Upregulation of IGF1R by Mutant RAS in Leukemia and Potentiation of RAS Signaling Inhibitors by Small-Molecule Inhibition of IGF1R

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Abstract

Purpose: Activating mutations in the RAS oncogene occur frequently in human leukemias. Direct targeting of RAS has proven to be challenging, although targeting of downstream RAS mediators, such as MEK, is currently being tested clinically. Given the complexity of RAS signaling, it is likely that combinations of targeted agents will be more effective than single agents.

Experimental Design: A chemical screen using RAS-dependent leukemia cells was developed to identify compounds with unanticipated activity in the presence of an MEK inhibitor and led to identification of inhibitors of IGF1R. Results were validated using cell-based proliferation, apoptosis, cell-cycle, and gene knockdown assays; immunoprecipitation and immunoblotting; and a noninvasive in vivo bioluminescence model of acute myeloid leukemia (AML).

Results: Mechanistically, IGF1R protein expression/activity was substantially increased in mutant RAS-expressing cells, and suppression of RAS led to decreases in IGF1R. Synergy between MEK and IGF1R inhibitors correlated with induction of apoptosis, inhibition of cell-cycle progression, and decreased phospho-S6 and phospho-4E-BP1. In vivo, NSG mice tail veins injected with OCI-AML3-luc+ cells showed significantly lower tumor burden following 1 week of daily oral administration of 50 mg/kg NVP-AEW541 (IGF1R inhibitor) combined with 25 mg/kg AZD6244 (MEK inhibitor), as compared with mice treated with either agent alone. Drug combination effects observed in cell-based assays were generalized to additional mutant RAS-positive neoplasms.

Conclusions: The finding that downstream inhibitors of RAS signaling and IGF1R inhibitors have synergistic activity warrants further clinical investigation of IGF1R and RAS signaling inhibition as a potential treatment strategy for RAS-driven malignancies. Clin Cancer Res; 20(21); 5483–95. © 2014 AACR.

Introduction

RAS genes, which are the most frequent targets of dominant somatic mutations in human malignancies, encode a family of proteins (H-RAS, N-RAS, K-RAS; reviewed in ref. 1), and it is estimated that 20% to 25% of patients with acute myeloid leukemia (AML) have a RAS mutation (2). Of relevance, transplantation of mice with bone marrow transduced with retroviral vectors encoding oncogenic KRAS or NRAS has been shown to lead to AML (3–5).

Mediation of the effects of RAS by major signaling pathways such as PI3K/PTEN/AKT/mTOR and Raf/MEK/ERK has prompted the development of targeted inhibitors of these pathways as a strategy to treat mutant RAS-driven malignancies. Despite its prevalence and significance with respect to transformation, direct molecular inhibition of mutant forms of RAS has thus far been difficult due to its biochemistry and structure (6), although KRAS (G12C) mutant-specific inhibitors, which depend on mutant cysteine for their selective inactivation of this mutant, have recently been reported and are in early stages of development (7, 8). So far, attempts to block RAS function, including inhibition of kinases associated with downstream effector pathways such as PI3K, AKT, MEK, and mTOR, have shown fairly modest clinical efficiency (9, 10).
Translational Relevance

Use of a multitargeted therapy approach for malignancies driven by the highly prevalent RAS oncogene is warranted in light of the elusiveness of direct RAS inhibition and limited clinical efficacy associated with targeted inhibition of key mediators of RAS signaling. A novel chemical screen using highly targeted agents to identify kinases important for RAS signaling more easily led to the discovery of synergism between MEK inhibition and inhibition of IGF1R against mutant RAS-positive leukemia. Elevation of functional IGF1R expression in mutant RAS-expressing leukemia cells further supports the role of this molecule as a viable target and highlights the potential for therapeutic intervention. A combinatorial chemical screen that seeks to identify molecules that synergize with the inhibition of bona fide RAS effectors may yield drugs with therapeutic potential and help researchers understand mechanisms of RAS transformation.

Inhibition of MEK, a prominent downstream effector of RAS, has been tested in mouse models of AML initiated by hyperactive RAS, resulting in initial response followed by relapse despite continued treatment, apparently by outgrowth of pre-existing drug-resistant clones (11). The development of “first-generation” allosteric MEK inhibitors, such as CI-1040 and PD0325901, was halted because of toxicity and minimal activity in RAS-mutant tumors (12).

While newer MEK inhibitors, such as AZD6244 (13), show less toxicity and more effectiveness against RAS-mutant-positive solid tumors, it is still unclear whether they are better than standard therapies. For example, a phase II trial of AZD6244 for patients with advanced AML showed only transient and modest effectiveness (14). As the limited efficacy of inhibitors of RAF/MEK/ERK signaling or PI3K/AKT in mutant RAS-positive cancer is believed to be due to negative feedback loops and compensatory activation of the different signaling pathways, the simultaneous testing of inhibitors of multiple effectors in mutant RAS-positive cancers is reasonable.

