Development of a Conditional In vivo Model to Evaluate the Efficacy of Small Molecule Inhibitors for the Treatment of Raf-Transformed Hematopoietic Cells

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Abstract
Conditionally active forms of the Raf proteins (Raf-1, B-Raf, and A-Raf) were created by ligating NH2-terminal truncated activated forms (Δ) to the estrogen receptor (ER) hormone-binding domain resulting in estradiol-regulated constructs (ΔRaf:ER). These different Raf:ER oncoproteins were introduced into the murine FDC-P1 hematopoietic cell line, and cells that grew in response to the three ΔRaf:ER oncoproteins were isolated. The ability of FDC-P1, ΔRaf:1:ER, ΔA-Raf:ER, and ΔB-Raf:ER cells to form tumors in severe combined immunodeficient mice was compared. Mice injected with ΔRaf:ER cells were implanted with β-estradiol pellets to induce the ΔRaf:ER oncoprotein. Cytokine-dependent parental cell lines did not form tumors. Implantation of β-estradiol pellets into mice injected with ΔRaf:ER cells significantly accelerated tumor onset and tumor size. The recovered ΔRaf:ER cells displayed induction of extracellular signal-regulated kinase (ERK) in response to β-estradiol stimulation, indicating that they had retained conditional activation of ERK even when passed through a severe combined immunodeficient mouse. The ΔRaf:ER cells were very sensitive to induction of apoptosis by the mitogen-activated protein/ERK kinase (MEK) 1 inhibitor CI1040 whereas parental cells were much less affected, demonstrating that the MEK1 may be useful in eliminating Ras/Raf/MEK–transformed cells. Furthermore, the effects of in vivo administration of the MEK1 inhibitor were evaluated and this inhibitor was observed to suppress the tumorigenicity of the injected cells. This ΔRaf:ER system can serve as a preclinical model to evaluate the effects of signal transduction inhibitors which target the Raf and MEK proteins. (Cancer Res 2005; 65(21): 9962-70)

Introduction
A goal of investigating signal transduction pathways is to better understand their precise function and susceptibility to inhibition by small molecular weight membrane permeable drugs (1). This information may aid in the identification of chemotherapeutic drugs which block specific enzymes essential for neoplastic, but not normal, cell proliferation. The BCR-ABL inhibitor (Gleevec, Imatinib) is one such example as it affects BCR-ABL and only a few other enzymes (e.g., c-Kit and platelet-derived growth factor receptor). Imatinib has been shown to be more toxic for neoplastic cells as compared with normal hematopoietic cells (1, 2). Inhibitors of the Raf/mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK and phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin pathways have been developed (3-7). Many of these kinase inhibitors bind the kinase domain and prevent its activation or activity. In addition, certain other kinase inhibitors may compete with the kinase substrate, which is often ATP. Alternatively, it may be possible to target cells containing mutant genes involved in the regulation of cell cycle and apoptotic pathways. Such targeting may be achieved by treatment of cells with low concentrations of drugs, which make the malignant cells extremely sensitive to other chemotherapeutic drugs but not affecting normal cells.

The proliferation of many hematopoietic precursor cells is promoted by interleukin-3 (IL-3), granulocyte/macrophage colony stimulating factor (GM-CSF), as well as other growth factors (8-10). Cell lines which grow in culture and remain cytokine dependent represent models to understand the proliferation and malignant transformation of hematopoietic precursor cells. The murine IL-3/GM-CSF–dependent FDC-P1 cell line was isolated from the bone marrow of a normal DBA/2 mouse and resembles cells with colony-forming unit granulocyte/macrophage morphology (8). Spontaneous factor–independent cells are rarely recovered from this cell line, which makes it an attractive model system to analyze the effects of various oncogenes on signal transduction and the transition to cytokine independence and leukemogenesis (8, 11-14). Oncogene-transformed cytokine-independent cells have been obtained by infection/transfection of this cell line. These cytokine-dependent and oncogene-transformed derivative cells represent appropriate tools to screen for novel therapeutic compounds as well as to evaluate their effectiveness in suppressing the growth and differentiation capacity of certain types of leukemias containing various oncogene mutations and chromosomal translocations.

