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Current status of the development of Ras inhibitors

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Despite the importance of *ras* as driver genes in many cancers, clinically effective anti-cancer drugs targeting their products, Ras, have been unavailable so far, which was in part ascribable to the apparently 'undruggable' nature of their tertiary structures. Nonetheless, recent studies in academia and industry have identified novel surface pockets accepting small-molecule ligands in both their active GTP-bound and inactive GDP-bound forms (Ras•GTP and Ras•GDP, respectively), which has led to a surge of investigations into the discovery of Ras-specific inhibitors particularly by utilizing their structural information for structure-based drug design (SBDD). We have been developing Ras inhibitors by SBDD targeting a novel conformation of Ras•GTP called state 1, possessing 'druggable' surface pockets, which emerges from the conformational dynamics. In this article, we will survey Ras functions from the structural biological point of view and summarize the current status of the development of Ras inhibitors including our own.

Keywords: anti-cancer drugs/NMR/Ras/structure-based/drug design/X-ray crystallography.

Abbreviations: c-Raf-1, conventional Raf homologue 1; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; ets-family protein, E26 transformation-specific (or E-twenty-six); FTase, farnesyltransferase; GAPs, GTPase-activating proteins; GEFs, guanine nucleotide exchange factors; GppNHp, guanosine 5′-(β, γ-imido) triphosphate; HBS, hydrogen bond surrogate; H-Ras, Harvey-Ras; ICMT1, isoprenylcysteine carboxymethytransferase-1; K-Ras, Kirsten-Ras; MEK, mitogen activated protein kinase-ERK kinase; M-Ras, muscle-Ras; N-Ras, neuroblastoma-Ras; PDEδ, phosphodiesterase 6 delta; PI3Ks, phosphoinositide 3-kinases; PTase, palmitoyltransferase; Raf, rapidly accelerated fibrosarcoma; Ral, Ras-like GTPase; RalGDS, Ral guanine nucleotide dissociation stimulator; Rap, Ras-related proteins; ras, rat sarcoma virus; RCE1, Ras-converting enzyme-1; Rheb, Ras homologue enriched in brain; R-Ras, related Ras viral oncogene homologue; RTK, receptor tyrosine kinase; SBDD, structure-based drug design; Sos, son-of-sevenless.

Functions of Ras Proteins and Their Regulation

The rat sarcoma virus (*ras*) proto-oncogene products Harvey-Ras (H-Ras), Kirsten-Ras (K-Ras) and neuroblastoma-Ras (N-Ras), collectively called Ras, are members of the Ras-family small GTPases, which also include Ras-related proteins (Rap), related Ras viral oncogene homologue (R-Ras), muscle Ras (M-Ras) (R-Ras3), Ras-like GTPase (Ral), Ras homologue enriched in brain (Rheb) and others (*1*). Ras function as a molecular switch by cycling between GTP-bound active and GDP-bound inactive forms (Ras•GTP and Ras•GDP, respectively) in intracellular signal pathways controlling cell growth, differentiation and survival (*2*). Interconversion between the two forms is reciprocally catalysed by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (*3*). For example, Ras activation is induced by epidermal growth factor (EGF) receptor tyrosine kinase (RTK) through plasma membrane recruitment of a GEF, Son-of-sevenless (Sos), which promotes the formation of Ras•GTP. The Ras-rapidly accelerated fibrosarcoma-mitogen activated protein kinase-ERK kinase-extracellular signal-regulated kinase (Ras–Raf–MEK–ERK) pathway is the best characterized Ras signalling pathway (*4*). Ras•GTP binds to Raf serine/threonine kinases, such as conventional Raf homologue 1 (c-Raf-1) and B-Raf and induces their translocation to the plasma membrane, where Raf is fully activated via phosphorylations and accompanying allosteric conformational changes. Raf phosphorylate and activate MEK1/2 dual specificity protein kinase, which subsequently phosphorylate and activate ERK1/2 mitogen-activated protein kinase. The activated ERKs translocate to the nucleus leading to gene expression through activation of transcription factors, such as E26 transformation-specific (or E-twenty-six) (Ets-family) proteins. In addition, Ras has multiple downstream effectors other than Raf, such as phosphoinositide 3-kinases (PI3Ks), Ral guanine nucleotide dissociation stimulator (RalGDS) family proteins and phospholipase Cε (*2*) (Fig. 1).