To address this, we designed a chemical screen to identify agents capable of potentiating the activity of the MEK inhibitor, AZD6244, against mutant RAS-dependent AML cells. In addition to the identification of inhibitors of well-known downstream mediators of RAS signaling, including inhibitors of mTOR, and PI3K signaling, the chemical screen also led to the identification of the small-molecule inhibitor, GSK1904529A, which selectively inhibits IGF1R with nanomolar potency and which exhibits potent antitumor activity (15). This finding prompted investigation of underlying mechanism(s) of synergy between IGF1R inhibition and MEK inhibition against mutant RAS-positive AML, as well as further exploration of IGF1R as a potential therapeutic target for this disease.

Materials and Methods

LINCS library chemical screen

We designed a chemical screen using the kinase inhibitor-focused library, LINCS, to identify selective kinase inhibitors capable of synergizing with the MEK inhibitor, AZD6244, against mutant NRAS-driven cells (see schematic, Supplementary Fig. S1). The LINCS library is available from Harvard Medical School/NIH LINCS program (http://lincs.hms.harvard.edu/) and contains 202 known selective and potent kinase inhibitors.

Cell lines and cell culture

IL3-dependent murine Ba/F3 cells, cultured with 3 ng/mL of mIL3, were transduced with NRAS G12D or KRAS G12D containing murine stem cell virus (MSCV) retroviruses harboring an IRES-GFP. After withdrawal of mIL3, these cell lines became growth factor–independent.

The human, mutant NRAS-expressing AML line, OCI-AML3, and mutant KRAS-expressing AML lines, SKM-1 (KRAS K117N), NOMO-1 (KRAS G13D), and NB4 (KRAS A18D), were obtained from Dr. Gary Gilliland. The wild-type (wt) RAS-expressing line, HEL, and mutant NRAS-positive line, HL60, were purchased from ATCC. The wild-type MOLM14 (16) was provided by Dr. Scott Armstrong and transduced with the FUW-Luc-mCherry-puro lentivirus (17).

FLT3-ITD-containing MSCV retroviruses were transfected into the IL3-dependent murine hematopoietic cell line Ba/F3 as previously described (18). Ba/F3:p210 cells were obtained by transfecting the IL3-dependent murine hematopoietic Ba/F3 cell line with a pGD vector containing p210Bcr-Abl (R2A2) cDNA (19–21). Ba/F3-NRAS-G12D, Ba/F3-KRAS-G12D, HEL, MOLM14, HL60, NOMO-1, NB4, and SKM-1 cells were cultured with 5% CO₂ at 37°C, at a concentration of 2 × 10⁵ to 5 × 10⁵ in RPMI (Mediatech, Inc.) with 10% FBS and supplemented with 2% l-glutamine and 1% penicillin/streptomycin. OCI-AML3 cells were cultured in alpha MEM (Mediatech, Inc.) with 10% FBS and supplemented with 2% l-glutamine and 1% penicillin/streptomycin. Parental Ba/F3 cells were cultured in RPMI with 10% FBS and supplemented with 2% l-glutamine and 1% penicillin/streptomycin, as well as 15% WEHI-conditioned medium (as a source of IL3).

We have authenticated the following cell lines through cell line short tandem repeat (STR) profiling (DDC Medical, Fairfield, OH): MOLM14, NOMO-1, HEL, SKM-1, OCI-AML3, and NB4. All cell lines matched >80% with lines listed in the DSMZ Cell Line Bank STR Profile Information.

Chemical compounds and biologic reagents

GSK1904529A, AZD6244, PD0325901, GSK2126458, GSK112021, AZD8330, PI-103, and ZSTK474 were purchased from MedChem Express Co. Ltd. NVP-AEW541 was synthesized by Novartis Pharma AG and was dissolved in DMSO to obtain a 10 mmol/L stock solution. Serial dilutions were then made to obtain final dilutions for cellular assays with a final concentration of DMSO not exceeding 0.1%.
Proliferation studies, apoptosis assays, and cell-cycle analysis

The trypan blue exclusion assay has been previously described (22) and was used for quantification of cells before seeding for Cell Titer Glo assays. The Cell Titer Glo assay (Promega) was used for proliferation studies and carried out according to manufacturer instructions. Cell viability is reported as percentage of control (untreated) cells, and error bars represent the SEM for each data point. Programmed cell death of inhibitor-treated cells was determined using the Annexin-V-Fluos Staining Kit (Boehringer Mannheim), as previously described (22). Cell-cycle analysis was carried out via propidium iodide staining and FACS analysis.

Mononuclear cells

Mononuclear cells were purchased from STEMCELL Technologies and cultured in the presence of DMEM with 10% FBS and supplemented with 2% l-glutamine and 1% penicillin/streptomycin.

