TARGETING RAS SIGNALLING PATHWAYS IN CANCER THERAPY

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The RAS proteins control signalling pathways that are key regulators of several aspects of normal cell growth and malignant transformation. They are aberrant in most human tumours due to activating mutations in the *RAS* genes themselves or to alterations in upstream or downstream signalling components. Rational therapies that target the RAS pathways might inhibit tumour growth, survival and spread. Several of these new therapeutic agents are showing promise in the clinic and many more are being developed.

Normal cellular behaviour in multicellular organisms is tightly controlled by a complex network of signalling pathways that ensures that cells proliferate only when they are required by the body as a whole — for example, during development or wound healing. Cancer occurs when normal growth regulation breaks down, usually because of defects in these signalling mechanisms. The RAS proteins were some of the first proteins identified that possessed the ability to regulate cell growth. They were discovered as proteins encoded by retroviral oncogenes that had been hijacked from the host genome by the Kirsten and Harvey rat sarcoma viruses. Searches for transfectable oncogenes in the genomes of human tumours also yielded the *RAS* genes. However, it soon became clear that these tumour- or retrovirus-derived forms of the *RAS* genes encoded proteins that were constitutively active because of point mutations in their coding sequences[1].

RAS proteins have essential roles in controlling the activity of several crucial signalling pathways that regulate normal cellular proliferation. However, human tumours very frequently express RAS proteins that have been activated by point mutation — ~20% of all tumours have undergone an activating mutation in one of the *RAS* genes[2]. In these tumours, the activated RAS protein contributes significantly to several aspects of the malignant phenotype, including the deregulation of tumour-cell growth, programmed cell death and invasiveness, and the ability to induce new blood-vessel formation[3].

Therapies that target the RAS proteins and the signalling pathways that they control would therefore be very valuable in treating tumours that have activating *RAS* mutations. However, their potential might be even greater, as many tumours that lack *RAS* mutations have found other ways to activate the same pathways. The RAS signalling pathways are now understood in great detail at the molecular level — as a result of two decades of intense study using genetics and molecular and cellular biology, and encompassing organisms from yeast to man via slime mould, worms, flies and frogs[4]. Structural information is also available on most of the key players, including many binary complexes of interacting proteins[5]. This knowledge is now being used to design rational therapies that target the RAS pathways.

There are almost 20 new therapeutic agents in clinical trials at present, and many more are being developed. Some of these should ultimately be effective treatments for some tumour types, particularly when used in combination with other agents.

So, what other proteins comprise the RAS signalling pathways, what is their role in normal cell growth and function, and how do they become activated during tumorigenesis? Several therapeutic agents have already been developed against the various components in the RAS pathways, so how successful are they in the clinic? Finally, are there less obvious ways of targeting the pathway that have not yet been exploited?

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doi:10.1038/nrc969

Summary

- RAS proteins, their regulators and the downstream enzymes that they control are activated in many tumour types by a variety of mechanisms, including oncogenic mutation of *RAS* genes.
- They are crucial mediators of several of the malignant characteristics of transformed cells and are therefore good candidates for tumour therapy.
- RAS proteins require post-translational modification by farnesylation to be biologically active. Farnesyl transferase inhibitors have some antitumour activity in the clinic, but they seem to act through targets other than RAS.
- Kinase inhibitors that block either RAF or mitogen-activated protein (MAP) kinase kinase MEK in the RAF/MAP kinase pathway downstream of RAS have been developed and show promise in early clinical trials.
- Inhibitors acting on epidermal growth factor (EGF) receptor and ERBB2 upstream activators of RAS have been developed. Antibodies directed against ERBB2 have been licensed for the treatment of breast cancer, whereas small-molecule EGF receptor inhibitors show potential against lung cancer in clinical trials.
- Other RAS-related therapies are in development, including inhibitors of AKT/PKB kinase activity, which is activated by *RAS* oncogenic mutation and by *PTEN* tumour-suppressor gene loss.

Normal RAS signalling pathways

RAS proteins: diversity and processing. The RAS proteins are members of a large superfamily of low-molecular-weight GTP-binding proteins, which can be divided into several families according to the degree of sequence conservation. Different families are important for different cellular processes — the RAS family controls cell growth and the RHO family controls the actin cytoskeleton. Three members of the RAS family — HRAS, KRAS and NRAS — are found to be activated by mutation in human tumours[6]. These three members are very closely related, having 85% amino acid sequence identity and, although they function in very similar ways, some indications of subtle differences between them have recently come to light. The HRAS, KRAS and NRAS proteins are widely expressed, with KRAS being expressed in almost all cell types. Knockout studies have shown that *Hras* and *Nras*, either alone or in combination, are not required for normal development in the mouse, whereas *Kras* is essential[7]. This might reflect different molecular functions of the three proteins, but is more likely to reflect the more ubiquitous expression of KRAS.

The normal function of RAS proteins requires them to be post-translationally modified. The purpose of this is primarily to localize them to the correct subcellular compartment — principally the inner face of the plasma membrane. RAS proteins that are mislocalized at other sites in the cell are inactive, probably because they cannot recruit their target enzymes. The fact that correct post-translational modification of RAS is required for its biological activity has made the enzymes involved in this processing very attractive targets for therapeutic intervention[8]. The steps in the normal post-translational processing of RAS are described in BOX 1 (REFS 9–11).

Signalling upstream of RAS. The activation state of RAS proteins depends on whether they are bound to GTP (in which case, they are active and are able to engage downstream target enzyme) or GDP (in which case, they are inactive and fail to interact with these effectors). In normal cells, the activity of RAS proteins is controlled by the ratio of bound GTP to GDP[4]. *In vitro*, purified RAS possesses a low level of intrinsic GTPase activity — bound GTP is slowly converted to GDP. It also has a slow rate of nucleotide exchange with the surrounding medium — bound GDP is gradually replaced by GTP. However, these processes are catalysed within the cell — the nucleotide exchange by guanine nucleotide exchange factors (GEFs) and the nucleotide hydrolysis by GTPase activating proteins (GAPs). Both of these activities involve large, considerably divergent families of proteins: it is the balance between these proteins that determines the activation state of RAS and its downstream target pathways. As the regulation of RAS has been reviewed in detail recently[12–14], only a brief overview is provided here.

