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New drugable targets in the Ras pathway?

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Abstract

Ras proteins are key elements in the regulation of cellular proliferation differentiation and survival. Mutational activation of Ras or of components of its effector pathways are detected in a third of human cancers and are essential for the genesis and maintenance of the tumoral phenotype. Colossal efforts have been dedicated to the development of therapeutic agents whereby aberrant Ras signals, and subsequently tumor progression, could be inhibited. However, many of these initiatives have proven less successful than originally expected. The present review summarizes the current status of some of these developments, the challenges that have arisen during preclinical and clinical stages and how novel venues of research on the Ras field make conceivable new strategies towards the development of antitumoral agents alternative to those currently in use. These new approaches would be aimed at disrupting key protein-protein interactions essential for the conveyance of Ras aberrant signals or be directed against new proteins recently demonstrated to be critical participants in Ras-regulated pathways.

Key words: Ras, Signal transduction, Cancer, Raf, MEK, ERK, MST2
Introduction

Three decades have passed since seminal discoveries demonstrated a causal link between the ras genes harbored in murine sarcoma retroviruses and cancer pathogenesis. In this time, we have recognized the ras gene products, the GTPases, H-Ras, N-Ras and K-Ras 4B/4A, as key signal transducers. We have learned the mechanisms whereby Ras is regulated by GEFs and GAPs. We have identified the constituents of the effector pathways through which Ras relays its signals to the interior of the cell and we have started to explore the peculiarities of Ras signals at the different cellular microenvironments where they ensue [1,2]. The unraveling of these biochemical milestones has progressed in parallel to the acquisition of a broad knowledge on the role played by Ras in cancer. Since the early 80s, in which Hras was identified as the first human oncogene and its activating mutations were defined, mutant alleles of the three ras genes have been detected in many human cancers. Analysis of over 40,000 tumor samples indicates an activating mutation rate of 22%, 8.2% and 3.7% for Kras, Nras and Hras respectively. If to this figure, we add those cases in which mutational activation is detected, in most cases in a non-overlapping occurrence, in components of Ras effector pathways, namely: Braf (22%) and p110α PI3K (12%), the proportion of human neoplasias exhibiting some hyperactive Ras-related pathway exceeds 50% (http://www.sanger.ac.uk/genetics/CGP/cosmic/Catalogue Of Somatic Mutations In Cancer; [3]. In addition, a vast body of data has been gathered substantiating the importance of Ras signals in cancer initiation and progression. Activated mutants, of Ras or of its downstream effectors, have been shown to induce malignant transformation in many cell types, due to unregulated proliferation, differentiation or survival. Pharmacologic and genetic inhibition of Ras signals have unveiled its necessity for the upbringing and maintenance of the transformed phenotype [1,3] and sophisticated animal models have endorsed the importance of Ras and its downstream routes for tumorogenesis in vivo [4].
With all this in perspective, it is no surprise that Ras has attracted enormous attention, both by academia and industry, for its potential as a target in cancer therapy. From the early days, gigantic efforts have been dedicated to strategies directed at curtailing Ras aberrant signals as a means of stopping tumor progression. Most of these initiatives have been aimed at either inactivating Ras or at inhibiting the activity of some of the kinases that populate its downstream pathways. The results have been of mixed nature. The attempts to inactivate oncogenic Ras have been mostly disheartening. In the case of the approaches towards inhibiting downstream kinases, mostly directed against Raf and MEK family kinases, several generations of inhibitors have now been under test. Some have made their way through the gradual phases of clinical trials. Others have been thwarted mostly by poor clinical efficacy and/or undesired toxic effects.

Are there alternative ways to aim at Ras signals to enable the generation of more efficient while less toxic inhibitors? New data have unveiled a plethora of proteins and processes that play critical regulatory roles on Ras signals, so as to severely impact on Ras biological outputs if interfered with. Moreover, novel functional liaisons have arisen lately, introducing new players into Ras-regulated pathways, which could pose potential new targets for therapeutic intervention. This review presents an overview of such novel findings that could provide new ways for interfering with Ras signals.
Old drugs for old concepts.