Antibodies, immunoblotting, and immunoprecipitation

The following antibodies were purchased from Cell Signaling Technology: phospho-4E-BP1 (S65) (rabbit, #9451), phospho--4E-BP1 (Thr37/46) (236B4) (rabbit mAb #2855), and total 4E-BP1 (53H11) (rabbit mAb, #9644) were used at 1:1,000. Phospho-AKT (Ser 473) (D9E) XP(R) (rabbit mAb, #4060) and total AKT (rabbit, #9272) were used at 1:1,000. Phospho-p44/p42 MAPK (T202/Y204) (rabbit, #9101) and total p44/p42 MAPK (Erk1/2) (3A7) (mouse, #9107) were used at 1:1,000. Phospho-S6 ribosomal protein (S235/236)(D57.2.2E) XP (R) (rabbit mAb, #4858) was used at 1:1,000. Total S6 ribosomal protein (SG10) (rabbit mAb, #2217) was used at 1:1,000. Phospho-IGF1Rβ (Y1135/1136/InsRbeta) (19H7) (rabbit mAb, #3024) was used at 1:1,000. Phospho-β-Tubulin (rabbit, #2146) was used at 1:1,000. Anti-GAPDH (D16H-11) XP (R) (rabbit mAb, #5174) was used at 1:1,000.

Anti-c-N-ras (Ab-1) (mouse, F155-227) was purchased from Calbiochem and used at 1:300. Total IGF1Rα (N-20): SC-712 (rabbit) was purchased from Santa Cruz Biotechnology and used at 1:1,000. Anti-β-actin (A1978, clone AC-15) (mouse) was purchased from Sigma-Aldrich and used at 1:1,000. Anti-KRAS (mouse, ab55391) was purchased from Abcam and used at 1:500.

Protein lysate preparation and immunoblotting were carried out as previously described (22).

Drug combination studies

For drug combination studies, single agents were added simultaneously at fixed ratios to cells. Cell viability was determined using the trypan blue exclusion assay to quantify cells for cell seeding and Cell Titer Glo for proliferation studies. Cell viability was expressed as the function of growth affected (FA) drug-treated versus control cells; data were analyzed by CalcuSyn software (Biosoft), using the Chou–Talalay method (23). The combination index $=$ $[D_x/D_y]_1 + [D_y/D_x]_2$, where $D_x$ and $D_y$ are the concentrations required by each drug in combination to achieve the same effect as concentrations $[D_x]_1$ and $[D_y]_2$ of each drug alone.

Knockdown of genes by shRNA

plKO.1puro lentiviral shRNA vector particles against KRAS and IGF1R were purchased from Sigma-Aldrich. Cells were incubated with the viral particles in the presence of 8 μg/mL polybrene for 24 hours, and the cells were selected with 1 to 2 μg/mL puromycin for 72 hours. Following selection, cells were used for the studies described. The sequences of shRNA are follows:

- GFP: ACAACAGCCACACGCTCATA
- IGF1: CITTAACTGACATGGGCCCTT
- IGF2: GGCAGAGATTTCACTACGAAA
- KRAS3: GCACAGCTATATGTATCATT

While knockdown of RAS and IGF1R inhibited the growth of mutant KRAS-expressing cells (data not shown), as compared with GFP control, it did not inhibit growth to the extent of prohibiting analysis of proliferation or validation of gene knockdown by Western blot analysis. Knockdown of RAS in HEL cells did not inhibit the growth of these cells (data not shown).

In vivo model of mutant NRAS-positive leukemia

Cells were transduced with a retrovirus encoding firefly luciferase (MSCV-Luc) and selected with neomycin to produce OCI-AML3-luc+ cells. Six-week-old female NSG mice were purchased from Jackson Laboratories. A total of 1.5 × 10⁶ OCI-AML3-luc+ cells were administered by tail vein injection. Mice were imaged and total body luminescence was quantified as previously described (22). One week after inoculation, treatment cohorts with matched tumor burden were established. Cohorts of mice were treated with oral administration of 30 mg/kg NVP-AEW541 per day, 25 mg/kg AZD6244 per day, or a combination of both. AZD6244 was reconstituted in 0.5% methylcellulose (Fluka) and 0.4% polysorbate (Tween80; Fluka). NVP-AEW541 was dissolved in 1 part N-methyl-2-pyrrolidone and then diluted with 9 parts PEG300. Tumor burden was assessed by serial bioluminescence imaging. Spleens were dissected and measured 1 week following the last imaging point (which was following 7 days of drug treatment). Major tissues were preserved in 10% formalin for histopathologic analysis. Studies were performed with Animal Care and Use Committee protocols at Dana-Farber Cancer Institute (Boston, MA).

AML patient cells

Mononuclear cells were isolated from samples from patients with AML identified as harboring mutant NRAS. Cells were tested in liquid culture (DMEM, supplemented with 20% FBS) in the presence of different concentrations of...
single and combined agents. All blood and bone marrow samples from patients with AML were obtained under approval of the Dana-Farber Cancer Institute Institutional Review Board.