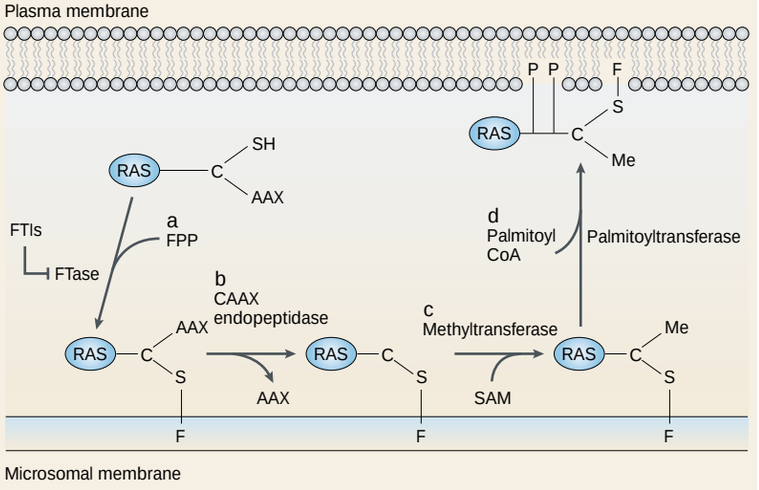
Several RAS-GEFs have been characterized. Most work has been done on SOS1 and SOS2, which were identified as mammalian homologues of the *Drosophila son of sevenless* gene product, a genetically identified RAS activator. Parallel developmental genetic analysis of RAS regulation in flies and in *Caenorhabditis elegans*, and biochemical and cell biological analysis in mammalian tissue-culture systems, led to the model of RAS regulation that is shown in FIG. 1. Following the activation of receptor tyrosine kinases such as the epidermal-growth-factor receptor (EGFR), the autophosphorylated receptor binds to the SH2 DOMAIN of the adaptor protein growth-factor-receptor-bound protein 2 (GRB2). Through its SH3 DOMAINS, GRB2 is bound to SOS, so activation of the receptor tyrosine kinase results in recruitment of SOS to the plasma membrane, where RAS is also localized as a result of farnesylation. The increased proximity of SOS to RAS results in increased nucleotide exchange on RAS, with GDP being replaced with GTP, which is the predominant guanine nucleotide in the cytosol. Many other receptor types, including the G-protein-coupled receptors, can activate RAS through stimulation of exchange factors. In some cases, this has been shown to involve transactivation of growth factor receptor tyrosine kinases[15].

SH2 DOMAIN
Src homology 2 domain. A protein domain capable of binding tyrosine phosphorylated sites.

SH3 DOMAIN
Src homology 3 domain. A protein domain capable of binding proline-rich motifs.

Box 1 | Post-translational processing of RAS proteins

Newly synthesized RAS is a cytosolic protein. Farnesyltransferase (FTase) catalyses the transfer of the 15-carbon isoprenoid chain from farnesyl pyrophosphate (FPP) to a cysteine residue that is close to the carboxyl terminus (C186 in human HRAS) (see **a**). This results in RAS associating with intracellular membranes via its farnesyl group (F). Farnesyltransferase inhibitors (FTIs) block this farnesylation, so RAS remains in the cytosol and is unable to stimulate its downstream targets. However, when FTase is inhibited, KRAS and NRAS, but not HRAS, can be geranylgeranylated — an alternative 20-carbon isoprenylation is added, and this is catalysed by geranylgeranyltransferase (GGTase) — resulting in rescue of processing of these RAS isoforms. Following isoprenylation, several other processing steps occur. An endopeptidase removes the end three amino acids from the carboxyl terminus of the protein (see **b**). The new carboxyl terminus is then methylated by a methyltransferase (see **c**). Following transportation to the plasma membrane, a final processing step (see **d**) occurs for HRAS and NRAS. Palmitoyltransferase catalyses the addition of two palmitoyl long-chain fatty acid groups (P) to a cysteine residue that is just upstream of the farnesylated carboxy-terminal cysteine. This stabilizes the interaction with the membrane. KRAS does not become palmitoylated, but its interaction with the plasma membrane is promoted by a group of lysine residues near the carboxyl terminus that interact with the negatively charged lipid head groups. The greatest drug discovery effort has gone into developing inhibitors of FTase, but other steps in the pathway might be worth pursuing. The failure of FTIs to block KRAS processing has proved to be a notable problem as KRAS is the most commonly mutated RAS isoform in human tumours. It is apparent that the effects of FTI on KRAS-transformed cells are due to inhibition of other farnesylated proteins, one of which is probably the RAS relative RHOB[41].



Although the above model is widely accepted, it is certainly an over-simplification. Other proteins are involved in the activation of RAS, such as SHC, which can mediate between receptors and GRB2 (FIG. 1). Several other non-receptor proteins can also become tyrosine phosphorylated and act as anchors for GRB2 interaction, and there are several other GEFs for RAS that are controlled quite differently[14,16,17].

The diversity of these exchange factors means that RAS is activated by a very varied collection of extracellular stimuli. This activation is opposed by the effects of the GAPs. These promote the hydrolysis of bound GTP by RAS by several orders of magnitude and normally ensure that RAS is rapidly inactivated after stimulation. Most of the analysis of RAS regulation has concentrated on the control of GEFs, but it is clear that GAPs are also regulated, at least in some circumstances[18]. At least six different GAP proteins exist that are active on RAS proteins[19].

Signalling downstream of RAS. GTP-bound RAS is able to bind and activate effector enzymes, and it is through these pathways that RAS controls cell proliferation, survival and other aspects of cell behaviour that can contribute to the transformed phenotype. Therapeutic interventions that target these enzymes might therefore be effective in treating tumours in which RAS is mutationally activated. The effectors of RAS have been reviewed recently in depth[3].

The first mammalian effector of RAS to be characterized — and still the most intensively studied — is the protein serine/threonine kinase RAF. A combination of genetic and biochemical evidence showed that GTP-bound RAS binds to, and contributes to the activation of, the three closely related RAF proteins, c-RAF1, BRAF and ARAF. This interaction causes RAF to be relocated to the plasma membrane, which seems to be crucial for its activation[20,21]. Downstream of this, activated RAF phosphorylates and activates mitogen-activated protein kinase kinases 1 and 2 (MEK1 and MEK2) — dual-specificity kinases that are capable of phosphorylating and activating the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 (extracellular signal-regulated kinases 1 and 2). Substrates for ERK1/2 include cytosolic and nuclear proteins, reflecting the fact that they can be transported into the nucleus following activation. The full range of effects of activating these kinases is yet to be determined, but most attention has, so far, focused on the regulation of transcription factors. ERK phosphorylates ETS family transcription factors such as ELK1, which forms part of the serum response factor that regulates the expression of FOS; in addition, ERK phosphorylates c-JUN. This leads to activation of the AP1 transcription factor, which is made up of FOS–JUN heterodimers[22]. As a result of stimulating these transcriptional regulators, key cell-cycle regulatory proteins, such as D-type cyclins, are expressed, which enables the cell to progress through the G1 phase of the cell cycle[23]. So, RAF activation can promote cell-cycle progression, at least in conjunction with other signals.