Since the early days, when it was found that amino acid substitutions at codons 12, 13 and 61 impaired Ras GTPase activity making it unresponsive to GAPs, substantial efforts were devoted to find means whereby Ras enzymatic activity could be restored, to no avail thus far. An alternative strategy was found in inhibiting Ras access to the plasma-membrane, an essential requisite for its biological activity [5], mainly by the use of farnesyltransferase inhibitors. These yielded spectacular results in mouse models that, unfortunately, were not repeated in human clinical trials, probably because K-Ras and N-Ras are also modified by geranylgeranylation, a process that becomes particularly active when farnesylation is blocked (for a review see [6]). Combined inhibition of farnesylation and geranylgeranylation showed very high toxicity at preclinical studies to be considered an option [7]. Another attempt pursued the blockade of Ras expression by using antisense oligonucleotide [8], but further progress was aborted probably due to concerns about the efficient delivery of these large molecules to tumour cells in vivo. As such, as of today, Ras remains a pharmacologically inaccessible target.

With Ras out of the central stage, the kinases included in several of Ras downstream pathways remained attractive candidates, in particular those forming the cascade leading to the activation of ERK MAP kinases [3] (Fig. 1). The Raf kinase family (ARaf, BRaf and CRaf) has emerged as an appealing target, after the discovery of activating mutations in BRaf in 60% of melanomas, 40% thyroid and 20% colorectal and ovarian tumors [9,10]. At this moment, four compounds: sorafenib, RAF265, PLX4032 and XL241 are being evaluated in different tumors (for reviews see [11,12]). These inhibitors function as ATP-competitive analogues that, by definition, tend to exhibit some degree of unspecificity. Thus, there have always been doubts on whether their anti-tumoral effects are due to “off target” effects as opposed to their anti-Raf activity. Such is the case for sorafenib, which also inhibits VEGF-2, VEGF-3 and PDGF-β, whose anti-oncogenic properties are independent of its inhibitory
effect on BRaf [13]. Another caveat is that tumours tend to acquire resistance to these inhibitors, probably due to “gatekeeper” mutations. For example, PLX4032: to which resistance is developed in a median time of 8-9 months [14]. Of major concern are recent discoveries demonstrating that Raf inhibitors can have effects opposed to those looked for, depending on the cellular context. In tumours harbouring oncogenic K-Ras, Raf inhibitors promote ERK activation in a Ras-dependent fashion, with subsequent stimulation of tumour growth [15,16]. Likewise, dead-kinase BRaf and oncogenic K-Ras cooperate to induce melanoma in mice [17]. These results speak aloud for the necessity of screening patients for BRaf and Ras mutations, in order to separate those likely to respond from those in which anti-Raf therapies could be harmful. Importantly, they also reduce the therapeutic arsenal available to treat the large number of tumours harbouring Ras mutations.

Inhibitors for MEK kinases (MEK1 and MEK2) are also currently available as a therapeutic option. Unlike Raf inhibitors, these are not ATP-mimetics and, consequently, exhibit an exquisite specificity. Their mode of action resides in binding to a unique “inhibitor-binding” pocket, locking the kinase in a closed, inactive form [18]. CI-1040, PD0325901, AZD6244 and XL518 have already undergone clinical evaluation. In the case of PD0325901 unacceptable toxicity has prompted its discontinuation [12]). CI-1040 demonstrated insufficient clinical activity, but its high safety profile has encouraged the development of derivatives [19]. AZD6244 has successfully gone through phase I in patients with advanced cancer [20] and is currently under phase II evaluation, in combination with other chemotherapeutic agents, in particular sorafenib and PI3K inhibitors, following promising results in mouse models [21,22]. Acquisition of resistance is also a concern for MEK inhibitors. Screening of tumors from relapsed patients following AZD6244 treatment, detected mutations in MEK that conferred resistance to the inhibitor [23]. Resistance can also arise in the absence of mutations in MEK itself, probably due to alterations in other key regulatory molecules. For example, K-Ras activation has been described to confer resistance to CI1040 [24].
The pharmacological agents currently available against Ras-mediated signals are not restricted to the Raf-ERK pathway. The PI3K pathway has also been the subject of intense therapeutic endeavours. Ras-GTP interacts with p110α and β catalytic subunits, activating a route that generates a strong antiapoptotic and pro-proliferative signal (Fig 1). The PI3K pathway is negatively regulated by the phosphatase PTEN [25]. Different components of the PI3K signalling pathway are deregulated in cancer. The loss of PTEN functions occurs in 30-40% of tumours (http://www.sanger.ac.uk/genetics/CGP/cosmic/) resulting the maintenance of downstream kinase AKT in a hyperactive status. Gain-of-function mutations have also been detected in p110α and amplifications are frequent in the gene encoding for p110β [26]. Finally, somatic mutations and amplifications of the AKT family genes have also been reported [27]. Unlike the Raf pathway, overlapping mutations in different components of the PI3K pathway do occur depending on the tumour type [28]: in endometrial cancers, Ras and PI3K mutation are mutually exclusive, suggesting that p110α is not necessary for the initiation of these tumours. Meanwhile, concomitant mutation of Ras and p110α are observed in 7% of colorectal cancers, indicating that these oncogenes synergise to confer a selective advantage in these cells [29]. The co-existence of such mutations is likely to be helpful to classify patients for treatment.

