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Development of combination therapies to maximize the impact of G12C KRAS inhibitors in lung cancer

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Abstract

KRAS represents an excellent therapeutic target in lung cancer, the most commonly mutated form of which can now be blocked using G12C KRAS mutant-specific inhibitory trial drugs. Lung adenocarcinoma cells harboring KRAS mutations have been shown previously to be selectively sensitive to inhibition of MEK and IGF1R signaling. Here we show that this effect is markedly enhanced by simultaneous inhibition of mTOR, while maintaining selectivity for the KRAS-mutant genotype. Combined mTOR, IGF1R, and MEK inhibition inhibits the principal signaling pathways required for the survival of KRAS-mutant cells and produces marked tumor regression in three different KRAS-driven lung cancer mouse models. Replacing the MEK inhibitor with the mutant-specific G12C KRAS inhibitor ARS-1620 in these combinations is associated with greater efficacy, specificity, and tolerability. Adding mTOR and IGF1R inhibitors to ARS-1620 greatly improves its effectiveness on KRAS-G12C mutant lung cancer cells in vitro and in mouse models. This provides a rationale for the design of combination treatments to enhance the impact of the G12C KRAS inhibitors which are now entering clinical trials.

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Introduction

Activating mutations in genes encoding the RAS subfamily of small GTP-binding proteins drive the formation of a large proportion of human tumors, with mutations often being associated with resistance to existing therapies (1, 2). In lung cancer, *KRAS* (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is mutationally activated in about 33% of adenocarcinomas (3). Targeting RAS proteins directly represents a clinical opportunity, as *RAS* mutant tumors frequently exhibit oncogene addiction (4, 5). However, direct pharmacological targeting of activated RAS proteins has been challenging and has not yet led to successful treatments in the clinic. Much effort has therefore focused on targeting tractable pathways acting downstream of RAS. For example, combined inhibition of ERK (extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase) signaling, two well-described RAS-controlled effector pathways, has shown some efficacy in mutant *KRAS*-driven mouse lung tumor models (6, 7). However, although this combination of pathway inhibitory drugs has been explored in a number of early phase clinical trials, it has not progressed due to marked toxicity and limited efficacy (8–10).

In an attempt to develop combination therapies capable of more specifically targeting RAS mutant cancer cells with less toxicity to normal tissue, we screened drug libraries against cell line panels for compounds with maximal selectivity for the RAS mutant genotype, finding not only MEK (mitogen-activated protein kinase) and RAF inhibitors, but also inhibitors of IGF1R (insulin-like growth factor 1 receptor) (11). Combined targeting of MEK and IGF1R shows some improved ability to constrain mutant *KRAS*-driven tumor cell growth in both lung cancer (11) and colon cancer (12) models. In addition, others have reported targeting of other receptor tyrosine kinases, including FGFR1 (13) and EGFR/ERBB proteins (14), in combination with the RAF/MEK/ERK pathway as having utility in various *RAS* mutant cancer models. In addition, targeting the SHP2 non-receptor protein tyrosine phosphatase may provide another approach to shutting off receptor tyrosine kinase signaling input to RAS and may show beneficial combination effects with MEK inhibition in RAS mutant cancers (15).

While combinations of therapies acting downstream of RAS have achieved some limited success in targeting RAS mutant cancers in model systems, marked progress has been made in developing agents that target RAS proteins directly (16, 17). Of the many different approaches attempted, the most striking advances have been made in the development of compounds that covalently bind to the novel cysteine residue present in the G12C mutant *KRAS* protein (18–21). The G12C mutation is the commonest alteration in *KRAS* in lung cancer, being found in 16% of all lung adenocarcinomas (3). Janes et al. reported the ability of the *KRAS*-G12C inhibitor ARS-1620 to inhibit the growth of human lung, pancreatic, and colorectal cancer cell lines bearing this mutation both in vitro and in vivo in mouse subcutaneous xenograft models (20). Two early stage clinical trials have recently started with other *KRAS*-G12C inhibitors, AMG 510 (NCT03600883) and MRTX849 (NCT03785249). However, while the results of taking agents such as these into the clinic are eagerly awaited, there is a strong likelihood that tumors may develop resistance to them, at least when used as single agents, either due to short-term signaling adaptation or long-term selection of minor variants.