In addition to the RAF/MAPK pathway, RAS has also been found to activate several other effector pathways, the best characterized of which are shown in FIG. 2. RAS can interact directly with the catalytic subunit of type I phosphatidylinositol 3-kinases (PI3Ks)[24,25], leading to activation of the lipid kinase as a result of its translocation to the membrane and conformational changes. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to produce phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) — a second messenger that binds to a large number of proteins through the pleckstrin homology and other domains. In this way, PI3K controls the activity of a large number of downstream enzymes. Much attention has been paid to the activation of the kinases PDK1 (3-phosphoinositide-dependent protein kinase-1) and

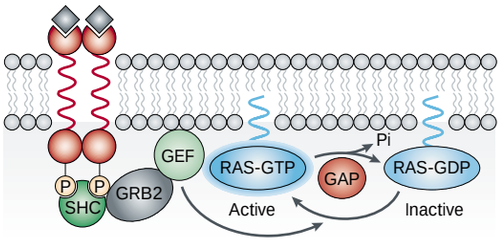


Figure 1 | **Signalling upstream of RAS.** The activation state of RAS is controlled by the cycle of hydrolysis of bound GTP, which is catalysed by GTPase activating proteins (GAPs), and the replacement of bound GDP with fresh GTP, which is catalysed by guanine nucleotide exchange factors (GEFs). The best-studied activation mechanism involves the assembly of complexes of activated, autophosphorylated growth-factor-receptor tyrosine kinases with the GEF SOS through the adaptor protein GRB2, and possibly SHC, resulting in the recruitment of SOS to the plasma membrane, where RAS is located. Several other GEFs exist that have distinct regulatory mechanisms. In addition, a wide range of GAPs have now been identified for RAS, some of which are also subject to regulation. RAS is also activated through GEFs in response to activation of a wide range of G-protein-coupled receptors.

AKT, (also known as PKB). PDK1 is important for the activation of a large number of protein kinases of the AGC family, including AKT/PKB, some PKCs, $p70^{S6K}$ and RSK. AKT/PKB has a strong anti-apoptotic function by phosphorylating various targets[26] and seems to be an important part of the survival signal that is generated by RAS[27]. In addition, PI3K activation leads to stimulation of RAC, a RHO family protein that is involved in the regulation not only of the actin cytoskeleton but also of transcription-factor pathways — for example, by activating nuclear factor-κB (NF-κB). RAC activation seems to be important in RAS-induced transformation and occurs through PI3K-dependent and -independent pathways[28,29].

A third well-studied effector family for RAS comprises three exchange factors for the RAS-related RAL proteins: RAL guanine nucleotide dissociation stimulator (RALGDS), RALGDS-like gene (RGL/RSB2) and RGL2/RLF. Through these proteins, RAS is able to stimulate RAL, resulting in activation of phospholipase D1 and activation of the CDC42/RAC-GAP-RAL binding protein 1 (RALBP1). The RALGDS pathway contributes, along with AKT/PKB, to the inhibition of the FORKHEAD transcription factors of the FoxO family[30]. These have been implicated in promoting cell-cycle arrest through induction of the cyclin-dependent kinase inhibitor KIP1 (also known as p27), and apoptosis through the expression of BIM and FAS LIGAND.

Phospholipase Cε is another RAS effector that has been reported recently. It has two RAS association domains and also a RAS-GEF domain, in addition to its phospholipase C domain, which promotes the hydrolysis of PtdIns(4,5)P_2 to diacylglycerol and inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3)[31]. Phospholipase Cε could link RAS to activation of PKC and calcium mobilization.

Through the combined action of these RAS-responsive signalling pathways, expression of activated mutant RAS in cells can promote several of the characteristics of malignant transformation. These include increased proliferation due to induction of cell-cycle regulators

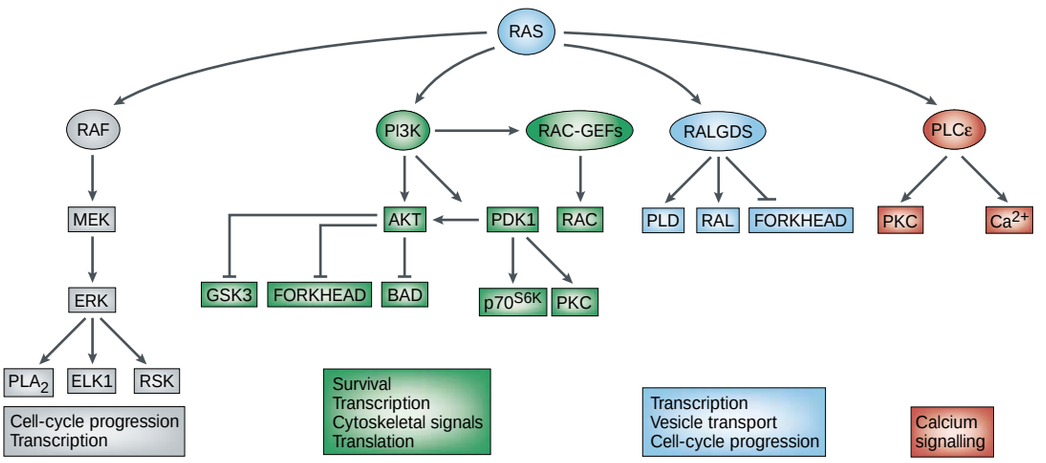


Figure 2 | **Signalling downstream of RAS.** Once in its active, GTP-bound state, RAS will interact with several families of effector proteins, resulting in stimulation of their catalytic activity. The main effectors are shown here. RAF protein kinases initiate the mitogen-activated protein (MAP) kinase cascade, which leads to ERK activation. This kinase has numerous substrates both in the cytoplasm and in the nucleus, including ETS family transcription factors such as ELK1 that regulate cell-cycle progression. Type I phosphoinositide 3-kinases (PI3Ks) generate second-messenger lipids, such as phosphatidylinositol-3,4,5-trisphosphate, which activate numerous target proteins including the survival signalling kinase AKT/PKB. RALGDS proteins are guanine nucleotide exchange factors (GEFs) for RAL, a RAS-related protein. Downstream targets include Forkhead transcription factors. Phospholipase Cε (PLCε) catalyses the hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol and inositol trisphosphate, resulting in protein kinase C (PKC) activation and calcium mobilization from intracellular stores. ERK, extracellular regulated kinase; GSK3, glycogen synthase kinase 3; MEK, mitogen-activated kinase/ERK kinase; $p70^{S6K}$, p70 ribosomal protein S6 kinase; PDK1, phosphatidylinositol trisphosphate-dependent kinase 1; PLA_2, phospholipase A_2; PLD, phospholipase D; RSK, p90 ribosomal protein S6 kinase.