IL-3 and GM-CSF exert their biological activity by binding to the IL-3 and GM-CSF receptors, respectively (9, 10, 15). These receptors activate a protein tyrosine kinase, Janus kinase (Jak)-2, which leads to the phosphorylation and dimerization of signal transducers and activators of transcription. In addition to Jak activation, receptor ligation promotes phosphorylation of the Src homology and collagen (Shc) protein. She then recruits the growth factor receptor binding protein 2/son of sevenless homologue complex to the p85 chain, resulting in stimulation of Ras. Activated Ras promotes the sequential activation of Raf, MEK, and MAP (ERK1 and ERK2) kinases as well as the phosphatidylinositol-3-kinase/Akt cascade.
Certain cytokines and IFNs stimulate Jak1 activity, which can activate the Raf kinase pathway (16). Thus, these signal transduction pathways are interconnected and often regulate each other.

There are three relatedraf genes in mammals: Araf, Braf, andraf-1 (17, 18). Raf proteins have three different functional domains: CR1, CR2, and CR3. The CR1 region has the binding site for an activated Ras protein (14). The CR2 region negatively regulates the Raf kinase domain (CR3), which is located in the carboxyl-terminal half of the Raf protein. Ras proteins transmit their regulatory signals to MEK1, a dual specificity serine/threonine and tyrosine kinase which phosphorylates downstream ERK1/2 (17, 18). ERK can phosphorylate other kinases (e.g., p90(RSK)) or transcription factors [e.g., cyclic AMP–responsive element binding protein (CREB), Elk, and c-Myc] which enter the nucleus and regulate gene expression (19, 20).

NH2-terminal deleted forms of protein (CREB), Elk, and c-Myc which enter the nucleus and regulate gene expression (19, 20). NH2-terminal deleted forms of Raf and MEK1 proteins, which remove the Ras binding domain and the negative regulatory sites in CR1 and CR2 in Raf and the negative regulatory domain of MEK1, result in activated oncoproteins due to the aberrant stimulation of downstream kinases, transcription factors, and molecules involved in the prevention of apoptosis (17, 18, 21). Conditional Raf and MEK proteins were developed by ligation of the 5′ deleted Raf and MEK cDNAs to the cDNA encoding the hormone-binding domain of the estrogen receptor (ER). The activity of the kinases encoded by these cDNAs is dependent on estrogen (β-estradiol; refs. 13, 14, 17). Conditional signal transduction proteins are in some cases better tools to understand the effects of these proteins on proliferation and apoptosis because once the regulator is taken away, the cells revert to the "cytokine-dependent" state. Thus, it is possible to compare the effects of a chemotherapeutic drug on a signaling pathway when the cells grow in response to this activated protein versus when the cells grow in response to the normal cytokine in the same clonal cell line.

Signal transduction pathways tend to be interwoven rather than function in linear cascades. Although the Ras/Raf/MEK/ERK cascade is often thought of as a linear cascade, it has many different sites of interaction with different signal transduction, cell cycle, and antiapoptotic cascades (22–31). For example, Ras can initiate signaling through multiple cascades, including Raf/MEK/ERK and phosphatidylinositol 3-kinase (PI3K). In addition, ERK can phosphorylate many substrates which are also regulated by the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (e.g., p90(RSK), p70(S6K), Bad, and CREB). Akt can, in some cases, phosphorylate Raf and lead to its inactivation, although other studies have shown that these pathways interact to prevent apoptosis (22–26). Thus, the Raf/MEK/ERK and phosphatidylinositol 3-kinase/Akt pathways regulate growth and apoptosis. The goal of the following studies was to develop a preclinical mouse model, evaluate the ability of cells transformed with conditionally activated Raf proteins to form tumors in immunocompromised mice, and investigate whether a MEK inhibitor would suppress their growth in vivo.

Materials and Methods

Cell culture and growth factors. Cells were maintained in a humidified 5% CO2 incubator with RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA). The IL-3–GM-CSF–dependent murine myeloid FDC-P1 cell line was cultured in medium supplemented with 10% WEHI-3B (D–) conditioned medium as a source of IL-3. Raf-responsive cells were cultured in RPMI 1640 + 10% FBS + 500 mmol/L β-estradiol (Sigma Chemical Co., St. Louis, MO) to activate the ΔRafER, as described (13). FD/BCR-ABL cells were cultured in RPMI 1640 + 10% FBS in the absence of IL-3 or β-estradiol. This ΔRaf-ER construct also contained a green fluorescent protein (GFP) tag which allowed identification of the protein by immunofluorescence (32).