Oncogenic potential of Ras is enhanced by point mutations mainly at Gly12 and Gln61, which not only reduce the intrinsic GTPase activity but also, more importantly, render Ras insensitive to the GAP activity, resulting in the constitutive activation of

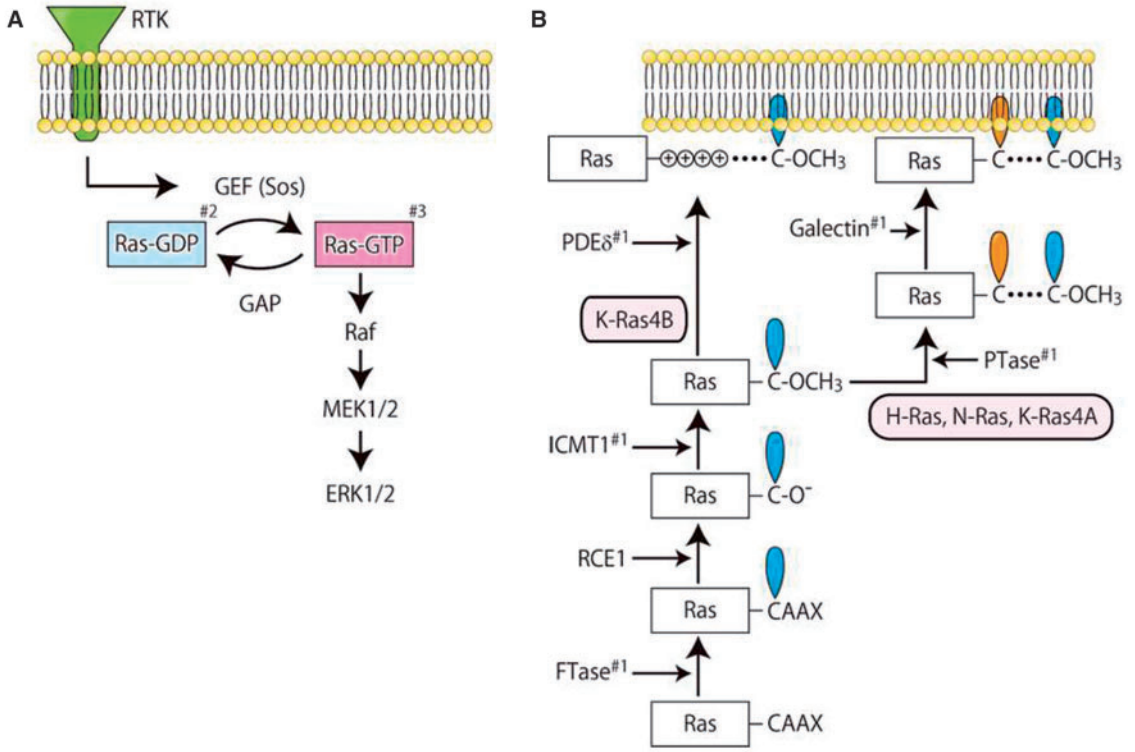

Fig. 1 Pathways for Ras-mediated signalling and post-translational modifications of Ras C termini. (**A**) The Ras–Raf–MEK–ERK pathway. RTK activation induces GEF-catalysed conversion of Ras•GDP to Ras•GTP, and Ras•GTP binds and activates Raf, which induces sequential activation of MEK and ERK through phosphorylations, leading to transcriptional activation. (**B**) Post-translational modifications of Ras C termini. Farnesyl moiety (blue rod) is covalently attached to Cys in the conserved C-terminal CAAX motif, which is followed by proteolytic cleavage of AAX and carboxymethylation of the newly formed C-terminal Cys by RCE1 and ICMT1, respectively. H-Ras, N-Ras and K-Ras4A are further modified by the attachment of palmitoyl moiety (orange rod) by PTases. The Ras-escort proteins, PDEδ and Galectin, bind to Ras by recognizing their farnesyl moiety and recruit them to the plasma membranes. #1, #2 and #3 represent the sites of action of various Ras inhibitors classified into the corresponding category numbers in Table I.

downstream signalling (*2*). Mutational activation of Ras is observed at the frequency of 15–20% in a variety of human cancers and the frequency goes up to 63–90% and 36–50%, respectively, in pancreatic and colorectal cancers (*2*, *5*, *6*). Cancer cells carrying activated oncogenes, such as *ras* exhibit a phenomenon known as 'oncogene addiction', where their survival becomes dependent on the activated oncogene functions (*5*). Indeed, functional inhibition of the activated Ras has been shown to reverse transformed phenotypes of cancer cells, leading to cell death and tumour regression (*6*, *7*). Although these observations make Ras some of the most promising anti-cancer drug target, there is no effective molecular targeted therapy for Ras-related cancers to date.