AKT/PKB
A family of three closely related serine/threonine protein kinases containing amino-terminal pleckstrin homology domains that bind to the lipid products of phosphatidylinositol 3-kinase (PI3K). They are activated on PI3K stimulation and provide anti-apoptotic signals to the cell.

FORKHEAD
A large superfamily of transcription factors, of which one family, FoxO, is phosphorylated and inhibited by AKT/PKB.

BIM
A member of the BCL-2 family of apoptosis regulatory proteins. BIM induces apoptosis by acting at mitochondria to promote the release of cytochrome *c* into the cytosol.

FAS LIGAND
An extracellular protein that binds and activates the death receptor FAS, also known as CD95 and APO1, leading to initiation of apoptosis.

Table 1 | Activation of RAS signalling pathways in different tumours

Defect or mutation	Tumour type	Frequency (%)
RAS mutation	Pancreas	90 (K)
	Lung adenocarcinoma (non-small-cell)	35 (K)
	Colorectal	45 (K)
	Thyroid (Follicular)	55 (H, K, N)
	Thyroid (Undifferentiated papillary)	60 (H, K, N)
	Seminoma	45 (K, N)
	Melanoma	15 (N)
	Bladder	10 (H)
	Liver	30 (N)
	Kidney	10 (H)
	Myelodysplastic syndrome	40 (N, K)
	Acute myelogenous leukaemia	30 (N)
BRAF mutation	Melanoma	66
	Colorectal	12
EGFR overexpression	Most carcinomas	>50
ERBB2 amplification	Breast	30
PTEN loss	Glioblastoma multiforme	20–30
	Prostate	20
	Pancreas	40
AKT2 amplification	Ovarian	12
	Pancreas	10
PI3K amplification	Ovarian	40

EGFR, epidermal-growth-factor receptor; PI3K, phosphatidylinositol 3-kinase. H, K and N refer to *HRAS*, *KRAS* and *NRAS*, respectively.

such as cyclin D1, which leads to inactivation of the retinoblastoma (RB) pathway, and suppression of cell-cycle inhibitors such as KIP1. In addition, cells become desensitized to apoptosis through AKT/PKB signalling and less well-defined mechanisms that are downstream of RAF. In addition to effects on cell proliferation and survival, RAS effector pathways also lead to the induction of angiogenesis, mainly by means of ERK-mediated transcriptional upregulation of angiogenic factors, and to increased invasiveness, through both ERK-mediated expression of matrix metalloproteinases and RAC-mediated effects on the cytoskeleton. Targeting RAS and its effector pathways could therefore have a potential impact on several different aspects of malignancy.

Aberrant RAS signalling in tumours

RAS mutation. Aberrant signalling through RAS pathways occurs as the result of several different classes of mutational damage in tumour cells. The most obvious of these is the mutation of the *RAS* genes themselves (TABLE 1). Some 20% of human tumours have activating point mutations in *RAS*, most frequently in *KRAS* (about 85% of total), then *NRAS* (about 15%), then *HRAS* (less than 1%). These mutations all compromise the GTPase activity of RAS, preventing GAPs from promoting hydrolysis of GTP on RAS and therefore causing RAS to accumulate in the GTP-bound, active form. Almost all RAS activation in tumours is accounted for by mutations in codons 12, 13 and 61 (REF. 2).

GAP deletion. RAS can also be activated in tumours by loss of GAPs. The most significant known example is the loss of neurofibromin, which is encoded by the *NF1* gene[32], which has all the characteristics of a tumour suppressor. One allele is lost in people with type I neurofibromatosis — a dominant syndrome that is characterized by large numbers of benign, and occasionally malignant, tumours in tissues of neural-crest origin. In the malignant tumours, both copies of *NF1* have been lost, resulting in activation of RAS.

Growth-factor-receptor activation. RAS signalling pathways are also commonly activated in tumours in which growth-factor-receptor tyrosine kinases have been overexpressed. The most common examples are EGFR and ERBB2 (also known as HER2/neu), which are frequently activated by their overexpression in many types of cancer, including breast, ovarian and stomach carcinomas[33] (TABLE 1). In addition, a mutation in the *EGFR* gene results in the expression of a truncated receptor that lacks part of the extracellular domain[34], and this mutated receptor is found to be overactivated in a significant proportion of glioblastomas and some other tumour types. EGFR-family tyrosine kinases are also commonly activated by the autocrine production of EGF-like factors such as transforming growth factor-α (TGF-α) in tumours. The exact frequency of this activation is hard to establish in human tumours, but it seems to be very high in tumours of epithelial origin.

Mutation or amplification of RAS effectors. Recently, it has emerged that BRAF is frequently activated by mutation in human tumours (TABLE 1) — in particular, in melanomas (~70%) and colon carcinoma (~15%). Mutations in *BRAF* occur in a very limited number of residues in the kinase domain, all of which result in kinase activation. The PI3K pathway is activated as a result of amplification of the *p110α* gene in a relatively small proportion of ovarian tumours, and also by the amplification of its downstream target *AKT2* in ovarian and breast tumours[35] (TABLE 1). However, the most significant direct activation of this pathway in tumours comes from deletion of the tumour suppressor gene *PTEN* (phosphatase and tensin homologue). This gene encodes a lipid phosphatase that removes the phosphate from the 3′ position of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, so reversing the accumulation of these second messengers that is caused by PI3K. *PTEN* is deleted in ~30–40% of human tumours[36] (TABLE 1), making it the second most significant tumour-suppressor gene after *TP53* (which encodes p53 in humans).

Cancer therapeutic agents targeting RAS

Farnesyltransferase inhibitors. The covalent attachment of the farnesyl isoprenoid group to the HRAS, KRAS and NRAS proteins is the first step in the carboxy-terminal post-translational modification of these proteins (BOX 1). Because this processing — which results in the stable

p110α
One isoform of the catalytic subunit of phosphoinositide 3-kinase (PI3K). Type I PI3Ks are made up of either α, β, γ or δ forms of p110, together with a regulatory p85 subunit (for p110α, β and δ) or p101 subunit (for p110γ).

