Tailoring Ras-pathway—Inhibitor combinations for cancer therapy

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Abstract

Constitutive activation of Ras pathways plays a critical role in cancer development and maintenance. Inhibitors of such pathways are already in use for cancer therapy, with significant but as yet only partial success in the most deadly types of human cancers, against which even combinations of Ras-pathway inhibitors with classic cytotoxic drugs or irradiation are insufficient. Combinations of farnesyl transferase inhibitors (FTI’s), inhibitors of Ras pathways, are now in use in clinical trials. In this review we analyze possible reasons for the limited efficacy — including the diverse and sometimes even contradictory effects of active Ras pathways in tumor cells — and propose possible alternative methods of tailoring Ras-pathway inhibitor combinations for cancer therapy. Such tailoring is now possible thanks to increased knowledge of the complexity of Ras pathways, their cooperation with other oncogenic pathways, and their “addictive” nature. We provide examples demonstrating that this knowledge can be translated into useful drug combinations that disrupt multiple oncogenic pathways and hit a weak point of a given tumor cell. One such example is combination treatment with a Ras inhibitor and a glycolysis blocker for pancreatic tumor cells. The future design of such potential drug combination therapies and the follow-up of their outcome will undoubtedly be facilitated by gene-expression profiling and proteomic methods.

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1. Introduction

Mutationally activated ras genes play a key role in oncogenesis and are hence considered to be important targets for cancer therapy (Cox and Der, 2002b; Downward, 2003). The four typical human Ras proteins, H-Ras, N-Ras, K-Ras 4A, and K-Ras 4B (encoded by three ras genes) are essential signal transducers that regulate cell growth and differentiation, survival, and death (Shields et al., 2000). They show a high degree of sequence homology and a common principal mode of activation and inactivation. Ras is turned on by extracellular signals that activate receptor protein tyrosine kinases, which in turn activate Ras guanine nucleotide exchange factors (RasGEFs) that induce exchange of GDP for GTP on Ras (Campbell et al., 1998; Corbett and Alber, 2001). The active, Ras–GTP complex assumes a conformation that has high affinity towards multiple downstream effectors that are thereby activated and trigger a diversity of intracellular signaling cascades which — along with other Ras-independent cascades — lead to changes in cellular behaviour (Mein et al., 2005; Shields et al., 2000). Normally Ras activation is transient; specific Ras GTase-activating proteins (RasGAPs) bind to the active Ras–GTP and induce its catalytic GTase domain to hydrolyze GTP thereby returning Ras to its inactive, GDP-binding conformation (Scheffzek et al., 1997).

The commonest mutational activations in the ras genes are missense mutations at codons 12 or 13, resulting in Ras mutants that are insensitive to RasGAPs, or at codon 61, resulting in Ras mutants with no catalytic activity. The outcome in either case is constitutively active Ras–GTP that acts independently of extracellular signals. The incidence of such chronically active Ras in human cancer is higher than the frequency of ras gene mutations (20–30%); this is attributable to high rate of activating mutations (20–30%); this is attributable to high rate of activating mutations, or over-expression of growth-factor receptors and their ligands resulting in Ras activation, or both (Bardeesy et al., 2005; Levitzki, 2003a,b). The chronically active Ras protein promotes oncogenesis through activation of multiple Ras effectors that contribute to deregulated cell growth, dedifferentiation, and increased survival,
migration and invasion of tumor cells (Downward, 2003; Mitin et al., 2005; Shields et al., 2000).

