Abstract

We discuss the biology of Ras signal transduction and the epidemiology of ras mutations in association with disease as a background for the development of a Raf kinase inhibitor, BAY 43-9006. Knowledge of Ras effector pathways has permitted genetic validation of numerous targets involved in the Ras signaling cascade. A key Ras effector pathway involves the kinase cascade RAF/MEK/ERK (MEK: MAP/ERK kinase; ERK: extracellular signal related kinase). Indeed, we present studies of cell lines stably expressing mutant MEK constructs, which point to Raf kinase as a target for therapeutics with selective anti-tumor activity. Finally, a small molecule drug discovery program based on inhibition of Raf kinase activity is outlined and the initial pre-clinical development process of the Raf kinase inhibitor BAY 43-9006 is discussed.

Ras epidemiology

In human tumors, mutant ras oncogenes are frequently associated with disease progression (Rodenhuis 1992, Sanchez-Cespedes et al. 2001). The epidemiology associating ras mutations with pathology has been explored in patients with colon and lung carcinomas, where disease progression and decreased patient survival correlate with ras mutational status. The aim of these studies was to clarify the association between Kirsten ras (K-ras) mutations, patient outcome and tumor characteristics.

In the case of colorectal cancer, 37.7% of 2721 patients scored positive for mutations in K-ras at codons 12 or 13 (Andreyev et al. 1998). Mutations were not associated with stage of disease, nor was there a difference in the frequency of K-ras mutations between sporadic colorectal cancer patients and those with a genetic predisposition to cancer. The rate of mutation in colorectal cancer did not appear to depend on the site of cancer, and there was no apparent difference in the specific amino acid changes associated with the distribution of tumors in the colon. Nevertheless, the presence of a K-ras mutation correlated with increased risk of recurrence (P<0.001) and death (P = 0.004), regardless of the Duke’s stage of the cancer.

In a follow-up study, 3439 colorectal cancer patients from 42 countries were entered into a multivariate analysis study that revealed that, of all the mutations analyzed, only a glycine-to-valine mutation found in 8.6% of patients had a statistically significant impact on failure-free survival and overall survival (H J N Andreyev, personal communication). In particular, patients with the more severe Duke’s C stage cancer had a 50% increased risk of relapse or death associated with the valine mutation. Therefore, of every 1 000 000 patients who contract colorectal cancer, 86 000 will harbor the K-ras V12 mutation, and the corresponding increased risk of recurrence or death from the cancer.

About one third (19/69) of adenocarcinomas of the lung harbor K-ras mutations (Slebos et al. 1990). The K-ras mutations at amino acid positions 12, 13 or 61 were found to be a strong and unfavorable marker of disease. In these adenocarcinomas, K-ras mutations were found to be associated with a very poor prognosis despite radical resection of the patients’ tumors. Twelve of the nineteen patients harboring K-ras mutations died during the follow-up period, as compared with 16 of the 50 patients without K-ras mutations. This difference in the prognosis of the disease was also reflected in the duration of disease-free survival, overall survival and in the number of deaths due to cancer. In fact when age, tumor size and differentiation of the tumors were taken into account, the K-ras mutation was the single most important prognostic factor, irrespective of disease stage. This difference might explain the relative resistance of these patients to either radiation treatment or cytotoxic drugs such as cisplatin.

These initial studies of lung adenocarcinomas were further extended by a study of 141 patients (Rodenhuis & Slebos 1992). In this study, K-ras mutations were also shown to be closely associated with smoking history. Of 141
patients with lung tumors, 41 (40%) of 101 smokers or ex-smokers were shown to be positive for K-ras mutations, whereas only 2 (5%) of 40 tumors of patients who had never smoked were positive. This suggests that K-ras mutations might arise as a direct result of exposure of the lung to one or more carcinogens from tobacco smoke. In an independent study from Japan, 13 (16%) of 89 adenocarcinomas scored positive for ras mutations, predominantly K-ras (Sugio et al. 1998). The 5-year survival rate in the ras mutation-positive group was 53.3%, which was significantly poorer than the 83.6% survival rate of the ras mutation-negative group. In a separate, combined analysis of almost 900 patients gathered from eight published studies in the literature, the presence of a K-ras mutation was shown to be associated with an almost doubled risk of death at two years from non-small cell lung cancer (Huncharek et al. 1999). In another study, 365 patients newly diagnosed with lung cancer and treated with potentially curative resection over a 4-year period, were studied. In this case, mutations were found only in smokers. Comparison of Kaplan-Meier curves indicated a strong association between K-ras mutation and decreased patient survival and this association was statistically significant only for stage I tumors. There also appeared to be a statistically significant association between female sex and K-ras mutation. It was also noted that there was no association of the K-ras mutation with duration of smoking or intensity of smoking, suggesting that tobacco carcinogens induce essentially all of the K-ras mutations and that they occur quite early in the clonal evolution of this disease.