Table 2 | Selected RAS-directed therapies in clinical trials

Target	Class	Drug	Trial phase	Trial results
RAS processing: farnesyltransferase inhibitors	CAAX peptidomimetic	R115777* (Zanestra)	III	No effect on colorectal cancer; no effect on survival in combination with gemcitabine in pancreatic cancer
	CAAX peptidomimetic	SCH66336 (Sarasar)	II	No effect on transitional cell cancer; less effective alone than gemcitabine on pancreatic cancer
	CAAX peptidomimetic	L778,123	II	No benefit on NSCLC; discontinued due to adverse cardiac effects
	CAAX peptidomimetic	BMS-214662‡	I	Some stable disease
HRAS mRNA	Antisense oligonucleotide	ISIS 2503	II	No effects
c-*RAF1* mRNA	Antisense oligonucleotide	ISIS 5132	II	No effects
MEK	Kinase inhibitor (non-ATP site)	CI-1040 (PD184352)	I	Some partial responses
RAF	Kinase inhibitor (ATP site)	BAY 43-9006	I	Some partial responses
EGFR	Kinase inhibitor (ATP site)	ZD1839 (Iressa)	III	Disappointing results in combination therapy against NSCLC, despite promising single-agent results in Phase II
EGFR	Kinase inhibitor (ATP site)	OSI-774 (Tarceva)	III	Some responses against NSCLC, ovarian, and head and neck cancer
EGFR	Chimeric antibody	IMC-C225 (Cetuximab, Erbitux)	II	Some responses in colorectal cancer
ERBB2	Humanized antibody	Herceptin (Trastuzamab)	III	Licensed for use on breast cancer

*REF. 86. ‡REF. 87. See text for remaining references. Where not specifically referenced, information is derived from ASCO abstracts (www.asco.org). EGFR, epidermal-growth-factor receptor; NSCLC, non-small-cell lung cancer.

localization of RAS to the plasma membrane — is essential for the biological activity of RAS, it was an obvious early target for the design of new rational therapies against the RAS pathway.

Several strategies have been developed to inhibit the farnesylation of RAS, the most common being the design of compounds that mimic the carboxy-terminal CAAX motif of RAS and compete for binding to farnesyltransferase. The earliest of these compounds were peptides, which were subsequently modified to give better drug properties (CAAX peptidomimetics). Another strategy has been to make compounds that compete with the farnesyl pyrophosphate group. Yet another group of drugs share properties of both farnesyl pyrophosphate and CAAX, so mimicking a transition state in the farnesylation process; these drugs are known as bisubstrate analogues.

Through a massive effort by many leading pharmaceutical companies, a large number of highly effective farnesyltransferase inhibitors (FTIs) have been identified, mostly through high-throughput screening of compound libraries, and developed as potential cancer therapies[37,38]. These were shown to efficiently inhibit the farnesylation of HRAS in cells in culture, which led to high expectations that they would be effective against the 20% of human tumours that have activating mutations in *RAS* genes. In an early experiment, transgenic mice expressing activated *HRAS* under the MMTV promoter were treated with a peptidomimetic FTI. These mice develop frequent mammary carcinomas, which showed very impressive reversal by the FTI[39]. In addition, the FTI caused little general toxicity. This work showed great promise for FTIs in the treatment of human cancers.

Unfortunately, this early potential has not been realized. The mode of action of FTIs has become increasingly unclear, and the initial spectacular successes that were achieved in mouse models have not been repeated in human patients. The root of the problem lies in the fact that, although HRAS is exclusively modified by farnesyltransferase, KRAS and, to a lesser extent, NRAS can also be modified by geranylgeranyltransferase (GGT). This results in the transfer to RAS of a different isoprenoid group — with 20 carbon atoms, rather than the 15 that are found in the farnesyl group — that is still able to support the biological activity of RAS. Geranylgeranylation of KRAS and NRAS becomes important only when farnesylation is blocked. As the vast majority of *RAS* mutations in human tumours are in *KRAS*, followed by *NRAS*, with very few in *HRAS*, it is likely that inhibition of mutant RAS farnesylation is not responsible for any antitumour effects of FTIs. Attempting to inhibit the function of KRAS and NRAS by using FTIs and GGTIs together has failed because of the very high toxicity that is associated with this combination[40]. Indeed, it is likely that the lack of toxicity of FTIs is due to the fact that they fail to inhibit effectively the function of all endogenous RAS proteins, which are known to be essential for normal cell growth.

The FTIs that have been developed to target RAS also inhibit the farnesylation of a great many other proteins. An argument has been put forward repeatedly that the ability of FTIs to inhibit tumour-cell growth and survival is due to the induction of aberrant modification of the RAS-related protein RHOB with geranylgeranyl rather than farnesyl groups, resulting in an altered function of this protein[41]. Although this model has some compelling features, strong arguments to the contrary have also been made[37], so this issue is still unresolved at present.

Despite uncertainty about their mechanism of function, FTIs do have marked effects on the growth and survival of some tumour cell lines *in vitro* and on xenografts in nude mice, although not necessarily those expressing activated RAS. Because FTIs inhibit the growth of some oncogenic KRAS-expressing human tumour cells as xenografts in nude mice, despite the fact that KRAS processing is not blocked, they clearly have potential as anti-tumour drugs, even though the mechanism of action is uncertain. The effects of FTIs in these pre-clinical systems have been reviewed extensively recently [42,43]. These data have prompted the launch of several clinical trials using FTIs, even though their real target is still controversial. The success or otherwise of these trials will determine whether there is a future for the use of FTIs as cancer therapy, and for what type of tumours they might be most suitable. The FTIs for which trials have been reported so far are listed in TABLE 2.

Despite the rebranding of FTIs as signal-transduction, rather than RAS-specific, inhibitors, they are yet to show great promise in the clinic, particularly in Phase II and III trials on common cancers. However, some more encouraging results have emerged from early trials on leukaemias[44]. It is certainly possible that they could eventually prove to be beneficial in certain tumour types or in combination with other agents, but it seems unlikely that they will live up to the early high expectations generated by the pre-clinical models.

Antisense oligonucleotides against RAS and RAF. A different approach to the therapeutic targeting of the RAS pathways is to inhibit the expression of HRAS and its downstream target c-RAF1; this strategy has already reached clinical trials. The inhibition has been achieved by using short antisense synthetic oligonucleotides that are specific for sequences in the mRNAs for these proteins[45]. On binding to cRNA, these oligonucleotides can inhibit protein production by several mechanisms: one is to promote degradation of the mRNA by directing RNaseH to the RNA–DNA duplex; another is to interfere with translation. Early trials in nude mouse models showed promise in reducing the growth of KRAS-transformed lung tumour cells[46]. Isis Pharmaceuticals have been successful in developing several stabilized phosphorothioate derivatives of oligonucleotides that effectively reduce the expression of *HRAS* (ISIS2503) or c-*RAF1* (ISIS5132) when added to cells[47,48] (TABLE 2).