Results

Inhibitors of PI3K and mTOR synergize with AZD6244

We screened the LINCS kinase inhibitor–focused chemical library, which is made up of highly selective small-molecule inhibitors, in an effort to find compounds that positively combine with the MEK inhibitor, AZD6244 (24) against mutant NRAS-expressing cells (see schematic, Supplementary Fig. S1). A number of interesting LINCS library compounds, including selective inhibitors of MEK and PI3K, were identified in the chemical screen as showing strong selective single-agent activity against Ba/F3-NRAS-G12D cells and the human mutant NRAS-expressing AML cell line, OCI-AML3, with little-to-no activity against parental Ba/F3 cells (known to be RAS independent for proliferation; Supplementary Fig. S2). AZD6244 was observed to synergize with LINCS library inhibitors of PI3K and mTOR, the targets of which mediate mutant RAS signaling, against mutant NRAS-expressing cells, Ba/F3-NRAS-G12D and OCI-AML3, but not wt RAS-expressing HEL cells (Fig. 2 and Supplementary Fig. S3). These results validate the suitability of the design of the chemical screen to identify inhibitors relevant to mutant RAS signaling as able to enhance the effects of AZD6244.

The IGF1R inhibitor GSK1904529A synergizes with AZD6244 against mutant RAS-expressing cells

Interestingly, the chemical screen led to the identification of the IGF1R inhibitor, GSK1904529A, as able to selectively potentiate the effects of AZD6244 against mutant NRAS- or KRAS-expressing Ba/F3 cells with no apparent combination effect observed against parental Ba/F3 cells (Fig. 1A–C and Fig. 2). This suggests that drug effects are targeted to mutant RAS, as exogenous expression of mutant RAS in growth factor–dependent Ba/F3 cells confers growth factor independence and renders cells solely dependent on mutant NRAS; selective inhibition of mutant RAS in this system would be expected to cause cells to die in the absence of growth factor. Target specificity of IGF1R and MEK inhibition was further investigated by treating Ba/F3-NRAS-G12D cells with IGF1R and MEK inhibitors, alone and combined, in the absence and presence of 15% WEHI-conditioned media (used as a source of IL3) in parallel. As expected, potent suppression of Ba/F3-NRAS-G12D growth was observed following drug treatment in the absence of

Figure 1. Identification of GlaxoSmithKline (GSK) IGF1R inhibitor GSK1904529A as able to potentiate the effects of MEK inhibition in mutant NRAS- and KRAS-expressing cells; characterization of IGF1R protein expression in mutant NRAS- and KRAS-expressing cells. A–C, approximately 3-day proliferation studies performed with GSK1904529A and AZD6244 against Ba/F3-NRAS-G12D cells. AZD6244 + GSK1904529A studies against Ba/F3 (n = 3), Ba/F3-NRAS-G12D (n = 6), and Ba/F3-KRAS-G12D cells (n = 2). D and E, IGF1R expression in parental Ba/F3 versus Ba/F3-NRAS-G12D and Ba/F3-KRAS-G12D cells. F, comparison of IGF1R expression in Ba/F3, Ba/F3.p210, Ba/F3-FLT3-ITD, and mutant RAS-expressing Ba/F3 cells. Shown in parentheses adjacent to the symbol keys are estimated IC50 values (nmol/L) corresponding to individual drugs or drug combinations.
15% WEHI; however, partial IL3 rescue was observed for each single agent and the potency of the combined inhibitors was substantially lower when Ba/F3-NRAS-G12D cells were cultured in the presence of 15% WEHI (Supplementary Fig. S4). These results suggest that in the Ba/F3 system, IGF1R inhibition and MEK inhibition selectively block RAS signaling without nonselectively interfering with IL3-mediated signaling.

Ba/F3-NRAS-G12D cells were observed to overexpress NRAS as compared with parental Ba/F3 cells; however, neither single-agent treatment nor drug combination treatment altered mutant RAS expression in mutant RAS-expressing Ba/F3 cells (Supplementary Fig. S5A and S5B). Elevated levels of IGF1R were also observed in Ba/F3-NRAS-G12D or Ba/F3-KRAS-G12D cells, as compared with parental Ba/F3 cells (Fig. 1D and E). There was no effect of short-term (1 hour) treatment with AZD6244 or GSK1904529A, alone or combined, on levels of IGF1R protein or activity in mutant RAS-expressing Ba/F3 cells (Supplementary Fig. S5C–S5E). IGF1R protein was observed to be phosphorylated in both parental Ba/F3 cells and Ba/F3-NRAS-G12D cells, with IGF1R phosphorylation in parental Ba/F3 cells possibly associated with the IL3 responsiveness of the cells (Supplementary Fig. S5F). In a report by Soon and colleagues (25), IL4 was shown to stimulate IGF1R phosphorylation to a similar extent to IGF1 in growth factor–dependent parental 32D cells. The link between IL3 and IGF1R activity is illustrated in a study in which IL3 simulated IGF1 in inducing cell-cycle progression of IL3-dependent FDC-PI cells made to conditionally proliferate in response to IGF1R activation (26). IGF1R and downstream molecules characteristically transduce antiapoptotic signals; IL3 also induced antiapoptotic pathways in these cells. It is possible, therefore, that we are observing a similar association between IL3 and IGF1R activity in parental Ba/F3 cells cultured in the presence of IL3, as these cells are resistant to IGF1R inhibitor and MEK inhibitor–induced apoptosis.