For ERK inhibition studies, cells were harvested in log-phase growth and exposed to the selective MEK inhibitor CI1040 (PD184352, 2-[2-chloro-4-iido-phenilamino]-N-cyclopropylmethoxy-3,4-difluoro-benzamide; ref. 33), which was kindly provided by Dr. J.S. Sebolt-Leopold (Cancer Molecular Sciences, Pfizer Global Research and Development, Ann Arbor, MI) or to a matched concentration of vehicle (DMSO). Effects of CI1040 on cell viability were evaluated by triplicate counting of trypsin blue–excluding cells under a light microscope.

Determination of extracellular signal-regulated kinase 1 and 2 activation. Cells were washed twice with cold PBS containing 5 mmol/L Na2EDTA and lysed on ice in Gold lysis buffer (Xenogen), as described (14). Western blots were probed with a phosphospecific anti-ERK antibody (Cell Signaling, Beverly, MA) or with antibodies which recognized total ERK, β-actin, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA), as described (14). In some cases, cells were stimulated with β-estradiol, IL-3, 0.1% DMSO (Sigma), or 20 mmol/L phorbol 12-myristate 13-acetate (Sigma) for 5 to 30 minutes before protein extracts were isolated for Western blot analysis.

Apoptosis assays. For Annexin V staining, cells were washed twice with binding buffer [10 mmol/L HEPES, 140 mmol/L NaCl, and 5 mmol/L CaCl2 (pH 7.4); all from Sigma] and stained with phycoerythrin-conjugated Annexin V (Caltag Laboratories, Burlingame, CA) for 15 minutes at room temperature. Annexin V fluorescence was determined using a FACScan flow cytometer. Controls were unstained parental FDC-P1 cells (GFP negative), unstained FD/GFPΔRafER (GFP positive), or stained with Annexin V only, which were used to calibrate the FACScan analyzer for each experiment.

Xenograft studies in severe combined immunodeficient mice and in vivo imaging. Four- to six-week-old C.B.17 severe combined immunodeficient (SCID) mice (Harlan Sprague-Dawley, Madison, WI) were implanted with 1.7 mg, 60-day release, 17β-estradiol pellets (Innovative Research, Sarasota, FL). The next day, 5 × 106 leukemic cells suspended in 200 μL PBS were injected s.c. in the right flank or i.v. via the lateral tail vein. For s.c. cell injection, tumor diameters were serially measured with calipers, and tumor volumes were calculated by the following formula: volume = width2 × length / 2.

For i.v. administration, mice were sacrificed when they became moribund or unable to obtain nourishment, libate, or if they lost >20% weight. For histologic analysis, liver, spleen, lungs, and/or excised tumors were embedded in paraffin, sectioned, and stained with H&E. Engraftment analysis. For visualization of the native GFP protein in genetically modified mice, mice were injected with AdLux-F/RGD (1,000 viral particles/cell) or AdLux-F/ER (1,000 viral particles/cell) for 4 hours at 37°C. For i.v. administration, mice were sacrificed when they became moribund or unable to obtain nourishment, libate, or if they lost >20% weight. For histologic analysis, liver, spleen, lungs, and/or excised tumors were embedded in paraffin, sectioned, and stained with H&E.

In vivo Model of Leukemia Driven by Activated Raf
fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with H&E. Additional sections were used for immunocytochemical detection of GFP, as described (36). Briefly, antigen retrieval was achieved by boiling with 0.01 mol/L nitric acid at 95°C to 100°C for 45 minutes. Before staining the sections, endogenous peroxidase was quenched using 3% H2O2 and nonspecific binding was blocked using 10% normal goat serum incubated for 1 hour at room temperature. The slides were incubated with anti-GFP polyclonal antibody (Clontech Laboratories, Palo Alto, CA) overnight at 4°C. Tissue was rinsed in PBS/0.025% Triton X-100 and subsequently exposed to biotinylated secondary antibody (anti-rabbit immunoglobulin G conjugate B-7389; Sigma) for 3 hours. The immunostaining was visualized by the avidin-biotin-peroxidase complex (ABC) method using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) as a substrate.