Several conceptual difficulties exist with the use of these reagents as cancer therapies. One problem concerns the very high level of specificity of these agents, which could mean that they do not target the most important proteins in the tumours — for example, mutation of *HRAS* is very rare in tumours, so 'removing' its expression is likely to be less effective than targeting *KRAS*. Antisense oligonucleotides against *KRAS* (ISIS6957) that are effective in cell culture have been developed, but have not been taken into clinical trials[47]. Similarly, c-RAF1 might not be the most important mediator of RAS-induced ERK/MAPK activation, as this function is effectively provided by BRAF[49]. Another problem is the challenge of effectively delivering these relatively large molecules to tumours and ensuring that they are taken up by the malignant cells. In addition, several problems with nonspecific toxicity of antisense oligonucleotides have been encountered.

Despite this, ISIS2503 and ISIS5132 — which target *HRAS* and c-*RAF1*, respectively — are now in Phase II trials, having been proved to be relatively non-toxic in Phase I trials. A Phase II trial of ISIS5132 showed no efficacy against non-small-cell lung carcinoma (NSCLC)[50], but it has been difficult to achieve a reduction in target protein levels in peripheral blood lymphocytes in these patients. Whether or not antisense therapy is likely to prove effective as a mainstream tumour treatment remains unclear.

Kinase inhibitors targeting RAS effector pathways. In recent years, the pharmaceutical industry has become increasingly adept at developing effective inhibitors of protein kinases. The most impressive example of such inhibitors in the clinic has been imatinib (Glivec) — the inhibitor of BCR–ABL that has provided a great leap forward in the treatment of chronic myeloid leukaemia[51]. Inhibitors of kinases that are involved in several RAS signalling pathways have been under development for some time, and some have now entered clinical trials. These include inhibitors that target both upstream regulators of RAS, such as growth-factor receptors, and downstream effectors, such as the components of the RAF–MAPK pathway.

Drugs that act downstream of RAS, targeting the RAF–MAPK pathway at two different points, are in trials at present. The RAF–MAPK pathway is activated in ~30% of human tumours, as determined by the phosphorylation status of the MAPKs ERK1 and ERK2 (REF. 52). Inhibitors were developed several years ago against MEK1 and MEK2 — the closely related dual-specificity kinases that are substrates of RAF and that phosphorylate and activate ERK1 and ERK2. PD98059 (REF. 53) and U0126 (REF. 54) are fairly specific, non-ATP-competitive MEK inhibitors that have been widely used for research purposes. They effectively inhibit ERK activation and can inhibit the proliferation, survival and motility of some tumour cell lines under certain conditions[55]. MEK inhibitors have proved to be effective in inhibiting the growth of tumours in immunodeficient mice; for example, the related inhibitor PD184352 has been used in a large study of colon carcinomas[56]. The orally active PD184352, which has now been redesignated CI-1040, has recently undergone Phase I clinical trials (TABLE 2). It seems to be well tolerated at doses that

Box 2 | Other approaches related to RAS in the clinic

Signalling pathways, by their nature, form interlinked networks. So, defining precisely what is, and what is not, a RAS signalling pathway is, to some extent, inevitably an issue of semantics. Although the agents described in the main text target the core of the RAS signalling module, several other drugs in clinical trials are directed at more peripheral components of these pathways, or pathways that show significant cross-talk with RAS. Such drugs include CCI-779 and RAD-001, inhibitors of mTOR — the mammalian target of rapamycin protein that is activated by the phosphatidylinositol 3-kinase (PI3K) pathway, another downstream effector of RAS[79]. Another is the geldanamycin derivative 17AAG, which inhibits the heat-shock protein HSP90: this results in the destabilization of a range of signalling proteins, including AKT/PKB, c-RAF1 and ERBB2 (REF. 80).

Several therapies target proteins that are transcriptionally induced by activated RAS, especially through the RAF–mitogen-activated-protein-kinase pathway. For example, activation of this pathway induces expression of matrix metalloproteinases and angiogenic factors[66]. Several inhibitors of matrix metalloproteinases have been developed with the hope of inhibiting tumour invasion and metastasis[81]. Several inhibitors of angiogenic signalling, particularly inhibitors of the vascular endothelial growth factor (VEGF) receptor tyrosine kinase, are also being tested in the clinic[82].

Other therapies that are targeted at RAS include gene therapy and immunotherapy approaches. Gene-replacement therapy has provided few, if any, indications as yet that it is likely to be effective in the treatment of cancer, although gene-directed enzyme prodrug therapy, sometimes called suicide gene therapy, shows more promise[83]. More excitement has been generated by oncolytic viruses that target cells in which the p53 or RB pathways have become deregulated[84].

inhibit ERK activation in patients' peripheral-blood mononuclear cells. Given the large number of tumours that have an activated ERK–MAPK pathway, it is certainly possible that this drug could prove to be effective against several tumour types. The results of further trials will be awaited with interest.

The other drug targeting the ERK–MAPK pathway that has reached clinical trials is BAY43-9006, an orally active inhibitor of c-RAF1 that targets the ATP-binding site[57] (TABLE 2). It is also active against BRAF, so it is likely to be effective at reversing ERK activation that is caused by RAS or BRAF mutation, which is evident in most melanomas[58]. This provides an important advantage over the exclusive targeting of c-RAF1 that is achieved by the antisense approach (see above). BAY43-9006 is well tolerated at doses that result in inhibition of phorbol-ester-induced ERK phosphorylation in patients' peripheral-blood lymphocytes. As with CI-1040, the performance of this drug in Phase II and III trials will be of considerable importance.

Kinase inhibitors targeting pathways upstream of RAS. Even in tumours in which RAS is not mutationally activated, RAS might well be stimulated by aberrant activation of upstream signalling pathways. The components of the regulatory system for RAS that have proved most amenable to therapeutic intervention are the growth-factor-receptor tyrosine kinases. In particular, the EGFR and its close relative ERBB2 are able to stimulate RAS through GRB2 and SOS. Targeting EGFR or ERBB2 might be effective in the types of tumours that overexpress them, resulting in failure to activate endogenous wild-type RAS and several other downstream proliferative and anti-apoptotic pathways. However, this approach could also have potential against tumours that carry activating mutations in *RAS* itself because these cells typically produce large amounts of autocrine growth factors of the EGF family as a result of activation of the RAF–ERK pathway[59]. Importantly, these growth factors might be able to activate other proliferative and anti-apoptotic pathways beyond what RAS alone can do. Data from transgenic mice show that skin tumour formation in response to moderate levels of RAS activation, due to expression of constitutively activated SOS, is dependent on autocrine activation of EGFR[60].