Anatomy of the Function and Regulation of Ras from the Structural Biological Point of View

Tertiary structures of Ras

Comparative analysis of the amino acid sequences between Ras and heterotrimeric G proteins, followed by site-directed mutagenesis studies, in 1980s identified functional domains named G domains comprising five G motifs (G1 to G5), which are conserved among heterotrimeric G protein Gα subunits and small GTPases. In 1988, de Vos *et al.* (*8*) reported the first tertiary structure of Ras, *i.e.* H-Ras•GDP, by a crystallographic approach. In the next year, the crystal structure of H-Ras•guanosine 5′-(β, γ-imido) triphosphate (H-Ras•GppNHp), where a non-hydrolysable GTP analogue, GppNHp, replaced the bound GTP, was determined by Pai *et al.* (*9*). The overall structure of H-Ras showed a Rossmann fold consisting of a hydrophobic core of 6 stranded β-sheets and 5 α-helices, which are connected by 10 loops (Fig. 2). Five of these loops play essential roles for GDP/GTP binding and hydrolysis of the bound GTP. Structural comparison between H-Ras•GDP and H-Ras•GTP revealed that the guanine nucleotide exchange predominantly induces conformational changes in the two flexible regions, named Switch I (residues 32–38) including the G2 motif and Switch II (residues 60–75) including the G3 motif (*10*). In Ras•GTP, the hydrogen bonding network across the γ-phosphate of the guanine nucleotide, Thr35 in Switch I and Gly60 and Gln61 in Switch II, stabilizes the conformation, whereas the corresponding interactions are totally missing in Ras•GDP because of the absence of the γ-phosphate group (Fig. 2).

Regulation of Ras activity by GEF and GAP

The structural basis for the GEF- and GAP-mediated regulation of the Ras activity was figured out by Wittinghofer, Kuryan and co-workers in the late 1990s through determination of the crystal structures of H-Ras in complex with the catalytic (cell division