We observed IGF1R levels in Ba/F3 cells transformed by FLT3-ITD or BCR-ABL to be similar to those observed in parental Ba/F3 cells and considerably less than those observed in Ba/F3-NRAS-G12D and Ba/F3-KRAS-G12D cells (Fig. 1F). These data suggest that not all oncogenes that are capable of conferring growth factor independence in Ba/F3 cells have the same inductive influence on IGF1R and support the notion that IGF1R overexpression in mutant RAS-expressing Ba/F3 cells is associated with mutant RAS expression.

Figure 2. Combination indices corresponding to proliferation studies investigating effects of MEK inhibition combined with LINCS chemical library inhibitors against AML cell lines. Values less than 0.9 indicate synergy and are in shades of red. (Darker shades signify higher synergy). Values greater than 0.9 do not indicate synergy and are colored white. Values less than 1 indicate synergy, whereas values greater than 1 indicate antagonism. CalcuSyn combination indices can be interpreted as follows: CI values < 0.1 indicate very strong synergism; values 0.1–0.3 indicate strong synergism; values 0.3–0.7 indicate synergism; values 0.7–0.85 indicate moderate synergism; values 0.85–0.90 indicate slight synergism; values 0.9–1.1 indicate nearly additive effects; values 1.0–1.2 indicate slight antagonism; values 1.20–1.45 indicate moderate antagonism; values 1.45–3.0 indicate antagonism; values 3.3–10 indicate strong antagonism; values >10 indicate very strong antagonism. Note: For some experiments, namely those in which there was no observed single-agent activity, combination indices could not be reliably calculated using the CalcuSyn software.
GSK1904529A and AZD6244 synergized against mutant NRAS-expressing cell lines OCI-AML3 and HL60, as well as active KRAS-expressing and dependent NOMO-1, NB4, and SKM-1 cells [Figs. 2 and 3A–E]. In contrast, GSK1904529A and AZD6244 did not positively combine against wt RAS-expressing HEL or MOLM14 cells (Fig. 3F and G) or normal mononuclear cells (Fig. 3H). Importantly, increased apoptosis and cell-cycle arrest and decreased soft agar colony formation were observed to correlate with combined drug treatment in mutant RAS-expressing AML cells, however, not wt RAS-expressing AML cells (Supplementary Fig. S6).

As leukemic cells in patients are exposed to growth factors, especially in the bone marrow, which could lead to drug resistance, we tested the ability of IGF1R inhibition and MEK inhibition to synergize against mutant RAS-positive AML cells in the presence of cytoprotective stromal conditioned media (SCM) derived from 2 different human stromal cell lines (HS-5 and HS27a). Despite a 15% to 20% cytoprotective effect of SCM on drug-treated cells, synergy was observed between GSK1904529A and AZD6244 that was similar to that observed between the drugs in the absence of SCM (i.e., the ED50 for GSK1904529A and AZD6244 against NOMO-1 the presence of HS27a SCM was 0.21917; Supplementary Fig. S7 and data not shown).

Of note, we observed a heightened response of mutant RAS-expressing HL60 cells to IGF, as compared with wt RAS-
expressing HEL cells (Supplementary Fig. S8). As some mutant RAS-positive cells may respond to IGF existing in serum, these results suggest that an IGF1R inhibitor may be more effective than an AKT inhibitor in suppression of PI3K/AKT signaling in mutant RAS-expressing cells. The data also support the use of HEL cells as a wt RAS-expressing control in studies involving IGF1R inhibition.

Ba/F3-KRAS-G12D cells treated overnight with an MEK inhibitor (AZD6244, 300 nmol/L) or PI3K inhibitors (BEZ-235, 1,000 nmol/L; PI-103, 1,000 nmol/L) showed a small reduction in IGF1R pror receptor and IGF1R β levels (data not shown). However, KRAS knockdown selectively led to substantial decreases in IGF1R levels in active KRAS-expressing cell lines, NOMO-1 and NB4 (Fig. 3I). The knockdown studies revealed these cell lines, as well as the active KRAS-expressing line, SKM-1, to be dependent on KRAS as KRAS knockdown resulted in a loss of cell viability (data not shown). This is in contrast to wt RAS-expressing HEL cells, for which KRAS knockdown did not lead to a loss of cell viability (data not shown).

**GSK1904529A synergizes with PI3K inhibitor ZSTK474 against mutant RAS-expressing cells**

To determine whether the positive combination observed between GSK1904529A and AZD6244 is unique or can be replicated by combining GSK1904529A with inhibitors of other downstream mediators of RAS transformation, we investigated the ability of GSK1904529A to positively combine with an inhibitor of PI3K signaling. The combination of GSK1904529A and the PI3K inhibitor, ZSTK474 (27), was antagonistic against parental Ba/F3 cells. However, a positive drug combination effect was observed against mutant NRAS- and mutant KRAS-expressing Ba/F3 cells (Supplementary Fig. S9).