In Table 1, a list of the more prevalent solid tumor types and their percentage of K-ras mutational involvement is presented. The numbers of expected new cases compared with the numbers of expected deaths from the diseases for the year 2000 describe poignantly the need for a tumor selective therapeutic agent that directly addresses the ras status of the tumors.

**Ras signal transduction**

The Ras proteins are expressed from three different genes, namely, Neuroblastoma (N)-ras, Harvey (H)-ras and Kirsten (K)-ras (Bar-Sagi 2001). The Ras proteins share high homology to each other and their ability to interact with regulators and effectors is similar. Nonetheless, the distribution of ras gene mutations does show some tissue specificity, with K-ras mutated most often in solid tumors, such as colon, lung and especially in pancreatic cancer, and N-ras usually mutated in hematopoietic tumors, predominantly acute myelogenous leukemia (Rodenhuis 1992).

Activating ras mutations are found in 50% of colon carcinomas, 30% of lung carcinomas, 80% of pancreatic carcinomas and approximately 20% of hematopoietic malignancies. In addition to mutations in the ras gene, activation of the Ras pathway may be mediated by several mechanisms. Either overexpression or amplification of growth factor receptors signaling through Ras, a reduction in expression or activity by the neurofibromatosis type-1 protein (NF-1), a GTPase activating protein, or an upregulation of the grb-2 adaptor protein may lead to higher than normal levels of active Ras in the cell (Weiss et al. 1999). Formal proof that Ras is directly causal to tumor progression arises from two independent observations. In one publication the gene encoding the oncogenic version of ras was disrupted by homologous recombination in two colon epithelial tumor cell lines (Shirasawa et al. 1993). The ensuing cell lines were morphologically altered, lost the capacity for anchorage-independent growth and grew more slowly both in vitro and in nude mice. More recently, expression of oncogenic alleles of K-ras was achieved in mouse strains through spontaneous recombination in the whole animal and this predisposition led directly to the formation of tumors in transgene animals, mainly lung tumors (Johnson et al. 2001).

Ras has been shown to regulate several pathways (see Fig. 1) that contribute to cellular transformation, including the Rac and Rho pathways, and the Raf/MEK pathway (Campbell et al. 1998). Ras has been shown to directly activate lipid kinases, specifically the conversion of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) to phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P3) by phosphoinositide 3–kinases. Generation of PtdIns(3,4,5)P3 results in recruitment of two kinases, phosphoinositide-dependent kinase-1, PDK-1, and protein kinase B, Akt/PKB, to the cell membrane via pleckstrin homology domains. Activation of phosphoinositide 3–kinases (PI3K) by Ras results in activation of pathways responsible for cellular migration, stress fiber formation and cytoskeleton rearrangements. Activation of Akt/PKB promotes cell survival and inhibits cellular apoptosis. Ras activates the Raf/MEK/ERK pathway by binding to and activating Raf kinase. There are three members of the Raf family of kinases (Raf-1, A-Raf and B-Raf). Raf activation requires a number of critical steps, including Raf-1 phosphorylation, binding of the Raf protein to Ras-GTP, oligomerization of Raf protein, association with other proteins (e.g. heat shock and 14–3–3 proteins), interactions of the Raf protein with membrane

**Table 1** Incidences of Ras mutations in solid human malignancies. The statistical numbers are quoted by the American Cancer Society for the year 2000.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>% Ras-</th>
<th>New cases*</th>
<th>Expected deaths*</th>
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<tbody>
<tr>
<td>Pancreatic</td>
<td>80</td>
<td>28 300</td>
<td>28 200</td>
</tr>
<tr>
<td>Colorectal</td>
<td>40</td>
<td>164 100</td>
<td>156 900</td>
</tr>
<tr>
<td>Lung</td>
<td>50</td>
<td>93 800</td>
<td>47 700</td>
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lips and Ras-induced conformational changes of the Raf protein (Avruch et al. 2001). Raf activated by Ras-GTP then phosphorylates and activates MEK1 and MEK2 kinases. These, in turn, phosphorylate and activate the ERK family members, ERK1 and ERK2. The activated ERK kinases translocate to the nucleus and stimulate pathways required for translation initiation and transcriptional activation and hence protein and gene expression leading to proliferation.