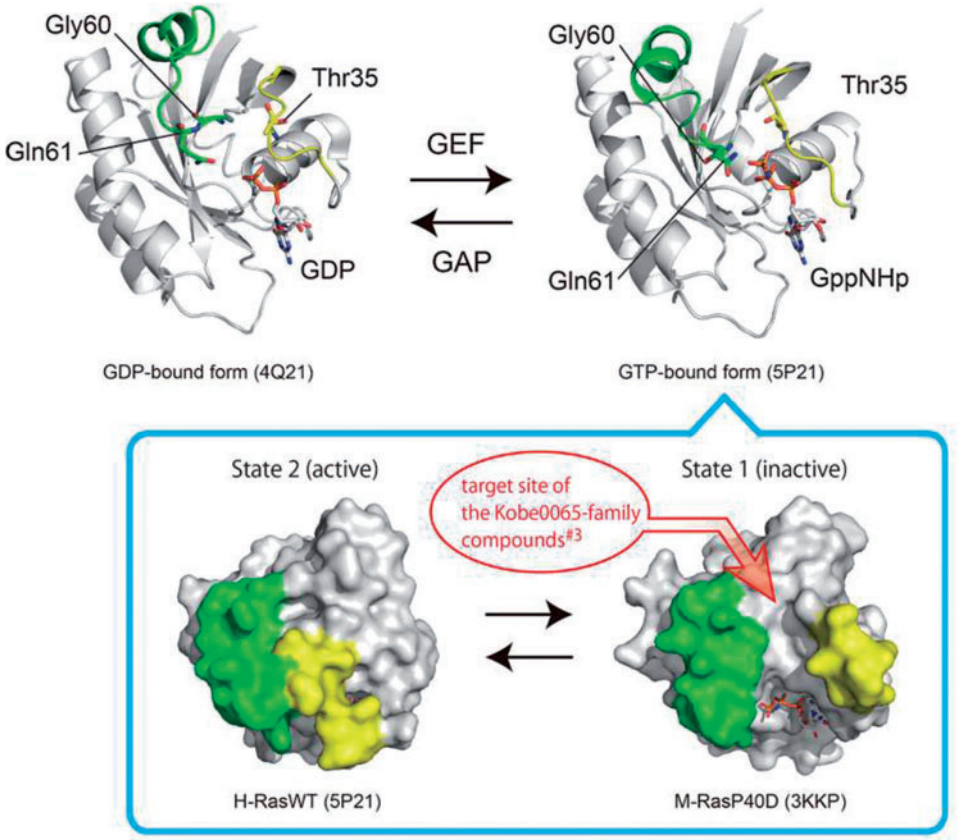

Fig. 2 Tertiary structures of H-Ras•GDP and H-Ras•GppNHp and the conformational dynamics of Ras•GppNHp. Ras functions as a molecular switch by cycling between GTP-bound active and GDP-bound inactive forms in various intracellular signal pathways. Interconversion between the two forms is reciprocally catalysed by GEFs and GAPs. Ras•GTP exhibits conformational equilibrium between two states, state 1 and state 2. State 1, corresponding to an inactive form, possesses a surface pocket, unseen in state 2, which can accommodate small-molecule compounds. Switch I and Switch II are coloured by yellow and green, respectively. Thr35, Gly60, Gln61 and guanine nucleotides are shown in stick representations. The protein data bank codes for H-Ras•GDP, H-Ras•GppNHp state 2 and M-RasP40D•GppNHp state 1 are 4Q21, 5P21, and 3KKP, respectively. #3 represents the category number of Ras inhibitor in Table I.

cycle 25 homologue) domain of Sos and p120GAP, respectively (*11*, *12*). During the Sos-mediated nucleotide exchange from Ras•GDP to Ras•GTP, the tight interaction of GDP with Ras is disrupted by the catalytic domain of Sos in the following two ways. First, the α-helical hairpin of Sos is inserted into Switch I of Ras, thereby displacing Switch I and subsequently opening up the nucleotide-binding site. Then, the side chains of the residues in the hairpin and those in Switch II, which assumes a disordered conformation resulting from the structural changes occurred at the first step, alter the chemical environments surrounding the binding site of the phosphate groups of the nucleotide and Mg^{2+}. Consequently, GDP binding is no longer favoured, and GDP is released from Ras. The resulting nucleotide-free form is preferentially converted to Ras•GTP rather than Ras•GDP because the cellular concentration of GTP is much higher than that of GDP.

Intrinsic GTP hydrolysis of Ras depends on the locations and orientations of the side chain of Gln61 in Switch II and of a catalytic water molecule activated by the Gln61 side chain to exert a nucleophilic attack on the γ-phosphate of GTP. The bulky Val12 side chain of the G12V mutant is thought to lower the GTPase activity through a steric interference over this catalytic process. Stimulation of the GTP hydrolysis by GAP is achieved in the following ways. First, binding of the variable α7 loop of GAP to Switch I of Ras•GTP establishes the pairing specificity between the two proteins. Then, a high-affinity interaction of Ras with the Phe-Leu-Arg (FLA) motif of GAP is established, which stabilizes the two switch regions. The Arg-finger loop of GAP, which is highly conserved among GAPs for various small GTPases, is inserted into an active site to neutralize developing charges in the transition state, thereby facilitating the Gln61/catalytic water-mediated GTP hydrolysis.

Effector recognition and the conformational dynamics of Ras

Crystal structure analyses of H-Ras•GppNHp in complex with the downstream effectors, such as Raf, PI3K and RalGDS (*13–16*), demonstrated that the backbone structures of H-Ras•GppNHp in the complexes were similar to that of H-Ras•GppNHp alone. Switch I and Switch II, containing flexible loops, constitute a principal binding interface for the effector recognition. Moreover, the flexible property of these regions is presumably instrumental in recognizing a variety of the effectors with substantial sequence diversity in their Ras-binding domains.

