Targeting Raf-kinase: molecular rationales and translational issues

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Target-based therapy has been a promising anti-cancer strategy in the preclinical setting, but its efficacy is still limited in clinical practice. The latter was probably due to the lack of identification of molecular targets in order to predict clinical response and for the existence of multiple survival compensatory downstream pathways. Therefore, the use of downstream targets could be useful in order to avoid these overcoming pathways. One of these targets is Raf-kinase. In this review we describe the structure and functions of the components of Raf-kinase family and their relevance in proliferation and survival of tumor cells. Moreover, we illustrate the signal transduction pathways regulated by Raf-kinases. The main preclinical and clinical results obtained with the use of the Raf-kinase inhibitor BAY 43-9006 or sorafenib are also described. The multi-target function of sorafenib is also explained and the disclosure of new therapeutic opportunities based on the dual inhibition of cancer proliferation and neo-angiogenesis is discussed. In conclusion, Raf-kinase appears an appealing therapeutic target, even if other preclinical and clinical studies are warranted in order to evaluate the activity of sorafenib both in monotherapy and in combination with other agents.

Key words: Raf-kinase, apoptosis, cancer therapy, BAY 43–9006, angiogenesis, VEGF-R

General concepts of molecular-targeted therapy of human cancer

In recent years the research for novel antitumor strategies has changed from a major focus on conventional cytotoxic drugs to the development of novel agents based on specific biological rationales. To date a large amount of molecular-targeted agents for the treatment of solid tumors as well as of hematologic malignancies has been developed in the preclinical setting and, finally, evaluated in clinical trials. Some of these agents, e.g. anti-CD-20 rituximab [1], anti-HER2 trastuzumab [2], anti-VEGF bevacizumab [3] monoclonal antibodies or the tyrosine kinase inhibitor imatinib-mesylate/Glivec [4], now have an established role in the treatment of B-cell neoplasms, breast cancer, colon cancer and chronic myeloid leukemia and have reached clinical practice. However, the results for most other agents have been disappointing in clinical trials although promising in the preclinical setting. The discrepancy between a highly promising preclinical and a low clinical effectiveness can be explained both on the basis of: (i) the use of experimental models with specific sensitivity to the investigational agents and, therefore, not resembling the clinical tumors; and (ii) on the inadequacy of the conventional mouse models based on human xenografts to recapitulate the clinical scenario.

An additional important point is the practical relevance of biomarkers predictive of therapeutic effectiveness. Agents with marginal activity in an unselected population may be very effective in a marker-selected group of patients. Trastuzumab is active when combined with chemotherapy in breast tumors with high expression of HER, assessed by standardized criteria in immunohistochemistry or harboring HER gene amplification in the tumor tissue [2], but has marginal activity in an unselected patient population. Unfortunately, prediction of antitumor activity cannot be achieved for most molecular targeted drugs in the clinical setting. This can be due to the loss of identification of true targets in tumor cells or on the inadequacy of analysis technologies. Moreover, the available data are mostly derived from retrospective studies and prospective trials are warranted. An additional important point needs to be considered. It is becoming clear that it is very important to identify high priority targets in survival-related as well as in transformation-related pathways. In fact, tumor cells are under a persistent stress and need to adapt to a different milieu in order to undergo local invasion as well as to activate the metastatic processes. While the early studies have mainly been focused on the mechanisms of uncontrolled cell growth, it is now clear that escape from apoptosis as well as adaptation to different microenvironments are critical processes and can offer important targets for selective antitumor strategies.

In a recent article we discussed the new concept of ‘addiction to survival factors’, which readily integrates the concept of ‘addiction to oncogenes’ [5]. Our general idea is that survival factors might not play an important role in the early steps of the
carcinogenetic event but might have a critical function at later points of the tumor progression. Their role as an adaptive response to the tumor microenvironment may suggest an ‘Achille’s heel’, which may be specific for the different sites of metastatic disease.

In this article, we will discuss the biological rationales and the clinical studies focused on the targeting of Raf-kinase, which is a critical molecule in the transformation as well as in the survival pathways in human tumor cells.

**Searching for New Targets: the Raf-Kinase Family Members**

The ras family of oncogenes and encoded proteins has been evaluated as a putative target for anticancer therapeutic development. These efforts have resulted in new insights into Ras-mediated cell signaling as it relates to human cancer. Ras plays a central role in an intricate array of signal transduction pathways, characterized by cross-talk, feedback loops and multicomponent signaling complexes [6]. One strategy to overcome the challenges inherent in developing therapeutics against signaling elements situated in redundant pathways is to target elements downstream of convergence points of critical signaling modules. This reasoning has led, in part, to interest in Raf-kinase, which is one of several downstream effectors of Ras, signaling modules. This reasoning has led, in part, to interest in Raf-kinase, which is one of several downstream effectors of Ras, as a target for therapeutic development against cancer.