In the current study we used a functional genomic screen to identify pathways whose inhibition would potentiate the selective effects of the MEK and IGF1R inhibitor combination on KRAS-mutant lung cancer cells that we investigated previously (11). We found that adding blockade of mTOR (mammalian target of rapamycin) to MEK and IGF1R inhibition has a profound inhibitory effect on KRAS-mutant lung cancers in vitro and in vivo, while showing selectivity relative to KRAS wild type cells. To avoid toxicities associated with the presence of a MEK inhibitor in this triple combination, we substituted a KRAS-G12C inhibitor, ARS-1620 (20), for the MEK inhibitor. We found that addition of mTOR and IGF1R inhibitor greatly increases the impact of ARS-1620 on KRAS-G12C mutant cancer cell lines both in vitro and in vivo, while maintaining selectivity relative to KRAS wild type cells and avoiding toxicities associated with MEK inhibition. This provides a clear direction for how better to use KRAS-G12C inhibitors in the clinic and how to attempt to circumvent the likely evolution of resistance to them when used as single agents.

Results

A whole-genome shRNA screen identifies sensitizers to MEK and IGF1R inhibitors

We have previously shown that non-small cell lung cancer (NSCLC) cell lines harboring KRAS mutations show an increased sensitivity to MEK and IGF1R inhibitors compared with KRAS wild-type counterparts, particularly in combination (11). However, the inhibition of cell growth in the presence of both drugs is incomplete at extended time points (fig. S1A). To identify combinatorial drug targets for the improvement of IGF1R and/or MEK targeting therapies, we adopted a pooled shRNA-drug screen strategy (Fig. 1A). We infected KRAS-mutant NSCLC H23 cells with a whole-genome shRNA library targeting 16,019 genes and cultured the cells in the presence or absence of the MEK inhibitor trametinib (GSK1120212), the IGF1R inhibitor linsitinib (OSI-906), or a combination of trametinib and linsitinib. We used relatively low doses of trametinib and linsitinib, which produced 40% and 25% reductions in cell viability, respectively (fig. S1B). After 6 days of treatment, changes in shRNA representation were determined by next generation sequencing (NGS) of genomic DNA. The resulting hit lists contained genes that, when silenced with at least two different shRNAs, sensitized cells to MEK and/or IGF1R inhibitors (fig. S1C and data file S1).

Representation of shRNAs targeting several mTOR pathway genes was decreased upon IGF1R inhibition with linsitinib (Fig. 1B). Both *MTOR* and Ras-related GTP-binding protein C (*RRAGC*) were top hits in the linsitinib and combination treatment hit lists (Fig. 1C and fig. S1D). *RRAGC* encodes the RagC protein, which forms heterodimers with RagA or RagB and is responsible for the translocation of mTORC1 from the cytoplasm to lysosomes, where it interacts with RHEB to become activated (22). mTORC1 is negatively regulated by TSC2 (tuberous sclerosis 2), which was the top hit producing resistance in cells treated with linsitinib plus trametinib (Fig. 1C, data file S1). We confirmed that knockdown of *MTOR* and *RRAGC* in H23 cells produced an enhanced sensitivity to linsitinib (Fig. 1D) as well as a more modest reduction in cell viability with trametinib treatment (fig. S1E and S1F). These results could be replicated with an alternative KRAS-mutant NSCLC cell line, H358 (fig. S1G and S1H). Moreover, inhibition of *TSC2* expression induced resistance to