2. Ras pathway activation and oncogenesis

Although the Raf–MEK1/2–ERK1/2 and the PI3-K–Akt cascades are the best characterized Ras effector pathways (Downward, 2003; Mitin et al., 2005; Pacold et al., 2000), we now know of many different functional classes of proteins that act as Ras effectors and contribute to Ras-dependent oncogenesis. These include:

i. The Raf family of serine/threonine kinases (Raf-1, A-Raf, and B-Raf) that phosphorylate MEK1/2 (Chong et al., 2005);

ii. The PI3-K catalytic subunit family (p110α, p110β, and p110γ) that catalyze the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Procko and McColl, 2005; Vanhaesebroeck et al., 2001; Wymann et al., 2000);

iii. The RalGEF family of proteins (RalGDS, Rlf, RGL2, and RGL3) that act as GEFs of the RalA and RalB GTases (Ferg, 2003; Feinstein, 2005);

iv. The RGL and Tiam family of proteins that act as GEFs of the Rho/Rac GTases (Minard et al., 2004; Sierra et al., 2000);

v. RIN1 that acts as GEF of the Rab5 GTase (Hu et al., 2005);

vi. NORE1 (RSSFS) that acts as an adaptor of the serine/threonine MST1 (Praskova et al., 2004);

vii. Phospholipase Cε that catalyzes the formation of diacylglycerol and inositol (1,4,5)-trisphosphate (Lopez et al., 2001); and

viii. IMP that acts as an E1 ubiquitin ligase and inhibits the MEK–ERK scaffold protein KSR (Matheny et al., 2004).

This incomplete list of Ras effectors illustrates the diversity of the Ras-signaling networks that regulate the expression of a variety of transcription factors controlling cell-cycle progression, for example, E2F1 (Berkovich and Ginsberg, 2001; Kaelin, 2003; Nevins, 2001), which regulates the activity and stabilization of cyclin D1 and of transcription factors such as Myc (Sears et al., 2000; Sears and Nevins, 2002), hypoxia-inducible factor-1α (HIF-1α) (Blancher et al., 2001; Sodhi et al., 2001), nuclear factor kappa-B (Amiri and Richmond, 2005; Ravi and Bedi, 2004), c-Jun (Behre et al., 1999; Janulis et al., 1999), NF-Y (Lee et al., 2005b), C/EBPbetαa (Sebastian et al., 2005; Shim et al., 2005), autoimmune regulator (AIRE) (Nagafuchi et al., 2005), STAT1 (Leaman et al., 1996; Song et al., 2002). These and other Ras-signaling networks also regulate cytoskeleton re-organization. Ample evidence from experiments – in which, inter alia, activated versions and dominant negative forms of Ras effectors and their downstream targets were employed – implicates Ras and its signaling pathways in oncogenesis.

This notion is strongly supported by the finding that a number of Ras effectors are frequently mutated in human tumors. For example, various human tumors are found to contain mutationally activated B-Raf (Davies et al., 2002) or to lack the tumor suppressor PTEN (Li et al., 1997; Teng et al., 1997). Because PTEN catalyzes the hydrolysis of PIP3, its loss leads to PIP3 accumulation, supporting the likelihood of PI3-K hyper-activation in human tumors. Mutational activation in p110α was recently reported in colorectal and other human cancers (Lee et al., 2005a; Li et al., 2005). In addition to the involvement of B-Raf or PI3-K activation in human cancers, there is strong evidence that Tiam1 links Ras with RhoGTPases and tumor cell invasion (Sander et al., 1998), that RalGEFs can mediate Ras transformation of human cells (Boettner and Van Aelst, 2002), that Raf and PI3-K mediate Ras survival signals (McFall et al., 2001), and that members of the RASSF family of Ras effectors act as tumor suppressors (Agathanggelou et al., 2005).

3. Only partial success in targeting Ras pathways for cancer therapy

Despite the strong evidence for the key role of active Ras and its effector pathways in human oncogenesis, efforts to target Ras or its upstream regulators and downstream effectors for cancer therapy have so far met with only partial success. There are a number of possible reasons for this.