**Statistical analysis.** For each group of mice (estradiol/no estradiol), a simple linear regression model was used to model tumor size as a function of time from day 13 to day 20. For each group of mice, a t test was used to determine whether the slope of the regression line was significantly different from 0. In addition, the difference in tumor size between the two groups of mice on days 13 and 20 was tested for statistical significance with a two-sample t test. The product-limit estimator of Kaplan and Meier was used to estimate the survival for each group of mice. The log-rank statistics was used to test for differences in survival distributions. P < 0.05 was considered statistically significant, and all statistical tests were two-sided.

**Results**

Previously, we have described the isolation of ΔRaF:ER-infected FDC-P1 cells, which proliferate in response to Raf activation (14). These cells absolutely require the activation of Raf for growth as removal of the β-estradiol from the tissue culture medium results in the rapid induction of apoptosis in these cells. We here determined the ability of these Raf-responsive cells to form tumors on injection into immunocompromised mice and their suitability as a model to test the effectiveness of small molecular weight inhibitors in the treatment of certain leukemias.

**Murine hematopoietic cells transformed by ΔRaF:ER genes are tumorigenic in immunocompromised mice.** To determine whether the cells which grew in response to the activated Raf protein would cause disseminated leukemia in immunocompromised mice, animals were i.v. injected with FD/GFPΔRaF:ER cells as well as two control cell lines, the parental cytokine-dependent FDC-P1 cells and cytokine-independent BCR-ABL–transformed cells. As documented in the Kaplan-Meier plot in Fig. 2A, mice injected with the FD/BCR-ABL cells rapidly succumbed to leukemia (100% of the mice had to be sacrificed by day 19), whereas no disease was observed in mice injected with the parental cytokine-dependent FDC-P1 cell line. Additional experiments indicated that the parental cytokine-dependent cells do not develop leukemia even after over 1 year of observation period. Implantation of β-estradiol pellets into mice injected with FD/GFPΔRaF:ER cells as well as two control cell lines, the parental cytokine-dependent FDC-P1 cells and cytokine-independent BCR-ABL–transformed cells. The survival curves for these two groups of mice, shown in Fig. 34, yielded the log-rank test P value = 0.003, suggesting a significant difference in survival between these two groups. Studies using different cell doses for i.v. injections showed that although the latency of disease was cell dose dependent, survival of mice carrying β-estradiol pellets was significantly shorter at all cell doses (data not shown).

To monitor leukemia dissemination, bioluminescence imaging was employed. Luciferase-expressing FD/GFPΔRaF:ER cells were injected i.v. in SCID mice implanted with β-estradiol release pellet. At indicated time points, mice were injected with luciferin and light emission was monitored using the In vivo Imaging System from Xenogen. These imaging studies showed that cells disseminated first to lungs (3 hours, 4 days) and later to spleen, liver, and lymph nodes. Mice were sacrificed on day 17, and organs were imaged in the dish, confirming whole-body imaging results, with accumulation of the leukemic cells in the spleen, liver, and lungs, but not in the kidney or brain (Fig. 2B). Necropsy of diseased mice revealed hepatosplenomegaly and increased immature/blast forms in the bone marrow and spleen accompanied by a dramatic disruption of splenic architecture (Fig. 2C). Abnormal dissemination of leukemic blastlike cells was also observed in liver and lungs (Fig. 2C). Together, these disease characteristics revealed that an acute myeloproliferative disease much like acute leukemia developed in mice injected with Raf-responsive cells, eventually resulting in early death.