Several approaches have been used to target the ERBB family, but by far the most progress has been made in two areas: small-molecule tyrosine kinase inhibitors[61,62] and humanized antibodies against the receptor extracellular domains[63]. At least six small-molecule inhibitors of EGFR tyrosine kinase activity are now in clinical trials. These all effectively block the kinase activity of this receptor and might also inhibit other members of the ERBB family to some degree, either as a result of cross-reactivity with their closely related kinase domains or due to inhibition of their transphosphorylation by EGFR in heterodimers at the cell surface. The two drugs in this class that are at the most advanced stage of development are ZD1839 (Iressa) from AstraZeneca and OSI-774 (Tarceva) from OSI/Genentech (TABLE 2).

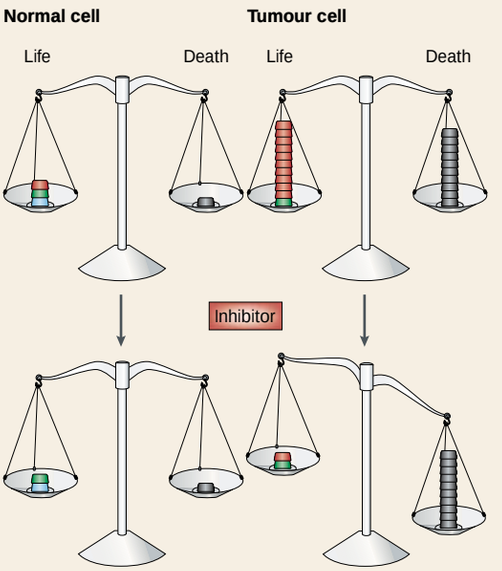
ZD1839 has been through several Phase I trials, with the most common toxic side effect being skin rash and diarrhoea. Four Phase II trials have also been completed. Promising single-agent clinical antitumour activity has been reported in advanced NSCLC, head and neck cancer and prostate carcinoma[64]. No effect was seen against metastatic renal-cell carcinoma. Phase III randomized trials of ZD1839, in combination with gemcitabine and cisplatin or with paclitaxel and carboplatin, for the treatment of NSCLC proved disappointing, given the expectations from earlier trials, leading to re-evaluation of treatment regimens.

OSI-774 is similar to ZD1839 in its pharmacological characteristics, with around a tenfold lower IC_{50} against EGFR kinase activity *in vitro*[65]. The two drugs had similar toxicities in Phase I studies, whereas Phase II trials using OSI-774 as a single agent produced promising results against NSCLC, ovarian, and head and neck cancer. Other EGFR-directed small-molecule tyrosine kinase inhibitors in early-stage trials include PKI116 (Novartis), GW2016 (GlaxoSmithKline), EKB-569 (Genetics Institute/ Wyeth-Ayerst) and CI-1033 (previously PD183805; Pfizer). Overall, this class of drug seems to show real potential, especially in the treatment of lung cancer. A comparison of clinical results against *RAS* gene mutation status has not been made as yet, although *RAS* mutations are common in this type of tumour. It will be interesting to find out whether tumours with *RAS* mutations are more or less sensitive to EGFR inhibitors than tumours without the mutations. An increase in sensitivity would indicate that autocrine EGF signalling is important in mutant RAS-driven cancer progression in the clinic.

IC_{50}
The concentration of a drug giving a 50% inhibition of the activity of a target enzyme.

Box 3 | Survival signals in tumour cells: an Achille's heel?

The issue of the degree of the therapeutic window that will be provided by drugs that target the RAS pathways is a crucial one. All cells use RAS signalling pathways to some extent, so there is a danger that inhibitors will have severe effects on normal cells as well as tumour cells. Potent inhibition of RAS function through the expression of dominant-negative mutants or microinjection of neutralizing antibodies has long been known to block normal cell proliferation. Although, ultimately, each drug target has to be validated experimentally for its differential effect on tumour versus normal cells, there are conceptual reasons for believing that certain types of signalling inhibitors, in particular those that inhibit survival pathways, might selectively disadvantage tumour cells. As represented in the figure by the 'scales' of life and death signals that the cell is experiencing, a normal cell requires a continuous low level of survival signal to remain alive[85]. These survival signals emanate from various different sources, including adhesion to extracellular matrix, soluble factors in the extracellular environment and interactions between cells. Each of these acts to instruct the cell that it is in an appropriate environment. The cells are also exposed to a low level of cell-death signals, perhaps due to occasional DNA damage or oxidative stress, but the balance of signals favours survival. In the tumour cells, microevolutionary processes have led to the selection of cells with greatly increased survival signalling — for example, by the loss of *PTEN*. Once a mutation has given a cell a survival advantage, it is then able to tolerate more death signals. For example, this allows it to survive notable DNA damage as a result of loss of cell-cycle control and rapid proliferation, and to be less easily killed by hypoxia and by immune system attack; all of these events result in the tumour cell's increasingly antisocial behaviour. It can also afford to dispense with previously required survival signals that were provided by matrix and other cells and to grow in a completely independent manner that is characteristic of tumour cells. This disorganized growth is therefore occurring at high levels of both apoptotic and survival signals, with the cells being dependent on one or a few strongly activated survival pathways, compared with a more complex pattern of survival signals for the normal cell. If both cell types are now treated with a survival-pathway inhibitor that targets the pathway on which the tumour cell is reliant, rapid death of the tumour cell will result, driven by the damage it has accumulated and formerly been able to ignore. The normal cell, by contrast, might survive, having much lower intrinsic levels of death signals and receiving a wider range of survival signals.



The ability of activated RAF to promote cell growth and survival via autocrine EGF signalling, without the possibility of direct activation of PI3K (which exists for mutant *RAS*)[66], might make tumours in which RAF proteins are activated by mutation attractive targets for EGFR inhibitor therapy[58].

The other important class of therapeutic agent that is directed against ERBB-family receptors are humanized monoclonal antibodies. These agents bind to the extracellular domain of the receptors, inhibit their activation by ligand, and promote receptor internalization and downregulation. In addition, they are thought to induce a cytotoxic immune response against the tumour cells[67]. The most advanced of this drug type against EGFR is a chimeric antibody — IMC-C225 — which is developed by ImClone Systems and Bristol-Myers Squibb (Cetuximab, Erbitux) (TABLE 2). Phase I trials showed that the drug was reasonably well tolerated[68], and a Phase II trial showed promising results in advanced colorectal carcinoma. Unfortunately, widely reported regulatory difficulties have delayed further development of this potentially important drug.