First, unlike in the case of receptor tyrosine kinases (RTKs), where inhibitors that act by blocking kinase activation (e.g. antibodies) or kinase activity are good candidates for anti-tumor drugs, the ideal Ras “inhibitor” would be a compound that leads to gain of function, or in other words, it would strengthen the enzymatic (GTPase) activity of oncogenic Ras. In this sense, the design of such compounds is analogous to and as difficult as the design of compounds that would lead to gain of function in mutated inactive tumor suppressors. We do not have such Ras “inhibitors”. The currently available useful inhibitors of Ras functions are farnesyl transferase inhibitors (FTIs) that inhibit farnesylation of Ras as analogues of and as difficult as the design of compounds that would lead to gain of function in mutated inactive tumor suppressors. We do not have such Ras “inhibitors”. The currently available useful inhibitors of Ras functions are farnesyl transferase inhibitors (FTIs) that inhibit farnesylation of Ras (Cox and Der, 1997, 2002a), farnesyl thioisaliclyclic acid (FTS), which interferes with Ras-membrane anchorage (Kloog and Cox, 2000), and compounds that inhibit Ras methylation, such as cysmethynil (Winter-Vann et al., 2005). Of these inhibitors, so far only FTIs have been tested in clinical trials where they exhibited some anti-tumor effects (Alsina et al., 2004; Cortes et al., 2005; Kim et al., 2005; Renter et al., 2000). However, the most frequently mutated K-Ras and N-Ras oncogenes undergo alternative prenylation and remain oncogenically active, indicating that the anti-tumor effects of the FTIs cannot be attributed to Ras inhibition (Cox and Der, 1997, 2002a; Lebowitz and Prendergast, 1998). Despite these limitations, the results obtained with FTS (Kloog and Cox, 2000) and with cysmethynil (Winter-Vann et al., 2005) show that targeting of Ras for cancer therapy is feasible. In this connection
Table 1: Examples of the effects of combinations of inhibitors of the Ras-signaling pathway and chemotherapeutic agents in pre-clinical experiments

<table>
<thead>
<tr>
<th>Drug combination</th>
<th>Effect</th>
<th>Cell line</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTI SCH56582 vincristine</td>
<td>Synergistic cell growth inhibition</td>
<td>Rat renal KNRK cells</td>
<td>Suzuki et al. (1998)</td>
</tr>
<tr>
<td>FTI L-744832 Paclitaxel</td>
<td>Synergistic cell growth inhibition</td>
<td>MCF7, MDA-MB468</td>
<td>Moasser et al. (1998)</td>
</tr>
<tr>
<td>dasatinib, crizotinib, vinblastine, 5-fluorouracil</td>
<td>Additive cell growth inhibition</td>
<td>MDA-MB468, DU-145, LNCaP, BxPC3, DLD1, NCI-H690, PaC1, IAPcA2, PANC1</td>
<td>Shi et al. (2000)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Synergistic cell growth inhibition</td>
<td>Non-small lung cancer cells A549, H1299</td>
<td>Marcus et al. (2005)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Synergistic cell growth inhibition and apoptosis</td>
<td>A549, T98G</td>
<td>Adjei et al. (2001)</td>
</tr>
<tr>
<td>Melphalan</td>
<td>Additive cell growth inhibition</td>
<td>A549</td>
<td></td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>Cell growth inhibition, less than additive</td>
<td>A549, MCF7, H1299, BxPC3</td>
<td>Lee et al. (2004)</td>
</tr>
<tr>
<td>Adenoviral IGFBP-3</td>
<td>Synergistic anti-tumor</td>
<td>H1299, H96, A549, H460, H322</td>
<td>Lee et al. (2004)</td>
</tr>
<tr>
<td>Proteasome inhibitor (bortezomib)</td>
<td>Synergistic cell death</td>
<td>MM, primary MM plasma myeloma cells</td>
<td>David et al. (2005)</td>
</tr>
<tr>
<td>FTI R115777 pamidronate (PAM), zoledronic acid (ZOL)</td>
<td>Synergistic cell growth inhibition and apoptosis</td>
<td>Human oropharyngeal epidermoid carcinoma KB, lung cancer H1355, Mia-PaCa2</td>
<td>Prevost et al. (2001)</td>
</tr>
<tr>
<td>FTS dacarbazine</td>
<td>Reduction in tumor growth</td>
<td>Human melanoma 607B cell line</td>
<td>Halscheid-Wiener et al. (2003)</td>
</tr>
<tr>
<td>Taxotere</td>
<td>Synergistic reduction in tumor growth</td>
<td>Panc-1</td>
<td>Gama-Weyn et al. (1997)</td>
</tr>
<tr>
<td>Pemetrexid (Alk inhibitor)</td>
<td>Reduced tumor proliferation</td>
<td>Mouse glioblastoma</td>
<td>Momota et al. (2005)</td>
</tr>
<tr>
<td>BIBX1382BS (EGFR tyrosine kinase inhibitor)</td>
<td>Decrease in post-irradiation cell survival</td>
<td>A549, MDAMB231</td>
<td>Toulany et al. (2005)</td>
</tr>
</tbody>
</table>