The raf family of genes was first identified as oncogenes in retroviruses that are the causative vectors of tumors in mice and chicken. The first raf gene to be identified, v-raf, the transforming gene of the mouse sarcoma virus 3611, induces fibrosarcomas and erythroleukemia in newborn mice, and C-raf (also called raf-1) is its proto-oncogene homolog [7]. A-raf and v-Rmii were next identified as the transforming gene, C-Rmii corresponded to a third mammalian raf gene, B-raf, which was also shown to be an oncogene [8]. Therefore, the mammalian raf family consists of the following three genes: A-raf, B-raf, and C-raf. The raf proto-oncoproteins encode three 68–74-kd cytosolic proteins, termed A-Raf, B-Raf and C-Raf (Raf-1), which share highly conserved amino-terminal regulatory regions and catalytic domains at the carboxyl terminus. Each Raf species has a distinct expression profile in tissues, which suggests that individual Raf isoforms perform clearly defined functions [9]. C-Raf is ubiquitously expressed in most tissues. Both A- and B-Raf have more restricted expression profiles than C-Raf, with A-Raf overexpressed in urogenital tissues (e.g. kidney, ovary, prostate and epididymis) and B-Raf overexpressed in neural, testicular, splenic and hematopoietic tissues [10].

The main signal transduction pathway involving Raf kinase is the mitogen-activated protein (MAP) kinase cascade that lies in a three-kinase-signaling module involved in transmitting membrane signals to the cell nucleus. A MAPK module consists of MAP kinase or extracellular signal-regulated kinase (ERK) activated by a MAP/ERK kinase (MEK or MAPKK) which, in turn, is activated by a MEK kinase (MEKK or MAPKKK). One such MEKK, which is the well-characterized downstream effector of Ras, is the serine–threonine kinase Raf-1. This protein is recruited by Ras-GTP to the plasma membrane, where Raf is activated by an as yet unknown factor [11]. Localization of Raf to the plasma membrane is essential for its activation.

Both A-Raf and C-Raf undergo localization to the mitochondria, which supports the notion that Raf regulates apoptosis, but the specific proportions of Raf isoforms that are localized to the mitochondria are not known [12]. This localization may be a result of isoform-specific lipid- or protein-binding partners, which recruit Raf to distinct membrane rafts. In fact, it has been reported that Raf-1 stimulates phosphorylation of the pro-apoptotic protein BAD. In other cell systems plasma membrane targeting of Raf-1 activates the classical MEK1/Erk (MAPK) cascade but does not protect cells, whereas mitochondrial targeting of Raf-1 protects cells from apoptosis. The anti-apoptotic signals from Raf-1 can be either MEK-independent or MEK-dependent, the latter through a MEK/Erk/ribosomal S6 kinase cascade. The MEK-independent signal is not well defined and additional studies are required for solving the role of Raf-1 in BAD phosphorylation [12].

C-Raf is the strongest candidate for the mitochondrial targeting of Raf-1 because it was shown that interference knockdown of Bcl-2 reduced Raf-1 mitochondrial localization. Therefore, Raf-1 translocation to mitochondria could displace Bcl-2 from Bad, activating the anti-apoptotic activity of the former. Moreover, it has been demonstrated that Raf-1 co-immunoprecipitates with Bcl-2 in several experimental models [13].

For a schematic representation of the pathways regulated by Raf see Figure 1.

**Preclinical Studies on Raf-Kinase Inhibition as Anticancer Strategy**

BAY 43–9006 (Sorafenib®) is a novel bi-aryl urea that has previously been shown to inhibit Raf-1 and tumor cell line proliferation and tumor growth in several human tumor xenograft models [14]. Moreover, it has been demonstrated that BAY 43–9006 inhibits another member of the Raf family, wild-type (wt) B-Raf and V599E B-Raf. In addition, BAY 43-9006 demonstrates potent inhibition of certain proangiogenic RTKs, including vascular endothelial growth factor receptor (VEGFR)-2, platelet-derived growth factor receptor (PDGFR) and VEGFR-3. BAY 43-9006 also substantially inhibits tumor growth of several human tumor xenograft models, even in the absence of MAPK pathway inhibition. Taken together, these data suggest that BAY 43-9006 functions as a novel dual-action RAF kinase and VEGFR inhibitor targeting both the RAF/MEK/ERK pathway and RTKs that promote tumor angiogenesis. Moreover, it also has to be considered that recent evidence suggests that C-Raf and B-Raf participate in the regulation of endothelial apoptosis and, therefore, angiogenesis, a process essential for tumor development and metastasis. Selective delivery of mutant C-Raf to tumor blood vessels induces endothelial cell apoptosis, which inhibits angiogenesis and results in regression of established tumors. Mice deficient in B-Raf or C-Raf die during embryogenesis because of severe vascular defects and increased apoptosis that could be due, in part, to effects on endothelial cell survival [15].