**Inhibition of ERK1/ERK2 phosphorylation predicts responsiveness to GSK1904529A and AZD6244 in RAS-transformed cells**

In an attempt to better understand the mechanism underlying the observed synergy between AZD6244 and GSK1904529A against RAS-transformed cells, we tested the single-agent effects of AZD6244 and GSK1904529A on both ERK1/ERK2 and IGF1R expression/activity. GSK1904529A was observed to inhibit phosphorylation of IGF1R in a concentration-dependent manner (Fig. 4A); however, it showed no inhibitory activity against phosphorylation of ERK1/ERK2 (Fig. 4B). Similarly, AZD6244 did not inhibit the activity or expression of IGF1R (Fig. 4C and D); however, it inhibited phosphorylation of ERK1/ERK2 in a concentration-dependent manner (Fig. 4E). These data suggest that GSK1904529A and AZD6244 are selective toward their respective molecular targets; the lack of target redundancy is supportive of the observed synergy between the 2 agents in this system.

We investigated whether or not a correlation exists between the heightened responsiveness of mutant RAS-expressing cells to simultaneous IGF1R and MEK inhibition and the basal expression/activity of integral mediators of the IGF1R or Raf/MEK/ERK signaling pathways. Higher basal levels of phospho-ERK1/ERK2 were observed in Ba/F3-NRAS-G12D and Ba/F3-KRAS-G12D cells, as compared with parental Ba/F3 cells (Supplementary Fig. S10A). Generally, high levels of phosphorylated Erk/Erk2 were observed in wt and mutant RAS-expressing human AML lines (Supplementary Fig. S10B). IGF1R was observed to be detectable (expressed) and phosphorylated (active) in wt and mutant RAS-expressing AML lines (Supplementary Fig. S10C and S10D); however, mutant NRAS-expressing OCI-AML3 and mutant KRAS-expressing SKM-1 showed comparatively elevated phospho/total IGF1R ratios in the panel of AML lines studied (Supplementary Fig. S10C).

We observed significant inhibition of ERK phosphorylation by AZD6244 in mutant NRAS- and mutant KRAS-expressing cells, which was sustained in cells treated with both GSK1904529A and AZD6244 (Supplementary Fig. S11 and data not shown). When compared with MOLM14 and HEL cells, OCI-AML3 cells treated with the same concentrations of AZD6244 and GSK1904529A showed substantially higher phospho-ERK inhibition by AZD6244 or the drug combination (Supplementary Fig. S11A). The effects of AZD6244, alone or combined with GSK1904529A, were similarly more strongly suppressive of ERK phosphorylation in mutant KRAS-expressing cells than in wt RAS-expressing HEL cells (Supplementary Fig. S11B). These results suggest that the inhibition of phospho-ERK1/ERK2 is likely required for an MEK inhibitor and IGF1R inhibitor to positively combine against mutant RAS-expressing or wt RAS-dependent AML cells.

**The selective IGF1R inhibitor, NVP-AEW541, synergizes with AZD6244 against mutant RAS-expressing cells**

As GSK1904529A is equipotent toward the insulin receptor (IR) and IGF1R receptor, and NVP-AEW541 (Novartis Pharma AG) shows far greater (100-fold) selectivity toward IGF1R over IR, we were interested in testing the ability of the more selective IGF1R inhibitor to synergize with AZD6244 against mutant RAS-expressing cells. Whereas the combination of NVP-AEW541 and AZD6244 against parental Ba/F3 cells was antagonistic, this combination showed synergy against Ba/F3-NRAS-G12D and Ba/F3-KRAS-G12D cells (Figs. 2 and 5A–C and Supplementary Fig. S4). NVP-AEW541, similar to GSK1904529A, inhibited the phosphorylation of IGF1R in a concentration-dependent manner; however, it did not inhibit phosphorylation of ERK1/ERK2 (Supplementary Fig. S12).

**Ribosomal protein S6 and 4E-BP1 as mediators of synergy between IGF1R inhibition and MEK inhibition in mutant RAS-expressing cells**

We did not observe enhanced inhibition of ERK1/ERK2 or AKT phosphorylation in mutant RAS-expressing cells treated with the combination of MEK and IGF1R inhibitors, as compared with either agent alone (Supplementary Fig. S13). Inhibition of phosphorylation of AKT, however, is clearly shown by both IGF1R inhibitors, NVP-AEW541 and GSK1904529A, which is expected as AKT is downstream of
IGF1R. The lack of enhanced inhibition of phospho-AKT by the drug combination is also not surprising, as AZD6244 selectively acts on the MAPK signaling pathway. The lack of elevated inhibition of phosphorylation of ERK1/ERK2 suggests that this factor may not contribute significantly to the observed synergy between IGF1R and MEK inhibition.