it is important to note that silencing of K-Ras by siRNA (Brummelkamp et al., 2002; Chen et al., 2005; Fleming et al., 2005) or by antisense K-Ras (Aoki et al., 2005) has been shown to result in reversal of the transformed phenotype and suppression of tumorigenicity of human cancer cells.

A second possible reason why the success of Ras-pathway inhibitors is only partial is that the targeting of a single oncogene in the presence of additional tumorigenic lesions might not be sufficiently effective against cancer. It therefore, seems reasonable to assume that targeting of Ras should be combined with the application of other anti-cancer drugs. Treatments with inhibitors of Ras-signaling pathways in combination with irradiation or cytotoxic drugs or other metabolic inhibitors have indeed been tested in preclinical studies (see examples in Table 1) and in clinical trials (see examples in Table 2), with some success.

Thirdly, since many of the pathways downstream of Ras are controlled not only by Ras, inhibition of Ras would not be sufficient to block them. The PI3-K pathway, for example, is activated by both Ras-dependent and Ras-independent mechanisms (Besset et al., 2000; Khwaja et al., 1998; Potempa and Ridley, 1998). In addition, in most cells over-lapping Ras-dependent and Ras-independent mechanisms are responsible for the control of cellular behavior, for example, cell-cycle progression (Kawasaki et al., 1996; Roche et al., 1996).

A fourth possible reason is the likelihood of negative interaction between Ras effector pathways; thus, activation of one pathway, for example, PI3-K, can inhibit another pathway, such as Raf-MEK-ERK (Guan et al., 2000; Reusch et al., 2001; Zimmermann and Moelling, 1999). In addition, the control of Ras-signaling pathways is far more complex than a linear cascade because positive and negative regulators can affect the strength and duration of the various Ras signals (Downward, 2003; Momin et al., 2005; Shields et al., 2000). For example, Ras activation of the Raf-MEK-ERK cascade...
### Table 2
Examples of the effects of combinations of inhibitors of the Ras-signaling pathway and chemotherapeutic agents in clinical trials