Several inhibitors against different elements of the PI3K pathway are currently under evaluation. LY294002 and wortmannin were the first PI3K inhibitors to be used in preclinical studies. These were highly unspecific and very toxic in animal models, but derivatives are being developed some of which are in early clinical trials [26,30]. The observation that the PI3K inhibitor PI-103 also inhibited mTOR [31] has led to the development of dual PI3K-mTOR inhibitors. The rationale behind is that inhibiting both components concomitantly could have a stronger antitumoral effect, though concerns for potential severe side effects are also present. Nevertheless, derivatives of PI-103, like GDC0941 and other dual
inhibitors are now at different phases of clinical trials [25]. With respect to AKT family kinases, two types of inhibitors have been developed: ATP-competitive inhibitors and non-catalytic inhibitors. Most of the ATP-mimetic drugs can inhibit all AKT isoforms, but also other members of the AGC kinase family. For this reason, isoform-specific inhibitors are being developed. The non-catalytic inhibitors function by masking the PH domain, thereby preventing AKT binding to the membrane. Some of these like perifosine [32] and MK2206 [33] are already in clinical trials. Finally, inhibitors for mTOR have been a valid option for some time now. Rapamicin is already approved for the treatment of renal carcinomas and several derivatives are well advanced, such as temsirolimus and everolimus [34]. One problem is that mTOR inhibitors can trigger the activation of PI3K through the inhibition of a negative feedback loop [35]. However this problem could be overcome by the use of dual PI3K-mTOR inhibitors. A major concern is that targeting the PI3K pathway alone may not be sufficient to stop tumour progression. However, as mentioned before, the use of these drugs in combination with treatments against other pathways could prove beneficial for cancer patients.

While we are to expect more of these “classical” inhibitors to be delivered in the near future, the question arises whether new concepts about Ras-regulated pathways, serve us to establish the foundations on which to base the development of alternative types of drugs to inhibit aberrant signaling. In this respect, there may be some room for optimism.

**The Ras-ERK pathway: couples to be torn apart.**

It is long known that ERKs dimerize in response to stimulation [36]. However, the biochemical and biological significance of ERK dimerization were unknown until recently, when we demonstrated that ERK dimers are formed using scaffold proteins as dimerization platforms. These scaffold-dimer
complexes are critical for relaying ERKs cytoplasmic signals, by ensuing the interaction of ERKs with their cytoplasmic substrates. In contrast, activation of ERKs nuclear substrates is mostly undertaken by ERK monomers. Importantly, The inhibition of ERKs cytoplasmic component, by preventing ERK dimerization, is sufficient to abrogate cellular transformation and proliferation, and tumor formation in xenografts of lung, colorectal and bladder carcinoma cells [37].

Dimerization seems to be a common theme in the ERKs cascade. BRaf and c-Raf heterodimerize in a Ras-dependent manner following stimulation [38,39], whereas oncogenic mutants do so constitutively. This process requires the participation of the protein 14-3-3 and is essential for c-Raf transactivation by BRaf [40]. Importantly, mutations that prevent dimerization of Drosophila Raf also impair its catalytic function [41]. Likewise, BRaf impaired for dimerization by similar mutations, is incapable of transactivating c-Raf, stimulating ERK phosphorylation [15] and inducing transformation [42]. MEK family kinases also dimerize. MEK1 and MEK 2 form stable heterodimers not regulated by growth factor stimulation. These heterodimers are critical for fine-tuning the amplitude and duration of ERK activation, by a mechanism that entails negative feedback regulation by ERK via phosphorylation of MEK1. In its absence, heterodimer formation is prevented and MEK2 phosphorylation and ERK activation are prolonged [43].

These findings highlight that the pathway leading to ERK activation is much more than phosphorylation affairs, critical protein-protein interactions must take place to ensure the flow of its signals. Some of these interactions offer, at least conceptually, attractive targets for future antitumoral drugs. We have demonstrated that inhibiting ERK dimerization by genetic means is sufficient for forestalling tumoral cell proliferation [37] and our recent findings suggest that inhibiting ERK dimerization potentiates the apoptotic effects of drugs such as cisplatin, taxol and adryamicin.
Following this promising lead, we are currently screening for compounds that administered to cells can prevent ERK dimerization.