As these results are consistent with each inhibitor selectively targeting its own independent signaling pathway, we decided to investigate drug combination effects on signaling mediators downstream of IGF1R and RAS that are influenced by both the PI3K//PTEN/AKT/mTOR and Raf/MEK/ERK signaling. We identified ribosomal protein S6 and 4E-BP1 as potential mediators of IGF1R and MEK inhibitor synergy, with the phosphorylation of ribosomal protein S6 and 4E-BP1 at Thr37/46 more greatly suppressed by the combination of IGF1R and MEK inhibition in mutant RAS-transformed cells (Fig. 5D–G). In contrast, we observed no enhanced inhibition of phosphorylation of 4E-BP1 at Serine 65 by the drug combination (Supplementary Fig. S14), suggesting that this phosphorylation site may not significantly contribute to the observed synergy between IGF1R and MEK inhibition. The enhanced suppression of S6 and 4E-BP1 (Thr 37/46) phosphorylation may thus add to the individual or parallel effects of both inhibitors in mediating growth inhibition.

Effects of IGF1R knockdown in mutant RAS-expressing cells

Knockdown of IGF1R in the mutant KRAS-expressing cell line, NB4, led to a decrease in cell growth (Supplementary Fig. S15A and S15E), suggesting that IGF1R is important for the viability of these cells. Single-agent and drug combination effects were modestly augmented in IGF1R knockdown cells as compared with control cells (Supplementary Fig. S15B). In contrast, IGF1R knockdown in wt RAS-expressing HEL cells had less of an inhibitory effect on cell growth and...
did not affect drug sensitivity of the cells (Supplementary Fig. S15C, S15D, and S15F). Interestingly, there was no apparent decrease in the phosphorylation of AKT or downstream signaling mediators, 4E-BP1 or S6, in IGF1R knock-down cells (data not shown). One possibility for this is that the more transient timing of inhibition of IGF1R and MEK through pharmacologic inhibition (2–3 days) as compared with that of IGF1R gene knockdown (over a week) may make the latter more subject to compensatory feedback loops characteristic of IGF1R signaling.

**In vivo efficacy of IGF1R inhibition and MEK inhibition in a mutant NRAS-positive leukemia mouse model**

We used a mouse model of mutant NRAS-positive leukemia to examine the in vivo effects of IGF1R inhibition combined with MEK inhibition. The combination of NVP-AEW541 and AZD6244 was observed to be significantly more effective in suppressing leukemia burden in mice tail vein injected with OCI-AML3-luc- cells, as compared with either agent alone following 1 week of drug administration (Fig. 6), and mouse spleen sizes, measured 1 week later, were observed to be smallest in drug combination–treated mice (Supplementary Fig. S16). It should be noted, however, that while a difference in engraftment was observed on day 7 following drug treatment between single-agent–treated and combination-treated mice, there was continued growth of leukemic cells in drug-treated groups.

**Efficacy of IGF1R inhibition and MEK inhibition against mutant NRAS-positive primary AML cells**

We also tested the combined IGF1R and MEK inhibition in 3 mutant NRAS-expressing primary AML patient cells,
using the same drug concentrations used for cell line–based studies (0, 75, 150, 300 nmol/L), and observed more cell death with the combination than either agent alone (Supplementary Fig. S17). Importantly, for patient sample AML3 (NRAS-G13V, with 92% bone marrow blasts), combination indices suggested nearly additive–synergistic effects for the combination of NVP-AEW541 and AZD6244: ED25 (0.98304, nearly additive); ED50 (0.21557, synergy); ED75 (0.14790, synergy); ED90 (0.26906, synergy). These data support the potential clinical utility of this drug combination for mutant NRAS-positive AML.

**Discussion**

In hematologic malignancies, NRAS and KRAS are each frequently mutated. In AML, NRAS codon 12, 13, or 61 mutations have been reported in 10% to 11% of cases, whereas KRAS mutations have been reported in 5% of cases (28, 29). Direct inhibition of mutant RAS with small molecules has proven difficult, and the effects of targeting downstream RAS effectors have been difficult to predict. This justifies targeting multiple downstream effectors of RAS or RAS-associated factors as a strategy designed to increase clinical efficacy and circumvent drug resistance in mutant RAS-positive malignancies.