linsitinib plus trametinib treatment (Fig. 1E). As expected, *MTOR* and *RRAGC* depletion reduced the activity of mTORC1, measured by the phosphorylation of the ribosomal protein S6, whereas knock-down of *TSC2* increased it (fig. S1I). The combination of linsitinib with *MTOR* or *RRAGC* knockdown resulted in a more profound inhibition of S6 phosphorylation (Fig. 1F). *MTOR* and *RRAGC* shRNAs induced the reactivation of the AKT pathway, probably due to suppression of the negative feedback loop from p70^{S6K1} onto IRS1 (23), but this was completely abolished by linsitinib. In summary, inhibition of the mTOR pathway components potentiates the effect of linsitinib or linsitinib combination with trametinib to strongly decrease the viability of KRAS-mutant cells, likely due to more efficient reduction of S6 and AKT phosphorylation.

Inhibition of IGF1R and MEK pathways in combination with mTOR inhibitors decreases the viability of KRAS-mutant NSCLC cells

mTOR has an important role in cancer biology, which has resulted in the development of several classes of mTOR inhibitors (24). We therefore decided to validate the results obtained in the shRNA screen using small molecule inhibitors of mTOR. Inhibition of mTORC1 using the rapalog derivative everolimus showed a reduction in cell proliferation when combined with the IGF1R inhibitor linsitinib in three KRAS-mutant NSCLC cell lines. The cells also exhibited an enhanced sensitivity to the combination of everolimus with a low dose of the MEK inhibitor trametinib (Fig. 2A). Combining the mTOR kinase inhibitor AZD8055, which blocks both mTORC1 and mTORC2 activities, with linsitinib also led to a striking reduction in cell viability. However, in contrast with the results obtained with everolimus, AZD8055 did not show a clear combinatorial effect with trametinib (Fig. 2B). These results could be replicated using alternative mTOR and IGF1R inhibitors (fig. S2A and S2B).

Triple drug combinations, using trametinib and linsitinib together with a rapalog or an mTOR kinase inhibitor, resulted in a profound inhibition of cell proliferation in the KRAS-mutant cell lines (Fig. 2A-B), which was accompanied by an enhanced induction of apoptosis (Fig. 2C, fig. S2C and S2D). The magnitude of the apoptosis induction produced by the three-drug combinations was considerably higher than the differences observed in cell viability after six days of treatment. We therefore decided to compare the double and triple drug combinations in long-term viability assays (Fig. 2D and fig. S2E). These results showed that although the two-drug combinations produced a strong reduction in cell viability after seven days of treatment, the three-drug combination caused a more profound and durable inhibition of proliferation at longer time points. Notably, these drug combinations produced only a modest effect on viability of a non-transformed lung cell line (Fig. 2E). The remaining cell growth observed in KRAS-mutant cells with the three-drug combination could be inhibited by increasing the dose of MEK inhibitor, with a minimal effect in wild-type cells. Use of a PI3K inhibitor instead of the IGF1R inhibitor could also block cell proliferation completely, but with higher toxicity and lower genotype selectivity (fig. S2F).

Next, we extended our findings using a larger panel of NSCLC cell lines. Consistently, the combination of either class of mTOR inhibitor with linsitinib potently reduced the cell

viability in the panel of cells harboring KRAS mutations (fig. S3A and S3B). Moreover, the addition of a low dose of trametinib decreased cell viability uniformly by more than 75% across the entire panel of mutant cell lines (Fig. 2F). In contrast, only the combination between AZD8055 and linsitinib showed an improvement in the response of the KRAS wild-type panel and the magnitude of this effect was considerably less than for the cells carrying KRAS mutations (Fig. 2G). Consistent with this, the synergy score of the combination between mTOR and IGF1R inhibitors, calculated using Chalice software (Horizon Discovery), was higher in the KRAS-mutant cells compared with the wild-type counterparts (fig. S3C). In summary, we had previously shown that KRAS-mutant cells are more sensitive than KRAS wild-type cells to the combination of linsitinib plus trametinib (fig. S3D) (11). Here we have further demonstrated that addition of mTOR inhibitors to the dual combination caused a prolonged inhibition of cell viability and enhanced the differential effect between KRAS-mutant and wild-type cells.