Similarly, Raf heterodimerization also shows potential as a target for therapeutic intervention. As demonstrated for Drosophila Raf [41] and mammalian BRaf-c-Raf dimers [15], preventing their formation significantly diminishes ERK signaling. Strategies for impeding the association between BRaf and c-Raf could be directly aimed at the dimerization interface, but also at the 14-3-3 binding sites, an essential interaction for dimerization to occur [40,42,44]. Unlike Raf and ERK dimers, MEK heterodimers seem not to be an option since blocking MEK1-MEK2 dimerization would result in enhancing ERK activation.

Importantly, dimerization interfaces and other protein-protein interaction motifs are probably unique regarding their molecular structure and interactions. Thus, they could yield drugs with much higher specificity, and subsequently less undesired, off-target, secondary effects, compared to those resulting from conventional strategies directed at inhibiting the enzymatic activities of kinases.

**Spatial regulators as therapeutic targets?**

Recent discoveries have established the concept that Ras signals are the sum of multiple, site-specified sub-signals [45]. Conceptually, searching for compounds that selectively block Ras sub-signals essential for tumor progression should produce drugs with reduced side effects, compared to compounds that block Ras signaling completely. Today, we know that within the PM, Ras is present at distinct microdomains [46]. In addition, Ras is also present in different endomembranes (for a review see [2]). At these sites, Ras is subject to site-specific control mechanisms undertaken by various regulatory proteins (for an extensive review see [47]). Recent findings have demonstrated that cellular
transformation can be prevented/reverted by the inhibition of specific, localization-defined sub-signals. For example, transformation by oncogenes like v-Src and Sis can be prevented by the inhibition of Ras signals coming from lipid rafts or disordered membrane [48]. Annexin A6, an ancillary protein that facilitates Ras inactivation via p120 GAP, suppresses Ras-induced transformation by recruiting p120 GAP specifically to non-raft PM microdomains [49]. Inhibiting Galectin-1, a protein essential to stabilize active H-Ras in non-raft PM “nanoclusters”, dislodges H-Ras from such structures and prevents fibroblast transformation [50]. These data illustrate that it is not necessary to suppress Ras signaling totally in order to obtain growth/transformation-suppressive responses. Thus, although conceptual at his moment, strategies directed at modulating the functions of some of these site-specific regulators could be a valid therapeutic option sometime in the future.

The same concept is applicable to events downstream from Ras. ERKs are cytoplasmic in unstimulated cells, an important fraction migrates to the nucleus, upon phosphorylation, where they perform essential functions [51]. However, ERKs extranuclear component is just as important, nearly half of the ~180 proteins identified as ERKs substrates, are non-nuclear proteins [52]. ERKs nuclear and cytoplasmic components are potential subjects for antineoplastic therapy. There is ample data showing that sequestering ERKs at the cytoplasm, thereby impeding ERKs nuclear signals, is sufficient for abrogating growth or provoking apoptosis in tumor cells [53-55]. ERKs nucleo-cytoplasmic shuttling is finely regulated. For efficient nuclear translocation, ERKs require: direct interaction with the nuclear pore complex; the participation of nuclear shuttles and a nuclear translocation signal, Ser-Pro-Ser, within the “insert” domain, that is phosphorylated upon stimulation, promoting ERKs nuclear translocation [56]. Conceptually, drugs aimed at masking this short sequence could represent an option to stop ERK nuclear translocation. As mentioned before, inhibiting ERKs cytoplasmic component by disrupting its dimerization is sufficient to prevent tumor progression [37]. Thus, the blockade of either of these subcellular components may be valid strategies for future therapeutic intervention.
The amplitude and intensity of ERKs signals are regulated by scaffold proteins, that assemble the components of the signaling cascade into a complex whereby signal optimization is achieved [57]. Scaffolds also play important roles in the spatial selectivity of ERKs, operating as transmitters of Ras signals, originated at different microenvironments, to specific ERKs substrates [58]. Apparently, distinct scaffolds operate in different subcellular localizations: KSR1 regulates ERKs signals at PM cholesterol-rich domains [59]; MP-1 at endosomes [60]; Sef at the GC [61]; Paxillin at focal adhesions [62]; and β-arrestins in clathrin-coated pits [63]. Scaffold proteins are candidates with an enormous potential to become site-specific therapeutic targets. Some of them, like KSR1 have no known function other than regulating ERKs, so no off-target effects should be expected. This could make KSR1 an ideal target for intervention. Indeed, mice deficient for KSR1 are normal though resistant to tumor development [64]. Unfortunately, much important structural information is still missing on, for example, how MEK and ERK dock onto KSR1. These sites harbor the potential to become hotspots for the design of drugs aimed at disrupting ERKs signal through competitive binding to scaffolds.

