in acute leukemias[12-17].

SIGNAL TRANSDUCTION PATHWAY

The first step in signal transduction is the binding of growth factors with receptors. This binding causes the activation of tyrosine kinase receptors by inducing dimerization and autophosphorylation. The Grb2 adapter molecule that is localized in the cytoplasm is bound through its SH3 domains to the carboxyl terminus of SOS. Upon ligand stimulation, Grb2 interacts through its SH2 domain with the phosphorylated tyrosine residues of the activated receptors. The translocation of the Grb2-SOS complex from the cytosol to the plasma membrane functions to draw SOS into proximity with the inactive Ras protein which is membrane bound[18,19]. SOS then activates Ras by promoting the release of GDP from the inactive Ras-GDP complex allowing free Ras to rebind GTP (Fig 1). When bound to GTP, the p21ras assumes an activated confirmation (amino terminus of Ras) which is necessary for the further transduction of the growth factor signal through MAP kinase cascade which relays signals into the cell[20,21].

Alternatively, Shc, an adapter protein, binds to the activated receptor and becomes phosphorylated. This Shc protein contains an SH2 domain at its carboxyl terminus, a central proline/glycine rich region that contains the major site of tyrosine phosphorylation at Tyr 317 and an amino terminus region. Tyr 317 forms a binding site for the SH2 domain of Grb2 that is inturn coupled with SOS through SH3 domains[22,23].

Ras then interacts with a protein kinase, Raf. Raf exists in 3 isoforms: A-Raf, B-Raf, and C-Raf 1[24]. Recent studies show that C-Raf 1 is the important

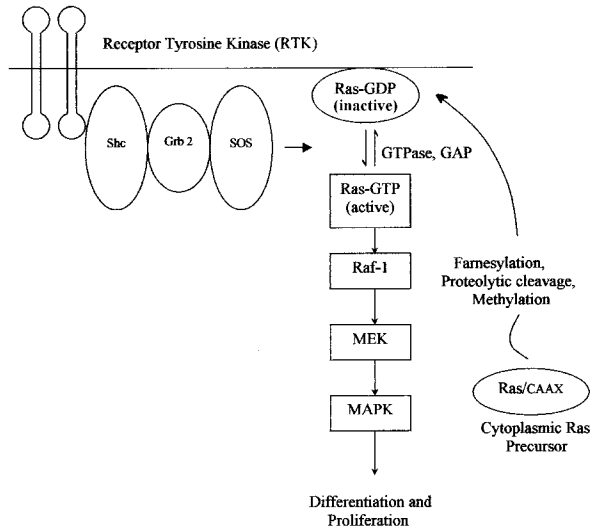

Fig 1. Model for receptor tyrosine kinase signal transduction through the Ras activated protein kinase cascade. Activation of the RTK leads to its phosphorylation of tyrosine residues. Shc binds to the activated receptor and becomes phosphorylated. Formation of the Shc-Grb2-SOS complex recruits SOS into proximity with the inactive Ras and activates it by catalyzing GDP/GTP exchange. Ras recruits Raf to the complex, allowing Raf activation and providing a means of activating the MAPK cascade.

signaling element down stream of Ras[25,26]. The A-Raf, B-Raf, and C-Raf 1 proteins share a common domain structure: the kinase catalytic domain occupies the carboxyterminal half of the polypeptide, where as the aminoterminal half contains two regions of high sequence conservation CR-1 and CR-2 which are regulatory[27]. All known Ras genes encode an identical stretch of nine amino acids in the amino terminal half (amino acids 32 – 40 in H-Ras) called the effecter loop that undergoes a major confirmation shift when Ras binds GTP[28,29]. GTP activated form of Ras binds to the regulatory region of Raf comprising amino acid residues 51 – 131 called the Ras Binding Domain (RBD)[30]. Activation of the kinase activity involves phosphorylation of serine or threonine residues on Raf.