As there is a high degree of uncertainty with respect to the exact contribution of RAS effectors to growth and survival of leukemia, screening of targeted small-molecule inhibitors can yield significant mechanistic and clinical insights. Seeing that only limited efficacy has been achieved with targeting downstream effectors of mutant RAS, such as MEK, we used a chemical screen approach, using the LINCS chemical library composed of highly selective kinase inhibitors, to identify agents able to effectively enhance MEK inhibition in mutant NRAS-expressing cells. LINCS library compounds were anticipated to be useful, because of their limited spectrum of kinase targets, to more easily identify kinase mediators of RAS signaling that could be exploited for the purpose of drug development. Consistent with what has been reported in the literature, we identified through this screen several inhibitors of mTOR and PI3K/AKT signaling, which function downstream of RAS, as able to potentiate the effects of the MEK inhibitor, AZD6244.
Importantly, however, we also identified the IGF1R/IR inhibitor, GSK1904529A, as able to potentiate the effects of AZD6244 against mutant RAS-expressing AML cells. While it has been proposed that dual inhibition of IGF1R and IR may result in greater therapeutic efficacy and overriding resistance to anti-IGF1R therapies (30–32), this dual inhibition may also lead to clinical limitations resulting from adverse effects/off-target toxicity. Importantly, potentiation of AZD6244 by an IGF1R inhibitor, NVP-AEW541, which shows 100-fold higher selectivity for IGF1R over IR, was also observed in our studies. This suggests that synergy with an MEK inhibitor can be achieved with either an IGF1R/IR inhibitor or a more targeted IGF1R inhibitor.

Potential clinical relevance of our findings is supported by the high degree of clinical investigation currently underway, namely, the preclinical or clinical development of approximately 30 IGF1R targeting agents and the close to 60 clinical trials evaluating such agents alone or in combination with other inhibitors (reviewed in ref. 33). The 2 primary approaches to inhibiting IGF1R are through small-molecule inhibition of the protein or antibodies, and at the present time, it is unclear which is likely to be more effective in patients (reviewed in ref. 33). While early clinical studies support the notion of IGF1R as being a viable therapeutic target for certain cancers, variability in patient responsiveness to IGF1R and toxicity associated with certain drug combinations warrant identification of predictive biomarkers to identify probable candidates and highlight a need for rational development of novel drug combination strategies focused on IGF1R-mediated signaling in combination with other targeted therapies. In response, we have identified mutant RAS-expressing leukemia as a promising disease target for therapy dependent on IGF1R inhibition coupled with targeted inhibition of mutant RAS signaling.

Several studies have implicated a functional association between RAS transformation and IGF1R. In an inducible NRAS (Q61K)-driven genetically engineered mouse model of melanoma, 4 days of genetic extinction of NRAS (Q61K) expression led to a substantial downregulation of IGF1R expression, according to reverse-phase protein array profiling (34). In addition, mouse embryo fibroblasts devoid of IGF1R cannot be transformed by Ha-RAS (35, 36). In addition to demonstrating synergy between selective IGF1R inhibition and targeted inhibition of downstream RAS signaling pathways, we present here the novel positive correlation between mutant RAS expression and IGF1R protein expression in mutant RAS-transformed hematopoietic cells. It is possible that the increased IGF1R levels observed in mutant RAS-expressing cells may be a hallmark prosurvival characteristic of RAS transformation that enables cells to thrive and disease to progress.

The combination of IGF1R inhibitors with inhibitors of RAS signaling, including BRAF or MEK, has been suggested for breast cancer in response to reports of MEK inhibitor induction of PI3K pathway signaling (37, 38), as well as KRAS-mutant colorectal cancer (39) and lung cancer (40, 41). Similarly, the combination of IGF1R inhibitors with inhibitors of AKT or mTOR has been proposed for solid tumors, including breast and prostate cancer, as a strategy to override drug resistance due to negative feedback loops associated with mTOR inhibition (42).

Previous reports have implicated IGF1R as a potentially important target in AML due to high IGF1R expression (30, 43, 44) and enhancement of AML cell survival and proliferation by IGF1R signaling (45). Of relevance, targeted small-molecule inhibition of IGF1R, such as by NVP-AEW541 or NVP-ADW742, or dual inhibition of IGF1R/IR receptor, such as by BMS-536924, leads to induction of apoptosis in AML cells (30, 42, 45, 46). mTOR inhibition causes induction of PI3K/AKT signaling through increased IGF1R signaling, and combined mTOR and PI3K/AKT inhibition leads to additive antiproliferative effects in AML (47). Furthermore, antibody targeting of IGF1R in combination with inhibitors of Raf/MEK/ERK or PI3K/AKT/mTOR signaling has been shown to be synergistic against hematopoietic cells engineered to express IGF1R (48).

Our findings are consistent with these studies and demonstrate a correlation between mutant RAS protein expression and IGF1R protein expression in mutant RAS-expressing AML. The present study is also the first to highlight—in mutant RAS-positive AML—the potential therapeutic benefit of dual suppression of IGF1R and RAS signaling. This novel combination strategy warrants further investigation as a therapeutic approach that could bypass some mechanisms of drug resistance associated with MEK inhibition and that therefore may be of potential clinical benefit in mutant RAS-driven leukemia.

Disclosure of Potential Conflicts of Interest

I.D. Griffin reports receiving a commercial research grant from Novartis. No potential conflicts of interest were disclosed by the other authors.

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Grant Support

N.S. Gray is supported by NIH LINCS grant HG006097.

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Received April 11, 2014; revised August 1, 2014; accepted August 6, 2014; published OnlineFirst September 3, 2014.
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