**Hematopoietic cells transformed by ΔA-Raf:ER and ΔB-Raf:ER cause leukemia in immunocompromised mice.** The ability of FDC-P1 cells transformed with either activated A-Raf (FD/ΔA-Raf:ER) or B-Raf (FD/ΔB-Raf:ER) to cause leukemia was also determined. Whereas five of five pellet-carrying mice injected...
with B-Raf–transformed cells had to be sacrificed (Fig. 3B), three of four β-estradiol–implanted mice injected with A-Raf–transformed cells had to be sacrificed (Fig. 3A). The median survival of B-Raf– and A-Raf–transformed cells was 21 and 25.5 days, respectively. In contrast, only one of five mice had to be sacrificed from the control group of non-β-estradiol-implanted mice. Thus, the ability of the cells to cause leukemia was linked to the phosphorylation status of the introduced Raf protein. Previous studies have shown that ΔB-Raf >> ΔA-Raf in terms of its ability to phosphorylate the downstream MEK1 molecule as a substrate (14). In both A-Raf– and B-Raf–transformed cells, β-estradiol induced activation of ERK (Figs. 3A and B, right). Necropsy of diseased mice injected with B-Raf–transformed cells revealed hepatosplenomegaly and infiltration of spleen, liver, and lungs with immature leukemic cells, similar to the histology of mice injected with activated Raf-1 oncogene (Fig. 3C).

Figure 1. FD/GFPΔRaf-1:ER cells form tumors in immunocompromised mice. A, five SCID mice were implanted with β-estradiol pellets, and the next day, 5 × 10⁶ FD/GFPΔRaf-1:ER cells were injected s.c. in the right flank. Additional five mice were injected with cells without prior administration of β-estradiol pellet. The tumor size was measured in each mouse on days 13, 15, 16, 19, and 20 following injection of the cells. The individual tumor sizes are shown, as well as the mean tumor size for each group on each day. The means are connected with a solid segmented line. A simple linear regression model (shown with a dotted line) was fitted to the data for each group of mice (pellet/no pellet). The slope of the regression line for the mice with the pellet is 266, suggesting that the tumor growth in this group is 266 mm³/d. The slope of the regression line for the mice without the pellet is 84, suggesting that the tumor growth in this group is 84 mm³/d. For the mice who received the pellet, the mean difference between the day 20 and day 13 tumor size is 1,920.81 (95% CI, 1,409.14-2,432.47). This difference is statistically significant with P < 0.001. For the mice who did not receive the pellet, the mean difference between the day 20 and day 13 tumor size is 558.34 (95% CI, 94.54 to 1,211.21). This difference is not statistically significant with P = 0.077. B, a representative example of the smear obtained from the extracted tumor. a, H&E staining; b, GFP immunofluorescence; c, anti-GFP immunohistochemistry. C, FDC-P1, FD/BCR-ABL cells, or cells isolated from tumors from two mice injected with FD/ΔRaf-1:ER cells were β-estradiol deprived for 24 hours and then exposed to DMSO or β-estradiol for the indicated time periods. Cell lysates were prepared, electrophoresed, and probed with phospho-ERK, total ERK, or actin antibodies.
Figure 2. Kaplan-Meier plot of FDC-P1, FD/GFP\DeltaRaf-1:ER, and FD/BCR-ABL. A, Kaplan-Meier curves representing time from i.v. injection of 5 × 10⁶ cells (day 0) to leukemia cell death. Comparison of leukemic latency of FD/GFP\DeltaRaf-1:ER cells in mice receiving \(\beta\)-estradiol (\(n = 5\), FD/GFP\DeltaRaf-1:ER + pellet) and in mice not receiving \(\beta\)-estradiol (\(n = 5\), FD/GFP\DeltaRaf-1:ER). The difference between FD/GFP\DeltaRaf-1:ER and FD/GFP\DeltaRaf-1:ER + pellet is statistically significant (\(P = 0.003\), log-rank test). The median survival for the mice without the \(\beta\)-estradiol pellet was 25 days, and with the \(\beta\)-estradiol pellet was 22 days. Mice injected with parental FDC-P1 cells were sacrificed at day 40 without evidence of leukemia. Five mice were injected with FD/BCR-ABL cells known to cause rapid leukemia in this model. B, FD/GFP\DeltaRaf-1:ER luc cells were injected i.v. into SCID mice carrying \(\beta\)-estradiol release pellet. Serial images of a representative mouse. Postmortem imaging studies showed dissemination of luciferase-expressing cells to lungs, spleen, and liver, but not to brain, heart, or kidney. C, histologic examination of spleen, liver, lungs, and bone marrow in the control SCID mouse (normal) and in a representative mouse injected with FD/GFP\DeltaRaf-1:ER cells. Cross sections are shown under ×10 and ×20 (liver and lung) or ×40 (spleen and bone marrow) magnification.
Recovered tumor cells are sensitive to mitogen-activated protein/extracellular signal-regulated kinase kinase 1 inhibitors. The recovered FD/GFPΔRaf-1:ER cells were examined for their sensitivity to the MEK 1 inhibitor CI1040. The Raf-responsive FD/GFPΔRaf-1:ER cells were more sensitive to the growth-inhibitory effects of CI1040 than the cytokine-dependent FDC-P1 cells (Fig. 4A). Growth inhibition was observed in the parental cells at high concentrations of CI1040; however, only very low levels of apoptosis were induced (Fig. 4B). In contrast, complete inhibition of cell growth and massive induction of apoptosis was observed in Raf-responsive FD/GFPΔRaf-1:ER cells (Figs. 4A and B). CI1040 completely abrogated Raf-induced ERK phosphorylation in FD/GFPΔRaf-1:ER cells but minimally affected IL-3–induced phospho-ERK in parental FDC-P1 cells (Fig. 4C). Thus, growth of Raf-1–transformed hematopoietic cells is MEK dependent and MEK inhibition results in complete growth inhibition due to massive apoptosis.