In parallel with the above crystallographic studies, in 1996, Geyer *et al.* (*17*) showed that H-Ras•GppNHp adopts two conformational states in solution, called state 1 and state 2 (Fig. 2) and that the two states exhibit an equilibrium by interconverting in a millisecond time scale through ^{31}P NMR analyses of the nucleotide phosphorus atoms. The two states were characterized by signals with different chemical shift values in the ^{31}P NMR spectrum acquired at a low temperature. The corresponding signals were also observed for H-Ras in complex with GTP or other non-hydrolysable GTP analogues, such as guanosine 5′-3-*O*-(thio) triphosphate (*18*, *19*). This dynamic structural feature is shared among the Ras-family small GTPases, including Rap, Ral and M-Ras, although the state distributions exhibit great variations (*20*). State 2 represents an active conformation because effector-binding induced an equilibrium shift towards state 2 (*8*) indicating that the previously solved crystal structures of Ras•GppNHp alone or in complex with the effectors corresponded to state 2. State 1 is regarded as an inactive conformation with a greatly impaired ability to bind to the effectors (*21*). In contrast to the extensive structural information accumulated on state 2, that on state 1 is very limited, which is presumably accounted for by a difficulty in determining a single stabilized conformation due to its flexible structural property. The structural analyses of state 1 were mainly conducted by utilizing artificial H-Ras mutants, such as T35S, T35A and G60A, and a Ras homologue, M-Ras, all of which predominantly adopt state 1 in solution (*21*–*23*). The first state 1 crystal structure of H-RasT35S•GppNHp solved in 2001 was unable to show its full figure because structural information on the two switch regions was completely missing (*21*). In 2005, we reported the state 1 crystal structure of M-Ras•GppNHp, however, structural information on five residues in Switch II was missing (*23*). In 2010, the complete state 1 structure was finally unveiled using M-Ras carrying an H-Ras-type amino acid substitution P40D at a high resolution of 1.35 Å (*24*). Next year, we addressed the mechanism for the state transition through the crystallographic and NMR analyses of M-RasD41E and H-RasT35S (*25*, *26*). As inferred from the previous studies (*27*), state 1 exhibits an open conformation accompanied by the loss of the hydrogen bonding networks across the two switch regions and the guanine nucleotide found in the state 2 structures, resulting in the formation of a surface pocket suitable for accepting small-molecule compounds (Fig. 2). Because the absence of such a pocket in the state 2 structures had rendered Ras 'undruggable', our discovery of the 'druggable' pocket shed a light on the structure-based drug design (SBDD) of specific inhibitors that allosterically block Ras activation by stabilizing the inactive state 1 conformation (*28*, *29*).

Post-translational modifications of Ras essential for the plasma membrane targeting

Post-translational lipid modifications of Ras are necessary not only for their plasma membrane targeting (Fig. 1B) but also for the full activation of the effectors such as c-Raf-1 (*30*). Although the sequences of the C-terminal 25 residues, called the hypervariable region, of the three Ras isoforms are highly divergent, they terminate with the CAAX (C: cysteine, A: aliphatic amino acids, X: any amino acids) motif sequence (*31*). A farnesyl moiety is covalently attached to the newly synthesized cytoplasmic Ras polypeptides by the enzyme farnesyltransferase (FTase). This prenylation reaction is followed by proteolytic cleavage of the AAX sequence, catalysed by Ras-converting enzyme-1 (RCE1), and further by carboxymethylation of the newly formed C-terminal Cys186 by isoprenylcysteine carboxymethytransferase-1 (ICMT1). In addition, a further modification step, where a palmitoyl moiety is attached to the Cys residues immediately upstream of the CAAX motif by the enzyme palmitoyltransferase (PTase), plays a crucial role in the membrane anchoring of the prenylated H-Ras, N-Ras and K-Ras4A. In the case of K-Ras4B, the polybasic region upstream of the CAAX motif substitutes for the palmitoylation for the membrane anchoring. The enzymes for a series of the post-translational modifications and their substrates are promising targets for the development of Ras inhibitors.

Current Status of the Development of Ras Inhibitors

In this section, we summarize the recent attempts to develop Ras inhibitors in academia and industry, which are classified into three categories based on the working concepts (Table I). First, we focus on the strategies aiming to block the membrane targeting of Ras, including the latest one targeting the prenyl-binding proteins that escort Ras to the plasma membrane. Second, we discuss the strategies aiming to prevent the formation of Ras•GTP by blocking the Ras–GEF interaction. Third, we discuss the strategies aiming to block the Ras–effector interaction, including those for the development of allosteric Ras inhibitors by targeting the conformational dynamics of Ras•GTP like our own.