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>Phase</th>
<th>Main end-point</th>
<th>No. of patients</th>
<th>Results with the experimental treatment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefitinib (EGFR tyrosine kinase inhibitor) with paclitaxel and carboplatin</td>
<td>II</td>
<td>Survival</td>
<td>1037</td>
<td>Negative (not better than chemotherapy alone)</td>
<td>Herbst et al. (2004)</td>
</tr>
<tr>
<td>chemotherapy, first line</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gefitinib (EGFR tyrosine kinase inhibitor) with gemcitabine and cisplatin</td>
<td>II</td>
<td>Survival</td>
<td>1093</td>
<td>Negative (not better than chemotherapy alone)</td>
<td>Giaccone et al. (2004)</td>
</tr>
<tr>
<td>chemotherapy, first line</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erlotinib (Tarceva) with carboplatin and paclitaxel chemotherapy, first line</td>
<td>III</td>
<td>Survival</td>
<td>1059</td>
<td>Negative (not better than chemotherapy alone)</td>
<td>Herbst et al. (2005b)</td>
</tr>
<tr>
<td>Bevacizumab with carboplatin and paclitaxel chemotherapy, first line</td>
<td>II</td>
<td>TTP, tumor response</td>
<td>99</td>
<td>RR = 31.5%</td>
<td>Johnson et al. (2004)</td>
</tr>
<tr>
<td>Bevacizumab (anti-VEGF Ab.) with erlotinib, pretreated</td>
<td>III</td>
<td>Tolerability, tumor response</td>
<td>40</td>
<td>TTP = 7.4 months; Favorable safety profile; RR = 17.5%</td>
<td>Herbst et al. (2005a)</td>
</tr>
<tr>
<td>FTI SCH66336 (lonafarnib) with paclitaxel,</td>
<td>II</td>
<td>Survival</td>
<td>33</td>
<td>48% experienced PR or stable disease; RR = 27%</td>
<td>Kim et al. (2005)</td>
</tr>
<tr>
<td>FTI R157777 (tipifarnib) with gemcitabine</td>
<td>III</td>
<td>Tolerability, survival</td>
<td>341</td>
<td>Negative (not better than chemotherapy alone); RR = 27%</td>
<td>Van Cutsem et al. (2004)</td>
</tr>
<tr>
<td>Erlotinib (Tarceva) with gemcitabine</td>
<td>III</td>
<td>Tolerability</td>
<td>285</td>
<td>Tolerated by most patients; RR = 23%</td>
<td>Karsten Witt, OSI Pharmaceuticals, Inc. (2005, FDA presentation)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Survival</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RR: response rate; TTP: time to progression; PR: partial response.
requires recruitment of KSR, which acts as a scaffold protein for the three kinases; at the same time, however, Ras activation of IMP will strengthen the signal because it results in auto-ubiquitylation of IMP, which would otherwise inhibit the signal through its interaction with KSR (Matheny et al., 2004). Another example is given by galectin-3, which interacts with active K-Ras and stabilizes the GTP-bound state of the protein while at the same time providing a signal that down-regulates active ERK (Elad-Sfadia et al., 2004). It is clear that in these cases direct inhibition of Ras would not simply result in the inhibition of all Ras pathways; the direct inhibition might in fact even lead to an increase in signaling through one pathway at the expense of another one (Elad-Sfadia et al., 2004; Shadom-Feenstein et al., 2005).

This kind of interaction might explain, for example, why in many pancreatic tumor cell lines that express activated K-Ras, activation of ERK is lower than would be expected (Yip-Schneider et al., 1999).

Fifth, because tumor cells are genomically unstable they might acquire compensatory genetic lesions after being treated with inhibitors of Ras pathways (Karlsson et al., 2003).

4. Combination of distinct Ras-pathway inhibitors as a concept for therapy

Awareness of possible reasons why the results of clinical trials with Ras-pathway inhibitors are only partially successful reinforces the idea that we need to seek alternatives to the classic strategy of combining Ras-signaling inhibitors with cytotoxic drugs or irradiation. Rather, we need to think of designing strategies based on the combination of different Ras-pathway inhibitors, for example, combining inhibitors of Ras and Raf, of Ras and PI3-K, or of Ras and receptor tyrosine kinases. Although such combinations might be too toxic for normal cells to tolerate, it is likely that tumor cells will exhibit relatively higher sensitivities to them. This possibility is strengthened by the phenomenon of “addiction to oncopgenes”, discussed in the next section. An alternative strategy would be to use a combination of different Ras-signaling inhibitors applied sequentially, for example, treatment with the RTK inhibitors followed by treatment with a Raf inhibitor.