Validation of Raf kinase as a target in cancer

In order to define the significance of the Raf/MEK/ERK pathway in tumor growth, we sought to selectively block that pathway in human tumor cells (Arboleda et al. 2001). Since Raf phosphorylates MEK on serines 218 and 222 (Zheng & Guan 1994), a MEK mutant in which these serines are substituted by alanines (MEK218A/222A) cannot be activated by Raf. This MEK mutant should block the Raf/MEK/ERK pathway, either by sequestering Raf or by sequestering ERK and blocking their wild-type activity (see Fig. 2).

To determine the relative contribution of the Raf/MEK/ERK pathway to the tumorigenic phenotype of human tumor cells, an epitope-tagged version of this MEK mutant (MEK218A/222A) was stably transfected into human tumor cell lines of epithelial or mesodermal origin (see Table 2).

The most extensively characterized cell lines were the MiaPaca-2 cell line, derived from a human pancreatic tumor, the DLD-1 cell line, derived from a human colon carcinoma, and the HT1080 fibrosarcoma cell line. They harbor activating K-ras mutations at codon 12 and 13, and N-ras at codon 61 respectively. Expression of this mutant MEK in each of these three cell lines (DLD-1, Mia-Paca-2 and HT1080) resulted in a reduction of endogenous MEK-1 activity when compared with vector controls. It was found that Raf-1 co-immunoprecipitated with this mutant MEK. In the cell lines assayed, there was a reduction in endogenous Raf-1 activity. Cumulatively, the results suggest this mutant MEK inhibits the endogenous Raf/MEK/ERK pathway by sequestering endogenous Raf.

To further explore the cellular effects of this mutant MEK in the cell lines, their ability to support anchorage-independent growth was examined. The MiaPaca-2 (see Fig. 3), the DLD-1 and the HT1080 clones expressing this mutant MEK showed statistically significant decreases in anchorage-independent growth compared with the vector control cell lines (see Table 3). The inability of the clones expressing this mutant MEK to support anchorage-independent growth correlates well with the reduction in MEK activity.

To explore their tumorigenic potential in vivo, DLD-1, MiaPaca-2, Panc-1 and HCT116 clones expressing this mutant MEK (MEK218A/222A) were injected...
subcutaneously into athymic mice. Tumor measurements were taken weekly over 7–10 weeks. At the end of this period, the mean tumor volume in the clones expressing this mutant MEK was 17–22% of parental vector controls. Furthermore, the vector control group developed palpable tumors within two weeks, whereas the MiaPaca-2 clones expressing this mutant MEK required six weeks to develop palpable tumors. Subcutaneous injection of DLD-1 clones expressing this mutant MEK showed a similar prolonged latency in the development of palpable tumors. Therefore, the reduced MEK activity observed in the clones expressing mutant MEK correlates with their decreased ability to support anchorage-independent growth and a significant decrease in their ability to form tumors in athymic animals.

The survival of athymic mice injected intraperitoneally with a MiaPaca-2 clone expressing this mutant MEK (MEK218A/222A), or a vector control, was examined. Pre- and post-mortem analyses included measurement of ascites accumulation, cachexia and tumor growth. Mice injected intraperitoneally with a clone expressing this mutant MEK lived at least twice as long ($P<0.001$) as the animals injected with vector control cells. Six of the eight mice in the group injected with cells expressing this mutant MEK lived without signs of disease until they were killed at the termination of
Figure 3 Example of soft agar experiments. In the left panel (a) is a typical soft agar picture of the MiaPaca-2 cell line expressing vector control. In the middle panel (b) and on the right (c) are two MiaPaca-2 clones stably expressing mutant MEK.

Table 3 Comparison of cell lines tested for growth in soft agar and their mean inhibition of growth in soft agar when compared with vector controls from each parental cell line.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Ras status</th>
<th>Clones analyzed</th>
<th>Inhibition in soft agar (%)</th>
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<tbody>
<tr>
<td>DLD-1</td>
<td>ras+</td>
<td>7</td>
<td>84.3±10</td>
</tr>
<tr>
<td>MiaPaca-2</td>
<td>ras+</td>
<td>8</td>
<td>72±28</td>
</tr>
<tr>
<td>HT1080</td>
<td>ras+</td>
<td>5</td>
<td>78.5±39</td>
</tr>
<tr>
<td>NCI H522</td>
<td>ras wt</td>
<td>4</td>
<td>92±15</td>
</tr>
<tr>
<td>C33A</td>
<td>ras wt</td>
<td>6</td>
<td>69±39</td>
</tr>
</tbody>
</table>

wt, wild-type.