Inhibition of the membrane targeting of Ras

The correct intracellular localization dependent on a series of the post-translational modifications is essential for the efficient Ras signalling. Thus, pharmacological inhibition of the enzymes catalysing various modification processes is expected to exhibit an antitumour effect towards cancer cells carrying the oncogenic Ras mutations by blocking the activated Ras signalling. A number of FTase inhibitors (FTIs) have been developed, and some of them have reached to the late stage clinical trials. However, it was eventually concluded that monotherapy with FTIs did not show any clinical efficacy to advanced solid tumours (*32*) presumably because an alternative prenylation, geranylgeranylation, of K-Ras4B and N-Ras catalysed by geranylgeranyl transferase I, circumvented the inhibitory effect of FTIs by functionally substituting for the farnesylation. However, recent studies reported promising efficacy of FTIs used as monotherapy or in combination with other conventional cytotoxic agents (*33*) while geranylgeranyltransferase inhibitors failed to show any clinical efficacy (*34*). Basically,

Table I. Classification of Ras inhibitors according to the working concepts

		References
#1: Plasma membrane localization		
FTase	TLN-4601	(*49*)
Galectin	Salirasib	(*37*, *38*)
APT-1/2	Palmostatin B, Palmostatin M	(*36*, *50*)
PDEδ	Deltarasin	(*39*)
ICMT	Cysmethynil	(*51*)
#2: GDP–GTP exchange		
Ras•GDP	SCH53239	(*52*, *53*)
	HBSs	(*44*)
	Andrographolide	(*54*)
	DCAI	(*42*)
	13	(*55*)
	Bisphenol A	(*56*)
K-RasG12C•GDP	SML-8-73-1, SML-10-70-1	(*43*)
	VSA9, AA12	(*44*)
#3: Downstream effector interactions		
Ras•GTP	Metal-cyclen complex, Metal-bis(2-picolyl) amine complex	(*57–59*)
	Antibody fragment	(*60*)
	Sulindac sulfide	(*61*)
	MCP compounds	(*48*)
	Kobe-family compounds	(*62*)

clinical trials for FTIs shall be focused on the treatment of H-Ras-dependent malignancies because only H-Ras is totally dependent on farnesylation for its membrane targeting. Inhibitors of RCE1 and ICMT were reported to show not so profound effect compared with FTIs, which is presumably accounted for by their non-specific activities towards other small GTPases (*35*). Inhibitors targeting the palmitoylation of H-Ras and N-Ras were shown to cause partial phenotypic reversion in an H-RasG12V-transformed fibroblast cell line, however, their anti-tumour effect on xenografts of human cancer cell lines remains to be shown (*36*). Collectively, the inhibitors targeting the enzymes for farnesylation and palmitoylation after all failed to effectively prevent the membrane targeting of K-Ras4B, mutationally activated most frequently in human cancers. Recently, blockade of the interactions between Ras and the prenyl-binding proteins, which escort Ras to the plasma membranes, has attracted attention for the development of K-Ras4B inhibitors. Salirasib, a farnesylcysteine mimetic interfering with the binding of farnesylated K-Ras4B to the Ras-escort protein Galectin, was reported to show efficacy in the early stage of clinical trials (*37*, *38*). Very recently, deltarasin, discovered by using the Alpha enzyme-linked immunosorbent assay technology in combination with SBDD, was reported to inhibit the interaction of K-Ras4B with the escort protein phosphodiesterase 6 delta (PDEδ) carrying a surface cavity that accommodates the farnesyl moiety of the Ras-family proteins (*39*). Although deltarasin displayed an activity to inhibit Ras-dependent signalling both *in vitro* and *in vivo*, further structural optimization would be needed considering its non-specific inhibitory activity towards other small GTPases, such as Rheb (*40*) (Table I, #1).