Raf then activates another kinase called mitogen extracellular signal regulated kinase (MEK) which inturn stimulates to activate yet another protein kinase called MAP kinase[31,32]. It was studied that phosphorylation of either Ser 218 or Ser 222 was sufficient to activate MEK[33]. Phosphorylation of MAP kinase occurs on threonine and tyrosine residues and its activation is believed to convey growth and differentiation signals to the

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 May；21 (5)
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nucleus, resulting in the induction of appropriate gene expression[34-36].

Recent investigations using mutated Ha-ras HaCaT cell lines demonstrate that Ha-ras oncogenes also contribute to the progression of epidermal tumors through increased production of vascular endothelial growth factor (VEGF)[37,38]. As the mutant Ras gene becomes resistant to GTPase activity resulting in uncontrolled cell growth and morphological transformation of normal cells, inhibition of the oncogenic Ras activity is thought to be useful for anticancer therapy in tumors in which Ras plays a role.

FUNDAMENTALS OF PROTEIN PRENYLATION

In order to perform both its normal and oncogenic functions, the Ras protein must be membrane associated. The carboxyl terminus of the proteins of the Ras family consists of a tetrad referred to as a 'CAAX' box, where C is cysteine, A is aliphatic amino acid, and X is a member of limited set of amino acids.

Membrane localization of Ras is the result of a three-step post-translational modification of its CAAX box, the initial step when X = Met, Ser or Gln involves farnesylation of the cysteine by farnesylproteintransferase (FPTase), proteolytic cleavage of the three C-terminal amino acid residues, and methylation of the resulting C-terminal farnesyl cysteine[39]. Of these steps farnesylation appears to be the crucial step to convert Ras from a cytoplasmic inactive precursor protein into a fully matured membrane associated protein[40-42]. This implicates FPTase as a preferred target for interrupting oncogenic Ras signaling.

Prenylated proteins share characteristic C-terminal sequences including CAAX, XXCC, or XCXC[43]. Addition of either a 15 carbon (farnesyl) or 20 carbon (geranylgeranyl) isoprenoid to the cysteine residue, proteolytic cleavage of the AAX peptide and methylation of the new C-terminal carboxylate are shown in Fig 2[40,41]. Some proteins may also have a fourth modification, palmitolylation of one or two cysteine residues N-terminal to the farnesylated cysteine[43]. Proteins terminating with an XXCC or XCXC sequence are modified by geranyl-geranylation and do not require an endoproteolytic processing step[44].

Many proteins have been identified as substrates for prenylation[45-47]. Besides Ras proteins many of these other substrates have been shown to be oncogenic or have roles in mitogenic signaling[46-50].