Next, the effects of in vivo administration of CI1040 on growth of FD/GFPΔRaf-1:ER cells in SCID mice were examined. FD/GFPΔRaf-1:ER cells infected with AdLux-F/RGD vector were injected i.v. into SCID mice carrying an h-estradiol release pellet. CI1040 or vehicle was administered at 100 mg/kg/mouse bid via gavage (from days 1 to 14), and leukemia dissemination was noninvasively monitored by bioluminescence imaging. Bioluminescence imaging showed that CI1040 suppressed the leukemic tumor burden by 28% on day 11 (P = 0.04; Figs. 5A and B). Whereas the survival of the control and vehicle-treated groups was identical (median survival, 12.5 and 13 days; P = 0.693), inhibition of ERK signaling by CI1040 slightly, but significantly, extended survival of mice in this most aggressive leukemia model (median survival, 15 days; P < 0.01, compared with control or vehicle-treated groups; Fig. 5C).

Discussion

One of the goals of these studies was to determine whether cells that conditionally grow in vitro in response to Raf activation would also form tumors in immunocompromised mice and retain their proliferative requirement for Raf. We determined that the Raf-responsive murine FD/GFPΔRaf-1:ER cells rapidly formed leukemias on injection into immunocompromised mice, homed to the spleen and bone marrow, and caused outright leukemia, which could rapidly be detected by bioluminescence imaging. Thus, this cell system is a useful model for tumor imaging to determine the ability of signal transduction inhibitors to inhibit the spread of leukemic cells.
The in vitro characteristics of the FD/GFP-Raf-1:ER cells indicated that they are strictly dependent on β-estradiol (13). In the in vivo experiments reported here, tumors developed more rapidly when mice that were injected with FD/GFPΔRaf-1:ER cells were administered a β-estradiol pellet as compared with those without β-estradiol. Moreover, the tumors were larger in mice implanted with β-estradiol pellets. In FD/GFPΔRaf-1:ER cells recovered from tumors, activation of the ΔRaER protein remained dependent on β-estradiol, indicating that in vivo growth of these cells did not alter their in vitro requirement for β-estradiol.

These Raf-responsive cells represent a relevant model to investigate the ability of different signal transduction inhibitors to target activated proteins involved in signal transduction and induce cell cycle arrest and/or apoptosis. In general, the signal transduction inhibitor CI1040 affected Raf-mediated more than cytokine-mediated proliferation and apoptosis. These results suggest that targeting Raf in Raf-responsive cells is more effective than targeting Raf in cells that grow in response to cytokines which can activate multiple signal transduction pathways. Thus, there is hope that targeting the particular pathway(s) affected in a transformed cell may specifically render these cells more susceptible to signal transduction inhibitors, whereas cytokine-dependent normal cells would not be as sensitive to these drugs. This becomes increasingly important as treatment of cells with multiple inhibitors may prove more effective in suppressing proliferation and inducing apoptosis. Lower drug concentrations could be used to suppress proliferation and induce apoptosis in the “transformed,” but not in cytokine-dependent, normal cells.