Inhibition of Ras•GTP formation by blocking the Ras•GDP–GEF interaction

It is not clear whether targeting the Ras•GDP–GEF interaction is an effective strategy for inhibition of the constitutively activated Ras mutants because they are likely to escape from the regulation by GEFs considering the great reduction of their GTPase activity and a vast excess of the free GTP concentration over that of GDP in cells. However, it might be effective for some cancer types, considering that the function of wild-type Ras is reported to be required for the growth of tumours carrying the oncogenic *ras* mutations (*41*). Fragment-based ligand screening by NMR targeting K-RasG12D bound to GDP or GppNHp was applied to identify ligand scaffolds, resulting in the isolation of 25 hit compounds. Crystal structure analysis of the complex between K-RasG12D•GDP and the compounds revealed a surface pocket capable of accomodating the small-molecule compounds (*42*). Among them, two compounds, called DCAI and DCIE, inhibited the Sos-mediated nucleotide exchange on K-Ras•GDP *in vitro*. However, there was no evidence for their inhibitory effect on Ras at the cellular level. An alternative strategy, oncogenic mutant-specific fixing of the GDP-bound form, was applied by two groups, Gray's and Shockat's (*43*, *44*). They selected K-RasG12C, an oncogenic mutant often observed in lung adenocarcinomas, as a target because the thiol group of Cys12 was very useful for covalent trapping of inhibitors via disulfide bond formation. Gray and co-workers screened GDP-derived analogues and identified SML-10-70-1, which was capable of functionally mimicking GDP when bound to Ras and showed antiproliferative activity towards K-RasG12C-bearing cells (*43*). However, similar efficacy of this compound towards K-RasG12S-bearing cells suggests the non-specific nature of its cellular activity. Shockat and co-workers performed fragment-based screening of small-molecule compounds that are capable of forming disulfide bond with Cys12 without relying on nucleotide analogues. Structure determination of the co-crystals of the resulting hit compounds with K-RasG12C•GDP led to the identification of a novel drug-binding site. Subsequent structure-based optimization of their derivatives resulted in identification of the most potent acrylamide AA12 (*44*). This compound modified K-RasG12C but not wild type *in vitro* and induced apoptosis of lung cancer cells carrying K-RasG12C. However, substantial dissociation of the IC_{50} values between biochemical and cell-based assays suggests its non-selective mode of action in cells. Synthetic hydrogen bond surrogate (HBS) peptides were designed by Bar-Sagi and co-workers as orthosteric inhibitors mimicking the α-helical domain of Sos. Among them, HBS3 disrupted the Sos–Ras interaction and down-regulated the Ras–Raf–MEK–ERK signalling in response to EGF stimulation despite its 10-fold

weaker binding affinity for Ras•GDP compared with parent Sos (*45*) (Table I, #2).

Inhibition of the Ras–effector interactions

Blockade of the interactions of Ras•GTP with the downstream effectors is the most promising strategy for inhibition of the dominant action of the constitutively activated Ras mutants in cancer cells. Sulindac, a non-steroidal anti-inflammatory drug, was reported to strongly inhibit H-Ras-induced malignant transformation of MDCK-F3 cells and the Ras-dependent Raf activation (*46*, *47*). Although an NMR study revealed its direct binding site in the vicinity of Switch I in H-Ras (*47*), lack of the experimental evidence for the efficacy on human cancer cell lines and for the antitumour effect suggests that further improvement of its potency, selectivity and drug-likeness would be a difficult task. MCP compounds were identified using the yeast two-hybrid system by Khazak, Tamanoi and coworkers. They discovered several hit compounds that inhibit the Ras–Raf binding and the GEF-mediated nucleotide exchange on Ras (*48*). The most potent compound MCP110 inhibited the Ras/Raf/MEK/ERK signalling in cell-based assays and showed *in vivo* efficacy. For all that, it is somewhat puzzling that target of the action of this compound, whether Ras or Raf, has not been unambiguously assigned (Table I, #3).

Allosteric inhibition of Ras•GTP, particulatly that targeting its conformational dynamics, is another promising strategy for inhibition of the Ras–effector interactions. As already mentioned, Ras•GTP exists in dynamic equilibrium between at least two distinct conformational states, state 1 and state 2, which correspond to inactive and active conformations, respectively, regardless of the presence or absence of the oncogenic mutations (*17*). Kalbitzer and co-workers discovered metal cyclen derivatives, which were capable of shifting the conformational equilibrium of H-Ras•GTP towards state 1 (*57–59*). NMR and crystallographic studies revealed two binding sites for them, which are located on the top of the α-phosphate and the Loop7. However, lack of the evidence for the cellular efficacy, low-binding affinity for Ras•GTP at a millimolar order and weak inhibitory effect on Ras–Raf binding may discourage their further optimization. As already mentioned, the state 1 structure of Ras•GTP possesses a surface pocket surrounded by Switch I, Switch II and GTP (Fig. 2). Based on the hypothesis that compounds which fit into this pocket and hold Ras to state 1 may block the Ras functions, we carried out *in silico* screening to discover such compounds. Based on the information on the state 1 crystal structure of M-RasP40D•GppNHp (*24*), computer docking simulation using the Molecular Mechanics Poisson-Boltzman surface area method was carried out to select candidate compounds from a virtual library containing more than 40,000 compounds. The selected 97 compounds were examined *in vitro* for their inhibitory effect on Ras–Raf binding, which resulted in the discovery of a hit compound Kobe0065 (*62*). Subsequent similarity search based on the core structure of Kobe0065, followed by the *in vitro* assay, yielded two Kobe0065 derivatives, Kobe2601 and Kobe2602 (*62*). The Kobe0065-family compounds inhibited Ras–Raf association in cells and reduced the phosphorylation levels of the downstream molecules, such as MEK and ERK. In addition to the inhibitory effect on the Ras–Raf–MEK–ERK pathway, they inhibited the Ras-PI3K-Akt and Ras-RalGDS-RalA pathways and moreover the Ras•GTP-dependent nucleotide exchange activity of Sos (*62*). Furthermore, the compounds inhibited the proliferation of several cancer cell lines, such as colorectal and pancreatic carcinomas, carrying the oncogenic *ras* mutations and showed tumour growth inhibition on a xenograft model (*62*). The NMR structure of a complex of Kobe2601 with H-RasT35•GppNHp, which predominantly adopts state 1, revealed a compound-binding pocket in the vicinity of the DCAI-binding site of K-RasG12D•GDP (*62*).