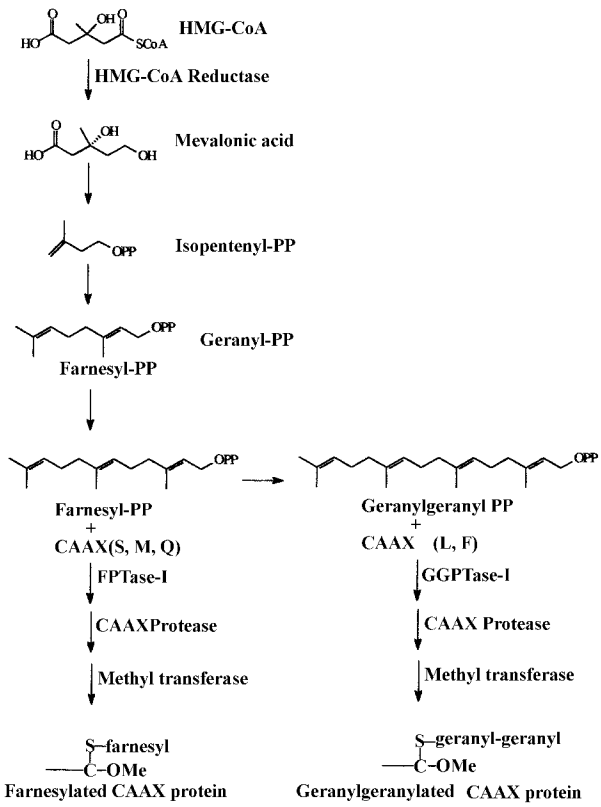

Fig 2. Post-translational modification of CAAX containing proteins. FPTase farnesylates CAAX substrates having C-terminal residues Ser, Met and Gln (S, M, and Q) GGPtase-I prefers CAAX substrates behaving a C-terminal Leu (L) or Phe (F). OPP: represents a pyrophosphate group.

ENZYME SYSTEMS

Three enzymes have been described that catalyze protein prenylation: farnesyl-proteintransferase (FPTase), geranyl-geranylproteintransferase type Ⅰ (GGPTase-Ⅰ) and geranyl-geranylproteintransferase type Ⅱ (GGPTase-Ⅱ). These enzymes are found in the soluble fraction of mammalian cells[40,41,44,51]. All three enzymes are Zn^{2+} metallo proteins and also require Mg^{2+} for activity[52]. Each of these enzymes selectively uses farnesyldiphosphate (FPP) or geranyl-geranyldiphosphate (GGPP) as the isoprenoid donor and selectively recognizes the protein substrate[53]. FPTase and GGPTase Ⅰ are α/β heterodimeric enzymes, that share a common α-subunit, the β-subunits are distinct, which are responsible for the differences in substrate selectivity seen for the two enzymes. FPTase consists of a 49-kDa alpha-subunit and a 46-kDa beta-subunit[54,55]. It was reported that

the phosphorylation of FPTase at the alpha and beta subunits plays a role in the regulation of farnesyltransferase activity *in vivo*[56].

FPTase farnesylates CAAX containing proteins that end most commonly with serine, metheonine, or glutamine. GGPTase-Ⅰ geranyl-geranylates CAAX containing proteins that usually end with leucine or phenylalanine. In addition to conferring specificity for FPTase or GGPTase-Ⅰ, the terminal residue of CAAX also influences affinity for the enzymes[57-60].

GGPTase-Ⅱ has different α and β subunits and complexes with a third component that presents the protein substrate to the α/β catalytic subunits[61]. GGPTase-Ⅱ modifies XXCC and XCXC proteins. Although these enzymes are highly selective for their respective substrates, the substrate specificities are not absolute. For example, FPTase can farnesylate CAAL containing proteins *in vitro*[62-65]. GGPTase can geranyl-geranylate normally farnesylated proteins such as K-RasB[66]. Moreover GGPTase can also farnesylate or geranyl-geranylate a single substrate[67]. However, recent investigation demonstrates that GGPTase-Ⅰ binds GGPP 300-fold tighter than FPP and that FPTase binds FPP 15-fold tighter than GGPP[68].

INHIBITORS OF FARNESYLTRANSFERASE

The greatest progress toward developing novel chemotherapeutics against Ras-induced cell transformation has centered on inhibiting the enzyme FPTase[69-74]. Several FPTase inhibitors have been discovered by random screening of natural products, chemical collections, and combinatorial libraries (Fig 3). The inhibitors can be subdivided into three broad categories: compounds competitive with FPP, compounds competitive with CAAX, and compounds competitive with both FPP and CAAX.