Combination of IGF1R with mTOR inhibitors results in a strong inhibition of PI3K/AKT and mTOR pathways

To investigate the mechanistic basis of the combinatorial effect of mTOR and IGF1R inhibitors in KRAS-mutant cells, we examined the effect of these drugs on the activity of PI3K/AKT and mTOR pathways. As expected, in three KRAS-mutant NSCLC cell lines, everolimus showed an efficient suppression of S6 phosphorylation, but it also produced a time-dependent increase of AKT phosphorylation at both phosphosites, Ser473 and Thr308 (fig. S4A). The kinase inhibitor AZD8055 caused a dose-dependent inhibition of S6 and AKT-Ser473 phosphorylation (targets of mTORC1 and mTORC2, respectively), but also resulted in an increased phosphorylation of AKT on Thr308 (fig. S4A). As we have previously shown, inhibition of IGF1R shows a marked suppression of AKT phosphorylation in KRAS-mutant cells (Fig. 3A) (11). Notably, linsitinib abrogated the reactivation of AKT phosphorylation produced by everolimus on both phosphosites (Ser473 and Thr308). Consistent with this, the combination also reduced the phosphorylation of the AKT substrate PRAS40 on Thr246 (Fig. 3A and 3B). On the other hand, when a low dose of AZD8055 was combined with linsitinib we observed a strong inhibition of S6 and AKT-S473 phosphorylation. Moreover, linsitinib was also able to completely block the reactivation of AKT on Thr308 produced by AZD8055 treatment (Fig. 3A and 3B). Therefore, the combination of rapalogs or mTOR kinase inhibitors with IGF1R inhibitors resulted in a strengthened inhibition of the PI3K/AKT and mTOR pathways, which could explain the combination effect observed when both drugs are combined (fig. S4B).

Next, we extended our findings to the panel of lung cancer cell lines to determine if there is a consistent differential response between KRAS-mutant and KRAS wild-type cells. Although there is a broad response across the cell lines, results showed that both genotypes exhibit a reactivation of AKT phosphorylation after mTOR inhibition (Fig. 3C, 3D and fig. S4C), which is a major mechanism of resistance to mTOR inhibitors (25). All cells carrying KRAS mutations showed a striking reduction in AKT phosphorylation when linsitinib was added in combination. In contrast, linsitinib only partially suppressed the induction of AKT phosphorylation caused by mTOR inhibitors in KRAS wild-type cells (Fig. 3D). This genotype difference could be explained by the fact that IGF1R inhibition alone produced a

more profound inhibition of AKT phosphorylation in cells carrying KRAS mutations compared with their wild-type counterparts (fig. S4D). Consistent with this and in agreement with the viability data, the panel of KRAS-mutant cells showed a robust and homogeneous reduction in AKT activity in response to the combination of mTOR and IGF1R inhibitors, whereas the KRAS wild-type cells exhibit a milder and more heterogeneous response (Fig. 3E). The ability of linsitinib to block AKT phosphorylation after mTOR inhibitor treatment correlated with a more profound inhibition of cell proliferation (fig. S4E).

Our results show that linsitinib plus everolimus treatment resulted in a strong inhibition of the PI3K/AKT and mTOR pathways in cells harboring KRAS mutations. However, addition of a MEK inhibitor to the combination was needed to achieve a higher induction of apoptosis and durable growth inhibition (Fig. 2). To investigate this mechanism, we analyzed the effect of the drug combination on the activity of the targeted pathways. As expected, the addition of a low dose trametinib to linsitinib plus everolimus led to a reduction of ERK phosphorylation but also abrogated the residual phosphorylation of S6 (Fig. 3F). Thus, the three-drug combination inhibited the two main pathways downstream of KRAS, PI3K/AKT and RAF/MEK/ERK, and completely suppressed S6 phosphorylation (Fig. 3F and fig. S4F), which is a key marker of responsiveness to MEK and PI3K inhibitors both in vitro and in vivo (26, 27). Of note, the inhibition of PI3K/AKT and the abrogation of S6 phosphorylation were specific to KRAS-mutant cells (Fig. 3G), which is consistent with the differences observed in cell viability between both genotypes.