Concluding Remarks

Elucidation of the important role of the *ras* oncogenes as core cancer driver genes prompted vigorous search for inhibitors of their protein products in academia and industry around the world. Although the first wave of this search resulted in the development of FTIs, the failure to show their clinical efficacy led to the development of a bunch of alternative strategies for targeting Ras, which are classified into three categories in this article. It is noteworthy that some of these strategies took advantage of new information on the tertiary structures of Ras, in particular the discovery of 'druggable' surface pockets, and the structural basis for their interactions with the effectors and GEFs.

Most of the strategies employed so far did not discriminate between wild type and the oncogenic mutant Ras. In this case, the principle of the selective action of Ras inhibitors on cancer cells rather than wild-type cells mainly relies on the 'oncogene addiction' phenomenon, whereby cancer cells become more sensitive to the pharmacological inhibition of the Ras function. Although cancer cells carrying the oncogenic Ras mutations have been shown to exhibit 'oncogene addiction' in various experimental models, it is not certain whether this phenomenon is enough to support sustained inhibition of the growth of naturally occurred cancers which are known to undergo active clonal selections and genomic mutations. In this sense, strategies specifically targeting the oncogenic Ras mutants, such as the G12V and Q61L mutants, are preferable, and those targeting RasG12C•GDP have been in progress as already mentioned. SBDD based on the tertiary structures of the G12V and Q61L mutants would be a promising task if their structures show significant differences from that of wild type.

Moreover, the individual strategies have their inherent problems arisen from the modes of action. The strategy targeting the membrane localization of Ras has a fundamental problem that there exist many other target proteins with vital functions for the post-translational modification machinery involving FTase, PTase and the escort proteins, which certainly harms the specificity of inhibition and potentially leads

to toxicity towards normal cells. Another strategy targeting the Ras•GDP–GEF interaction also has a fundamental problem of its own that it is unclear whether it is effective in inhibition of the constitutively activated Ras mutants in cancer cells, which are likely to escape from the requirements for GEFs in sustaining a high level of the GTP-bound form as already discussed. The strategy targeting the Ras•GTP–effector interactions was once thought to be difficult to take because of the apparent absence of any 'druggable' surface pockets on the state 2 crystal structures of Ras•GTP. Nonethelss, recent structural studies have identified novel surface pockets accepting small-molecule ligands in Ras•GTP, especially in its state 1 conformation. Because the binding sites on Ras•GTP of various Ras effectors are largely overlapped, compounds that inhibit interaction with one effector are likely to be effective for other effectors. On the other hand, our recent structural studies using X-ray crystallography and NMR have revealed that the state 1 pocket, flanked by the two switch regions, displays substantial structural flexibility resulting from complex intramolecular motions, which may add more difficulty in obtaining potent inhibitors.

In conclusion, the current status of the development of Ras inhibitors reviewed in this article undoubtedly indicates that Ras is no longer a synonym for 'undruggable target' and must be given a new look as a promising target for anti-cancer drug development. Adding to the current SBDD approaches, advanced structural studies in combination with high performance computing will certainly accelerate the discovery of Ras inhibitors with higher potency and specificity.

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Conflict of Interest

None declared.

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