The first reported cell-active FPTase inhibitors that are competitive with the FPP substrate of FPTase are manumycin and L-704, 272. Manumycin was identified in culture medium of a strain of streptomyces using the yeast assay and L-704,722 was rationally designed from FPP[75-77]. Chaetomellic acids A and B produced by *Chaetomela acutiseta* and zaraagozic acid A were identified by the direct FPTase assay[76]. These are potent inhibitors of FPTase with IC_{50} values in the nmol range. The fungal products gliotoxin and acetylgliotoxin were identified as FPTase inhibitors by the direct FPTase assay[78]. Pepticinnamins, the peptide inhibitors of FPTase were identified in culture media of a Streptomyces strain using the direct FPTase assay[78]. Recent studies in activated H-Ras-transformed NIH3T3 cells have led to the identification of J-104,871, a novel FPTase inhibitor which is competitive with FPP[79]. A more recent similar study showed that the compound TR006 inhibited the farnesylation of Ras in a Ha-ras transformed cell lines of smooth muscle[80,81]. A noteworthy feature of the *in vitro* evaluation by Eumer *et al*. on farnesyl phosphonyl phosphinate, a diphosphate-modified derivative of farnesyldiphosphate is ended with a very potent FPTase inhibitor[82]. Although these compounds have shown biological activity, compounds competitive with FPP need to overcome the high avidity of FPTase for its substrate FPP. FPTase binds FPP with low nanomolar affinity while cellular FPP concentrations are near micromolar. Thus FPTase would be prebound with FPP at the time when the CAAX substrate is presented for catalysis[83]. This scenario is also supported by enzymatic kinetic data[84,85]. Thus inhibitors of FPTase that are competitive with FPP require a very tight K_i. Besides, these compounds would have to be selective for FPTase over other FPP utilizing enzymes.

The first reported CAAX peptidomimetics L-731, 734, L-744,832, BZA-5B and B-581 were derived from synthesis using CAAX tetrapeptide sequence as a template[86-89]. Recently, through a high volume-screening program, the pentapeptide derivative PDO 83176 was identified as an inhibitor of FPTase in rat brain[90]. Since peptides are rapidly degraded by intracellular proteases, these compounds required modifications to eliminate the labile peptide bonds. FTI-265, L-745,631 and SCH- 44342 are examples of cell active nonpeptides[91-94]. These nonpeptidemimetics are potent inhibitors of FPTase and are highly selective for FPTase over GGPTase-Ⅰ. SCH- 44342 is the first reported cell-active non-thiol FPTase inhibitor that is competitive for the CAAX substrate[91]. Recently, a non-thiol derivative of BMS tetrahydroisoquinoline has been reported[95,96]. Experiments in H-ras-transformed rat embryo cells using the compound FTI-277 resulted in a higher degree of apoptosis after irradiation and increased radiosensitivity than control cells, suggesting that the development of drugs that can reduce radioresistance would potentiate the efficacy of radiation therapy[97]. Further insights in the design of FPTase inhibitors may derive from structural studies of ligands bound to the enzyme[98-102]. Compounds that mimic the transition state would be both highly