**Ras apoptotic route as a therapeutic target.**

It is well known that oncogenic Ras can trigger apoptosis in different cell types. Recent findings involve RASSF family proteins as critical pro-apoptotic effectors [65,66]. In response to Ras activation, members of this family, like RASSF1, NORE1 and RASSF2, activate the pro-apoptotic kinases MST1 and MST2, engaging a pathway through which cell survival is regulated (Fig 3) [67-69]. Contrarily, c-Raf inhibits MST2 in a kinase-independent fashion [70,71]. Thus, MST2 association with RASSFs promotes apoptosis whereas its liaison with c-Raf prevents it, resulting in aberrant growth. Even though many aspects about MST1/2 regulation are still unclear, targeting MST1/2 could be of use in the treatment of tumours harbouring Ras and, maybe, BRaf mutations. The development of
inhibitors for the interaction between c-Raf and MST2, thereby tilting the balance towards MST2 association with RASSF, should direct tumour cell to their disappearance by apoptosis.

**Other strategies to treat Ras tumours.**

Despite great efforts and investments devoted to the development of drugs aimed at the Ras pathways, little success has been obtained thus far. For this reason, new approaches are necessary. Recently, the concept of “non-oncogene addiction” has arisen, based in the observations that certain normal genes are necessary for the maintenance of the tumoral phenotype [72]. These genes can be directly regulated by an oncogene, but can also act in parallel pathways and, therefore, not appear as obvious candidates for pharmacological intervention. If these genes are inhibited, there is a synthetic lethality effect resulting in the activation of senescence or apoptotic responses, causing tumour regression. This concept has been use to identify genes necessary for the maintenance of “Ras-addicted” tumours. Using RNA-interference screenings PLK1, STK33, SYK, RON, TBK and integrin β6 have been identified as essential for the progression of tumours harbouring oncogenic Ras [73-75]. With all certainty, in the near future, more of these genes will be identified, some of which with the potential to be exploited for the development of new antineoplastic therapies.

**CONCLUSIONS**

The knowledge acquired during the last three decades about Ras has led to significant advances in tumor treatment. Unfortunately, many of the expectations deposited in Ras pathways-targeted drugs have not been fulfilled. High toxicity and resistance acquisition have hampered many of the drugs developed to date. While more of these “classical” inhibitors are to be expected, recent findings in the Ras field have put on the table new players and novel functional liaisons that provide an alternative
way to aim at Ras signals by targeting protein-protein interaction rather than enzymatic activities. These novel findings could pave a new era in drug discovery and in the development of new types of drugs for the treatment of tumours that present mutations in the Ras signalling pathways.
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These three publications unveil the "paradoxical" effect of Raf inhibitors on ERK activation and proliferation depending on the presence of oncogenic Ras, highlighting the need for patient screenings for Ras mutations prior treatment with Raf inhibitors.


Screening tumors from patients relapsed after treatment with MEK inhibitors identifies mutations within MEK drug-binding pocket that confer resistance to treatments.


These two studies demonstrate that mutations preventing BRaf-cRaf heterodimerization preclude ERKs activation.


Identifies MEK1 /MEK2 heterodimerization as an essential mechanisms for the regulation of ERK signal amplitude and intensity. In the absence of such heterodimerization enhances ERK signal.


Provides insights into how scaffolds confer signalling specificity to ERKs signals by relaying Ras signals from distinct compartments to different ERK substrates.


Explains the concept of non-oncogene addiction, define new hallmarks for cancer progression and propose new extrategies to develope for new cancer therapeutycs


These three publications identify new KRas genetic interactions with several new genes using RNAi screenings
Figure 1. “Classical” therapeutic targets in Ras pathways. A simplified representation of the Ras effector pathways harboring components whose enzymatic activities are being currently targeted for anti-tumor therapy. Therapeutic agents in black have been discontinued.
Figure 2. Novel strategies for targeting Ras pathways. A simplified model of Ras effector pathways harboring potential hot spots for therapeutic intervention, based in disrupting protein-protein interactions rather than enzymatic activities.