The MEK inhibitor CI1040 induced apoptosis when cells grew in response to Raf, but not to IL-3. This MEK inhibitor affected constitutive Raf signaling more than cytokine-mediated proliferation. CI1040 also suppressed leukemia in vivo, however, this resulted in only marginal improvement of survival of the mice. Rac usually transmits its signal through MEK; however, there are situations where the Raf signal does not pass through a MEK intermediate (31). For example, nuclear factor-κB activation by Raf is mediated through MEK kinase and is independent of MEK (37). Thus, MEK inhibitors may not always be able to suppress all effects elicited by Raf and provide further rationale for the development of Raf-specific inhibitors. Furthermore, Raf inhibitors, under certain conditions, may be more useful than Ras inhibitors. Although Ras lies upstream of Raf, Ras inhibition might result in inhibition of both Rac and phosphatidylinositol 3-kinase. In some cases, it may be more beneficial to inhibit Raf but leave the phosphatidylinositol 3-kinase pathway intact.

Although mutations at the Raf gene in human neoplasia have been detected, they have not, until recently, gained the clinical importance that Ras mutations readily achieved. Due to more innovative, high-throughput DNA sequencing, scientists have recently discovered that the B-Raf gene is frequently mutated in certain cancers, including hematopoietic tumors such as non-Hodgkin’s lymphoma and acute myelogenous leukemia (38, 39). Approximately 60% of the melanomas surveyed in one study were observed to have mutations at B-Raf (40). This result bestows relevance to investigating signal transduction pathways because by understanding how B-Raf is activated, through one Ras-dependent and one Ras-independent event, scientists could predict why a single missense mutation in B-Raf permitted ligand-independent activation, whereas similar mutation events would not be predicted to result in either Raf-1 or A-Raf activation as they required multiple activation events. Interestingly, in 22 melanomas and colorectal and non–small-cell lung cancer tumors examined, there were 10 with mutations at B-Raf and 10 with mutations at Ras. Two tumors had mutations at B-Raf and Ras and two did not have mutations at either gene. Thus, B-Raf transformation did not seem to require Ras and many tumors had mutations at one or the other, but not both, genes. Recently, it has been suggested that B-Raf mutations occur during the process of tumor progression as opposed to establishment (41). This was suggested by analysis of B-Raf mutations in different developmental stages of melanomas.

Raf inhibitors (ZM 336372 and Bay 43-9006) have been developed. The Bay 43-9006 inhibitor is in clinical trials and shows promise in the treatment of certain tumors (42, 43). Patients with many different types of tumors have been treated with this drug, including colorectal, ovarian, hepatocellular, breast, pancreatic, non–small cell, gastric, renal, cervical, head and neck, and skin. This drug is...
administered p.o., and over 60 patients have been treated in phase I clinical trials and, more recently, in phase II clinical trials in patients with colorectal cancer. The drug is well tolerated and no dose-limiting toxicities have been observed yet. Antisense mRNA Raf inhibitors have also been developed and have shown promise in inhibiting the growth of certain tumors. The ability to evaluate these and other Raf inhibitors in cells and tumor systems, which are known to proliferate in response to Raf, is essential.

Figure 5. Effects of MEK inhibition of Raf-induced leukemias. A, FD/GFP,3.Raf-1:ER luc cells were injected i.v. into SCID mice carrying 17b-estradiol release pellet. Mice were treated with either CI1040 (100 mg/kg/mouse p.o. bid, days 1-14, n = 9), vehicle (n = 6), or left untreated (control, n = 6). On days 1, 6, and 11, mice received injection of d-luciferin and placed for imaging in the In vivo Imaging System from Xenogen. B, results were averaged from the peak light-emitting exposure from each group and displayed as photons per second after normalizing for baseline luminescence intensity. C, Kaplan-Meier curves for these three groups of mice. Whereas the survivals of the control and vehicle-treated groups were comparable (median survival, 12.5 and 13 days; P = 0.69), inhibition of ERK signaling by CI1040 extended survival of mice in this aggressive leukemia model (median survival, 15 days; P < 0.01, compared with control or vehicle-treated groups, Kruskal-Wallis test).

References

Acknowledgments

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