The other important anti-receptor drug is trastuzamab (Herceptin), which was developed by Genentech. This humanized monoclonal antibody against ERBB2 has proved to be effective against breast carcinomas in which ERBB2 is highly expressed — that is, 20–30% of cases of metastatic breast cancer. In Phase III trials, it extended the median survival of patients from 20.3 months to 25.1 months[69]. Despite some problems with cardiac toxicities, trastuzamab has now been licensed in the United States and elsewhere as a single agent for treating metastatic breast cancer with ERBB2 overexpression. As *RAS* mutations are extremely rare in breast cancer, it is unlikely that trastuzamab is interfering with a mutant *RAS*-induced autocrine loop, although it is certainly capable of blocking the activation of endogenous wild-type RAS proteins in the breast cancer cells[70,71]. Despite the considerable success of antibody-based drugs against ERBB-family proteins, these agents are likely to remain expensive relative to small-molecule inhibitors — even after patent protection expires — due to the difficulty of making large amounts of proteins for clinical use.

Other therapeutic approaches that are more distantly related to RAS pathways are considered in BOX 2.

New directions in targeting of RAS pathways

A recurrent difficulty with targeting important growth and survival pathways, such as those controlled by the RAS signalling pathways, is the achievement of a sufficient therapeutic window to enable the elimination of tumour cells but not their normal neighbours. The pathways described above are used by normal cells as well as by tumour cells, although the tumour cells might develop a greater reliance on them (see BOX 3). The ideal cancer therapy would exclusively target transformed cells without harming normal cells. In practice, this is very difficult. The only treatments to attempt this for RAS signalling pathways are immunotherapies that aim to direct the immune response to cells that express specific mutations in the *RAS* oncogene[72]. However, the effectiveness of these drugs has yet to be shown.

Several other strategies are being used to inhibit signalling pathways that commonly show increased activity in tumours, even if they are also used by normal cells. The development of kinase inhibitors that are directed against AKT/PKB is, for example, receiving much attention. Some inhibitors have already been reported, although their specificity is unclear[73]. They might be very effective at removing the survival advantage of cells in which *PTEN* has been deleted or in which RAS has been activated, but their effect on normal cells will require further analysis. Along similar lines, PI3K inhibitors might also be useful, although given that they act higher up the pathway, they might have more widespread effects.

Additional approaches that target the post-translational processing of RAS after isoprenylation are also being pursued. RCE1, the CAAX endoprotease that removes the end three amino acids, and isoprenylcysteine carboxyl methyltransferase (ICMT), the methyltransferase that methylates the new carboxyl terminus residue, are both required for efficient function of RAS[74]. Inhibitors against these enzymes might be useful for treating tumours with mutant *RAS*, although existing RCE1 inhibitors have only modest effects on transformed cell growth. Localization of RAS to the plasma membrane can also be disrupted by farnesylthiosalicylic acid, which seems to remove correctly modified RAS from the membrane, resulting in reversal of the transformed phenotype of cultured cells that express activated RAS[75].

Very good structural information about the interaction of RAS with its effectors exists, with high-resolution structures being available for complexes of RAS with RAL, PI3K and RALGDS. This has led to interest in designing drugs that inhibit these protein–protein interactions. Early work focused on the use of peptides, but they yielded little success. More recently, small-molecule screens have been carried out. However, it is clear that the large size of the interaction site between the two molecules will make this approach very challenging. An RNA APTAMER has been reported that will effectively inhibit the interaction of RAS with RAF, but it remains to be seen whether this approach has potential for clinical application[76]. A small-molecule inhibitor of RAS interaction with RAF has recently been shown to revert RAS transformation of cells in culture[77].

A further target that might be suitable for therapeutic intervention is the GTP-binding site of RAS. In principle, it should be possible to make small molecules that bind to this site on RAS and inhibit its interaction with GTP, while maintaining it in an inactive conformation: this is analogous to the approach that has been successfully used for the ATP-binding site of many protein kinases. However, the very high binding affinity of RAS for GTP and GDP, together with the high concentration of GTP in the cell, might make this impractical. A more subtle variation on this idea is to develop GTP analogues that activated mutants of RAS can effectively hydrolyse to GDP. 3,4-Diaminobenzophenone-phosphoramidate-GTP (DABP-GTP) has been shown to be hydrolysed by codon 12 mutant RAS *in vitro* several hundred times more rapidly than GTP[78]. A further development could be imagined along these lines: a hypothetical drug capable of interacting with certain residues (such as 61) that are involved in the hydrophilic attack on the γ-phosphate of GTP could restore GTPase activity to the oncogenic RAS protein without affecting any other cellular function. Such a drug might convert oncogenic RAS proteins into normal molecules. These types of reagents could eventually prove suitable for clinical development and have the advantage of selectively targeting mutant RAS.

Conclusions

Just over 20 years on from the discovery that *RAS* was an activated oncogene in human tumours, we have learnt an enormous amount of molecular detail about how the RAS proteins function. The signalling network they control is not simple, with multiple points of bifurcation, cross-talk and feedback. We might be deluding ourselves in thinking that we have a reasonable understanding of this system, and surely much is still to be learnt, but we clearly have a great many leads that are worth pursuing in the search for effective cancer therapies. Issues of specificity will remain important: how specific should a drug be for its target, and can it be too specific, thereby failing to inhibit related molecules that might compensate for the true target? More importantly, the issue of how great a therapeutic window is likely to be offered by many of these rationally designed drugs remains uncertain. The RAS signalling pathways, in common with many of the other signalling pathways that are scrutinized by the pharmaceutical industry, are fundamental to the existence of normal as well as tumour cells, even if tumour cells might be more reliant on them. Even if these drugs and their successors are effective, the problem of resistance will inevitably arise quite quickly, given the strong selective pressure applied to the genetically unstable cancer cells. Ultimately, this is most likely to be overcome by taking a cue from the battle with

RNA APTAMER
A synthetic RNA molecule selected for ability to interact with a target protein.

infectious diseases and using many different effective drugs simultaneously, assuming that we ever have access to such an arsenal. In terms of the design of clinical trials, clinicians might need to revise the end points that are used to determine drug efficacy: if reduction of tumour mass by single agents is the only end point used, many useful drugs might be discarded before they have a chance to show their potential in combinations. The development of appropriate surrogate markers to monitor drug action will be crucial in this regard. Tumour stratification will also be important in selecting patients on which to test these inhibitors if drugs that are effective on relatively rare tumour types are to be identified. Although we are still only near the beginning of a very long and labour-intensive process, there is reason to be cautiously optimistic that eventually some of the drugs discussed here, or more likely their derivatives, will form the basis of truly effective treatments for many common cancers.

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