A prerequisite for the design of such strategies would be wide-range gene profiling of the tumor before and after treatment. This is indeed already being carried out in a number of trials, as well as in patients with disease recurrence who are being treated with approved drugs (Kane, 2005; Poccu, 2005). For example, in a number of trials patients are selected for a particular drug treatment according to their sensitivity to hormones, as in breast cancer and in prostate cancer (Centeno et al., 2005; Cleator and Ashworth, 2004; Greubberger et al., 2001; Kristensen et al., 2005; Body et al., 2005; Sorlie, 2004; Symmans et al., 2003; Trump, 2005; van’t Veer et al., 2002). Similarly, patients are selected for treatment according to the profile of the oncopgenes to be targeted, for example, over-expression of epidermal growth factor receptor (EGFR) for the treatment with the monoclonal antibody cetuximab (Erbitux®) or with the tyrosine kinase inhibitor erlotinib (Tarceva®) (Yang et al., 2005). ErbB2 over-expression for the treatment with the monoclonal antibody trastuzumab (Herceptin®) or with the inhibitor imatinib (Gleevec®) (Dressman et al., 2003; Yang et al., 2005), or over-expression of the anti-apoptotic protein Bcl2 for the treatment with the Bcl2 antisense (oblimersen, Genasense®TM) (Merseburger et al., 2003; Watson et al., 2005). The follow-up by expression profiling in such trials is of utmost importance because it allows us to gain information about the genes whose expression was up- or down-regulated by the treatment and might therefore contribute to drug resistance. Targeting of such genes, either alone or in combination with the original treatment, could be beneficial.

Follow-up in long-term treatments has also proved to be very important (Sklar et al., 2000; Stovall et al., 2004; Zebrack et al., 2004, 2002). An outstanding example is given by Philadelphia positive chronic myeloid leukemia (CML), a tumor that appears to be highly dependent on the constitutive activity of a single tyrosine kinase oncoprotein, the BCR–ABL chimera. This dependence is clearly evident by the complete regression of advanced tumors – which are refractory to cytotoxic drugs – after treatment with imatinib (Gleevec®), which inhibits BCR–ABL tyrosine kinase activity (Demetri et al., 2002; Druker et al., 2001), and from the recent observation that in recurrent disease the BCR–ABL often undergoes mutations that render the tyrosine kinase refractory to imatinib (Cortes and Kantarjian, 2005; Crossman et al., 2005). Interestingly, the transforming potential of BCR–ABL is absolutely dependent on activation of endogenous Ras (Burchett et al., 2005; Goga et al., 1995).

It is therefore, likely that in recurrent BCR–ABL, treatment with Ras inhibitors such as FTIs (Feldman, 2005; Gotlib, 2005) or with FTS could be a good second-treatment choice.

5. Addiction to Ras and to other oncopgenes

The Ras-related pathways seem to be a good example of the “addiction to oncopgenes” paradigm (Weinstein, 2002). This refers to the process in which maintenance of the transformed phenotype of cancerous cells becomes dependent on the persistent presence of a specifically activated or over-expressed oncogene. The addiction might also be a weak point of the cancer cell, which is likely to become more susceptible to a drug that targets the addiction. The cause of the addiction would therefore be the Achilles’ heel of the cancer cell, and could be targeted by selective drugs (Bachireddy et al., 2005). Thus, while activating mutations in Ras or in its upstream regulators and downstream effectors accelerate the development of cancer, they also would increase the sensitivity of the cancer cell to small-molecule inhibitors that target the addictive Ras machinery. We have indeed noticed that cancer cells with relatively high levels of active Ras are
Clearly more sensitive to the Ras inhibitor FTS than cells in which the levels of active Ras are relatively low (Kloog and Cox, 2000). It is likely that cancer cells with B-Raf mutations (Davies et al., 2002) will exhibit relatively high sensitivity to Raf inhibitors such as the BAY 43-9006 used in clinical trials (Awada et al., 2005; Moore et al., 2005; Richly et al., 2004) or to MEK inhibitors, and that cells with PTEN mutations will exhibit relatively high sensitivity to Akt inhibitors, glycolysis synthase kinase (Gsk)-3 inhibitors, or mTOR inhibitors such as rapamycin.