The dual function of sorafenib in inhibiting both cancer cell proliferation/survival and neo-angiogenesis is an intriguing issue that can disclose new therapeutic opportunities. In fact,
one of the most important limits of the target-based therapy is the strict specificity of the agents used that can be overcome by alternative survival pathways that can also be hyper-activated in cancer cells. This concept is based on the observation that survival pathways are pleiotropic and cancer cells have been developed in the host for several years having the opportunity of selecting more than one signaling in order both to escape apoptosis and to mediate proliferation. Therefore, the use of multi-target agents could be a useful tool to avoid these important limitations.

We have recently found that the treatment of colon cancer cells with GOLF, a recently published chemotherapy regimen, induces apoptosis and decreases the expression of C-Raf through the activation of its proteasome-dependent degradation [16]. On the basis of these data, we are evaluating if sorafenib can have a cooperative effect with GOLF on growth inhibition and apoptosis of colon cancer cells. We have found a preliminary cooperative effect between a low dose of sorafenib (0.1 μM) and GOLF that merits further investigations (M. Caraglia et al. AACR 2006). These data suggest a central role of C-Raf in the protection of these cells from apoptosis induced by a complex chemotherapy regimen. These data are also confirmed by other recent results obtained in our laboratory. In fact, we have demonstrated that interferon-α (IFN-α) induces apoptosis counteracted by an EGF-ras-C-Raf-Erk-dependent pathway in human epidermoid cancer cells [17]. We have recently demonstrated that C-Raf is activated by IFN-α and this activation induces the cytoplasmic co-localization of bcl-2 and its translocation to mitochondria where it phosphorylates Bad on Ser112. This event leads to the dissociation of Bad from Bcl-2 and the subsequent activation of the anti-apoptotic function of the latter. This survival response of tumor cells from the apoptosis induced by IFN-α is overcome by the addition of the farnesyltransferase inhibitor R115777 (Zarnestra) that, in fact, potentiates growth inhibition and apoptosis induced by the cytokine. It is possible, therefore, that the use of Raf kinase inhibitor could also be useful in potentiating the effects of IFN-α in this experimental model.

**clinical issues**

A phase I clinical and pharmacokinetics study of sorafenib has been performed by Strumberg et al. [18]. In their series of patients the drug was well tolerated by chronic administration and a 400 b.i.d. for phase II trials has been identified. Pharmacokinetics of sorafenib disclosed large interpatient variability. Higher dosages were precluded by diarrhea and skin toxicity. PMA-induced ERK phosphorylation in PBL was used...
as a pharmacodynamic surrogate and was completely suppressed at 400 mg b.i.d. schedule. This study provided proof of principle of antitumor activity of this compound.

In a phase III trial performed on advanced renal cell carcinoma progressing after first-line treatment sorafenib 400 mg b.i.d. was shown to prolong progression-free survival compared to placebo, with manageable toxicity (PFS 24 months for sorafenib versus 12 months placebo, HR 0.44, \( P < 0.00001 \)) [19]. The ongoing study follow-up will potentially demonstrate the achievement of the overall survival primary end point.

An interesting study has recently been published on the sorafenib-induced hypertension [20]. In this series 75% of patients experienced a >10 mm increase of systolic blood pressure which is evident within 3 weeks from beginning of treatment and persists for at least 18 weeks, without sodium retention or changes in nephro-vascular physiology, leading the authors to propose a direct vasculature effect of the compound. Several studies are ongoing on sorafenib-combined treatments.

**conclusions**

Raf-kinase appears an appealing therapeutic target, even it other preclinical and clinical studies are warranted in order to evaluate the activity of sorafenib both in monotherapy and in combination with other agents. The identification of a molecular signature of therapeutic response to Raf-kinase inhibitors by gene expression or proteomic analysis will be a critical issue for patient selection and treatment tailoring.

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**references**