The study at 35 weeks. Upon autopsy these mice revealed no tumor growth in the peritoneal cavity. In contrast, within four to eight weeks post-injection, clinical disease was evident in all the animals injected with the cells containing the vector control. In this group, the first death occurred at six weeks and all remaining mice were killed at week 15 due to severe manifestation of disease. Upon autopsy, all these mice revealed the presence of tumors in the peritoneal cavity. Collectively, the data strongly suggest that a reduction of endogenous Raf function inhibits both tumor growth and metastases, and improves the survival of athymic mice bearing human tumors.

We found that such genetic inhibition of the Raf-MEK pathway reduced the endogenous activity of the pathway, inhibited the anchorage-independent growth of tumor cells and blocked tumor progression in mice. Thus, we believe that an inhibition of the Ras pathway at the level of Raf kinase through small molecule intervention might prove to be of therapeutic value in the treatment of cancers such as those shown in Table 1.

Discovery of BAY 43-9006, a specific Raf kinase inhibitor

The lead series for the Raf kinase project can be broadly defined as bis-aryl ureas. Many thousand medicinal chemistry compounds, from either medicinal chemistry directed syntheses or numerous combinatorial libraries, were screened through the Raf kinase biochemical assay (Riedl et al. 2001). Compounds were first analyzed for in vitro activity against recombinant, activated human Raf kinase.

Active compounds (<500 nM) were then tested in a mechanistic cellular assay (Wilhelm et al. 2001). The activity of endogenous phosphorylated MEK was assayed by a high throughput immunoprecipitation assay of MEK in response to estradiol stimulation of an estrogen receptor (ER) fused to a Raf kinase construct within a mouse 3T3 cell line.

Compounds that demonstrated inhibition of Raf kinase-mediated MEK phosphorylation in cells were then analyzed for their ability to inhibit HCT116 tumor cell proliferation in vitro, as well as for inhibition of soft agar growth (both HCT116 and MiaPaca-2 cell lines), a hallmark of transformation. Finally, the lead compounds were also tested in a tumor cell-based mechanistic assay monitoring the inhibition of MEK and ERK phosphorylation in HCT116 tumor cells. Counterscreening was performed using biochemical assays for MEK and ERK activity, as well as a cell-based assay for insulin receptor function. This drug discovery effort led to the selection of BAY 43-9006 as a candidate for clinical development.

Most of the in vivo characterization of BAY 43-9006 was carried out in the HCT116 xenograft model (Gianpaolo-Ostravage et al. 2001). The HCT116 human colon tumor cell line contains a K-ras mutation. The tumor...
growth of this cell line has been shown to be dependent on K-ras; disruption of the activated K-ras gene inhibited cellular proliferation, soft agar growth and in vivo tumor growth (Shirasawa et al. 1993). BAY 43-9006 significantly inhibits tumor growth in this model in a dose-dependent manner (see Fig. 4). BAY 43-9006 also showed significant activity against two additional human tumor xenograft models with K-ras mutations: MiaPaca-2 pancreatic carcinoma and H460 non-small cell lung carcinoma (Gianpaolo-Ostravage et al. 2001). BAY 43-9006 has demonstrated oral in vivo activity in three human tumor xenograft models with mutant K-ras genes (HCT116, MiaPaca-2, H460) and one human tumor xenograft with a wild-type K-ras but exhibiting overexpression of growth factor receptors for epidermal growth factor (EGF) and HER 2 (SKOV-3).

Clinical testing of oral tablets of BAY 43-9006 in cancer patients commenced in July 2000 (Strumberg et al. 2001). To date, this compound has been well tolerated, and dose escalation is continuing. In contrast to the pharmacokinetics in mice, this compound enjoys a relatively long terminal half-life of 35 hours in humans. Preliminary clinical data is encouraging, as at least 37% of patients in this initial study had stable disease lasting longer than 12 weeks.

Conclusion

The Ras/Raf pathway is frequently activated in human tumors. Inhibition of this pathway with an interfering mutant of MEK results in reversion of the cancer phenotype, validating Raf kinase as an attractive target for anti-tumor therapeutics. BAY 43-9006 is an orally available potent inhibitor of Raf kinase with significant activity in four different human tumor types including colon, pancreatic, lung and ovarian tumors. Tumor growth was potently suppressed when BAY 43-9006 was dosed for 14 days, and this tumor suppression was maintained as long as dosing was continued. BAY 43-9006 also demonstrated significant anti-tumor activity against larger (400 mg – 1 g) colon or ovarian tumors, with some regressions during the dosing period observed. These data suggest that BAY 43-9006 may have potential clinically as a cancer therapeutic with a novel mechanism of action.

References