Other major oncogenes in addition to Ras could be considered as “addictive oncogenes”. These oncogenes can vary between different cancerous cell types and might cooperate with Ras and other oncogenes. Cooperation or dependence of “addictive oncogenes” would provide a strong rational background for combined targeted therapies that are likely to have minimal effects on normal cells. In such cases, targeting of an oncogene by a single drug might also affect a second oncogene that depends on the one that was targeted. This could be exploited for a combinatorial treatment in which the effect of the single drug would be enhanced. For example, cooperation between Ras and Myc contributes to cell transformation, suggesting that targeting of both oncogenes and/or their targets could be an important strategy for cancer therapy in cases of Myc gene amplification or Ras activation. The known dependence of the stability of Myc protein on Ras suggests that disruption of Ras functions might do away with Myc and its functions. We recently showed that that this occurs in human neuroblastoma with amplified MycN gene (Yaaari et al., 2005). Another example is provided by GBM cells where HIF-1α, whose stability is controlled by Ras, plays a critical role in up-regulation of metabolic pathways, enabling these deadly tumors to progress in conditions of extreme hypoxia (Korkolopoulou et al., 2004; Vordermark and Brown, 2003; Zagzag et al., 2000). Here too we found that targeting of Ras exposed GBM cells to the weak point of their “addiction” to processes regulated by HIF-1α (Blum et al., 2005). Thus, rather than directly targeting an oncogene of interest that serves as an “addiction oncogene” (Broxterman and Georgopapadakou, 2005), disruption of an essential upstream regulator might more effectively inhibit the oncogene’s regulatory pathways (Blum et al., 2005; Yaaari et al., 2005), as described below.

6. Targeting multiple oncogenic pathways via inhibition of a single critical oncogene

In human neuroblastoma the MycN gene is frequently amplified by more than 100 copies, leading to Myc overexpression (D’Cruz et al., 2001; Schwab, 1999). The larger the number of copies of MycN, the more aggressive the disease and the poorer its outcome (Schwab, 1999). Another alteration in Myc that is common in many human tumors is mutation of Thr58, which prevents Myc degradation (Sears et al., 2000). The Myc proteins are short-lived nuclear transcription factors that play a pivotal role in regulating cellular proliferation, cellular growth, differentiation, angiogenesis, adhesion, and apoptosis (Dang, 1999; Facchini and Penn, 1998; Pelegrinis et al., 2002; Sears and Nevins, 2002). A critical feature of the regulation of the Myc protein is that it must be phosphorylated to become activated and stabilized. Phosphorylation of Ser62 stabilizes and extends the half-life of Myc, whereas Thr58 phosphorylation targets the transcription factor for ubiquitin-mediated degradation. The phosphorylation of Myc is regulated by two independent Ras-downstream pathways that act synergistically to stabilize Myc. Acting through Raf-MEK-ERK, Ras induces phosphorylation of Myc at Ser62, thereby extending the half-life of Myc. Concurrently, Ras signals through the PD-1-K-Akt pathway block the activity of glycogen synthase-3, preventing phosphorylation of Myc at Thr58 and its proteosomal degradation (Sears et al., 2000; Sears and Nevins, 2002). Understanding these active Ras signal-transduction pathways (Sears et al., 2000; Sears and Nevins, 2002) that contribute to the progression of neurobladoma led us to utilize FTS, which inhibits Ras protein, in LAN1 neuroblastoma with amplified MycN gene. Treatment of LAN1 cells with FTS or expression of dominant negative Ras resulted in inhibition of Ras signaling and almost complete disappearance of MycN protein, leading to a strong reduction in LAN1 cell growth (Yaaari et al., 2005).