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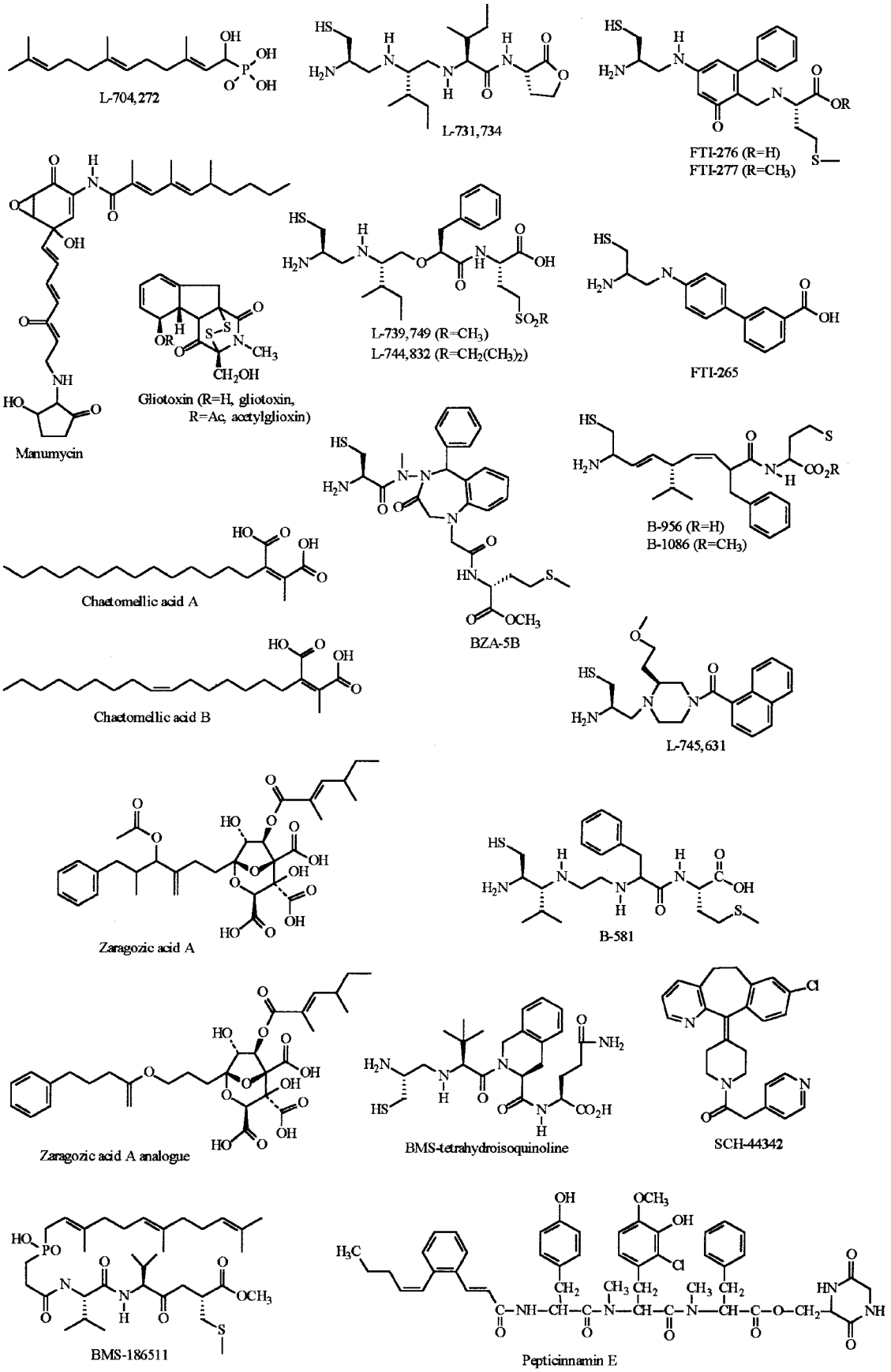

Fig 3. Examples of FPTase inhibitors.

potent and specific inhibitors of the enzyme revealed from the kinetic analysis of FPTase[103-105]. Although, bisubstrate analogs have shown utility in cell-based assays, further modifications of the analogs may improve potency beyond that achievable with FPP or CAAX mimetics alone.

CONCLUSIONS

The medicinal chemistry efforts have led to the novel and potentially useful compounds described in this review are founded on an increased understanding of the mechanistic principles that underlie cancer biology. Thus, these inhibitors represent true mechanism-based anticancer agents with more efficiency and little toxicity than conventional cytotoxic agents. In addition, since activated Ras expression has been shown to markedly increase radiation resistance, farnesyltransferase inhibitors may prove clinically useful as radio-sensitizers of tumors that depend on Ras function. Future research on the three dimensional structure of prenyltransferases facilitates the inhibitors of this class to become clinically available.

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 May；21 (5)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

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抑制 ras 法尼基转移酶：一种新的、安全的肿瘤化疗方法

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关键词 原癌基因蛋白 p21(ras)；二甲烯丙基转移酶；蛋白质异戊二烯化；Ca^{2+}-钙调蛋白依赖性蛋白激酶；抗肿瘤药；信号传递

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