This emerging therapeutic paradigm of targeting critical signaling pathways that are required to sustain the activation of critical oncogenes was demonstrated also in the case of human GBM (Blum et al., 2005). Here, HIF-1α plays a critical role in maintaining the malignant phenotype of GBM (Korkolopoulou et al., 2004; Vordermark and Brown, 2003; Zagzag et al., 2000). HIF-1α is a nuclear transcription factor, which regulates the expression of many enzymes that participate in glycolysis (the major energy pathway in GBM) and in many other types of human tumors (Seagroves et al., 2001). Studies have shown that the levels and activity of HIF-1α protein are controlled by Ras (Blancher et al., 2001) in a manner that resembles the above-mentioned Ras-Myc cooperation; Ras regulates the stability and transcriptional activity of HIF-1α (Blancher et al., 2001) by mechanisms that involve, respectively, GSK-3-dependent and ERK-dependent phosphorylation of the transcription factor (Sodha et al., 2001). We found that inhibition of Ras by FTS leads to destabilization of HIF-1α protein in U87 and other glioblastoma (GBM) cells. Concomitantly with the disappearance of HIF-1α, the expression of key enzymes of the glycolysis pathway and of other HIF-1α-regulated genes (including vascular endothelial growth factor [VEGF], Glut1, and CA9) were transcriptionally down-regulated by the FTS treatment. This led to glycolysis shutdown, resulting in a dramatic reduction in ATP and a severe energy crisis. As a consequence, U87 cell growth was arrested and the cells died (Blum et al., 2005). Importantly, inhibition of active Ras by FTS in these same U87 GBM cells also abrogated the Rb–E2F1 pathway, leading to down-regulation of the mitogenic transcription factor E2F1, which is essential for cell-cycle progression (Blum et al., 2005).
the expression of VEGF is controlled by HIF-1 in tumors that are highly dependent on angiogenesis, because consequently of HIF-1 of any given tumor will facilitate a rational design of drug

tailoring is already possible because of increased knowledge of the complexity of Ras pathways, their cooperation with other oncogenic pathways, and their “addictive” nature. We have provided a number of examples demonstrating that such knowledge can be translated into a useful drug combination, which disrupts multiple oncogenic pathways and hits a weak point of a given tumor cell; for example, combined treatment with a Ras inhibitor and a glycolysis blocker kills pancreatic tumor cells. This is only one of many possibilities that can be exploited for tailored cancer therapy with existing drugs that target well-known oncogenic pathways. The design of such drug-combination therapies and the follow-up of their outcome will undoubtedly be facilitated by the use of biological markers, gene expression profiling and proteomic methods.

Biological markers have a number of benefits. First, they can be used as quantifiable measures of the oncogenic cascade (Garcea et al., 2005; Ghaneh et al., 2002). Second, prognostic biological markers may become potential targets for drug treatment in themselves. Third, biological markers can be used as surrogate markers in tumor or in other tissues such as sera. This allows recording progression of the tumors or their response to drugs. Last, biological markers can be used for early diagnosis or for the identification of patients at-risk of cancer development (Garcea et al., 2005; Ghaneh et al., 2002).

Proteomics should provide a convenient platform for validating the efficacy of investigated drug combinations. In addition, the microarray technologies should make it possible to determine gene expression patterns before and after a single drug treatment or drug combination (Herbst et al., 2005a; Johnson et al., 2004).

Critical for the success of this technique is the identification of genes involved in conferring drug sensitivity and resistance, as well as predicting which patients are most likely to benefit from the drug treatment. Thanks to recent improvements in computational methods for promoter sequence analysis (Elkon et al., 2003) and the integration of “metagene” analysis to predict the activity of oncogenic pathways (Huang et al., 2003), we can now track down the activation status of
key transcription factors that govern specific gene expression. This dramatically improves our ability to find a gene product that is critical for the survival of a given tumor cell but is less important for the normal cell. The use of microarrays also generates database sets that should allow confirmation of the mechanism of action of a given drug. This technology is likely to play an important role in all phases of drug discovery and development. It includes identification and validation of new targets, profiling of on- and off-target effects during optimization of new drugs and of drug combinations, and prediction of side effects. In our laboratory we are currently utilizing a number of gene-profiling technologies with the aim of building up a comprehensive database of the many different types of human tumor cell lines treated with the Ras inhibitor FTS, either alone or in combination with other drugs. We expect this database will be useful in following up the current clinical trials with FTS, and will allow the future design of trials with rational drug combinations.

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