mTOR inhibition activates the IGF1R pathway in KRAS-mutant cells

Previous studies have shown that mTOR inhibition can lead to activation of receptor tyrosine kinase (RTK) signaling (25). To explore whether RTK activation could be responsible for the increase in AKT signaling in our cells, we performed phospho-RTK profiling. These results showed that, in cells harboring KRAS mutations, mTOR inhibition resulted in an induction of IGF1R and insulin receptor (InsR) phosphorylation (Fig. 4A and fig. S5A). We then confirmed by western blotting that both everolimus and AZD8055 increased IGF1R phosphorylation in three KRAS-mutant NSCLC cell lines, whereas the members of the human epidermal growth factor receptor (HER) family EGFR, ERBB2, and ERBB3 were not activated (Fig. 4B). The pattern of RTK activation was different when we extended our analysis to KRAS wild-type cells. H2170 cells showed an increased phosphorylation of ERBB2 and ERBB3, whereas in HCC95 cells mTOR inhibition activated IGF1R and c-MET (Fig. 4C and fig. S5B). The phospho-RTK profiling also suggested differences in the basal RTK activation between KRAS-mutant and wild-type cells. Western blot analysis confirmed that cells harboring KRAS mutations had generally higher activity of the IGF1R pathway, whereas wild-type cells had an increased activity of the HER kinase family members (Fig. 4D). This result was further validated using reconstituted RASless MEFs expressing either wild-type or mutant KRAS isoforms. Cells expressing mutant KRAS had higher IGF1R activity, and its inhibition partially reduced AKT phosphorylation, resulting in a better response to the combination of linsitinib, everolimus, and trametinib (fig. S5C and S5D).

To explore the mechanism of activation of the IGF1R pathway, we studied the possible role of the IGF1R ligands, IGF1 and IGF2. Most of the KRAS-mutant cells analyzed secreted

either IGF1 or IGF2. In contrast, no IGF1R ligand secretion was detected in the conditioned medium of KRAS wild-type cells (Fig. 4E). Taken together, these results suggest that cells harboring KRAS mutations have a strong basal activity of the IGF1R pathway and this pathway is critical for the activation of PI3K, both in basal conditions and after mTOR inhibition. This may explain why the combination of mTOR and IGF1R inhibitors is more efficient in KRAS-mutant cells than in their wild-type counterparts.

A three-drug combination causes tumor regression in KRAS-driven lung tumors

To determine the efficacy of the drug combinations *in vivo*, we used the KRAS G12D-driven lung tumor mouse model *Kras^{LSL-G12D};Tp53^{Flox/Flox}*, which develops aggressive lung adenocarcinomas. Whereas treatment with linsitinib or everolimus had a modest or no effect on tumor growth, a striking reduction in tumor progression was obtained when both drugs were combined (Fig. 5A). Nevertheless, the treatment failed to promote tumor regression over the four-week course of the treatment. In keeping with our *in vitro* findings, addition of trametinib to this combination led to marked regressions in volume of most of the tumors analyzed (Fig. 5A). The degree of regression was greater than with the double combination of trametinib plus linsitinib (fig. S6A). The triple drug combination was generally tolerated during the treatment period (fig. S6B). However, postmortem analysis in six mice detected stomach ulcers in four mice and one incident of adverse effects in the eye, which are consistent with the effects reported in patients treated with MEK inhibitors and rapalogs (28–30). Analysis of the activity of the targeted pathways in drug-treated tumors recapitulated the *in vitro* findings. Everolimus treatment induced a clear increase of IGF1R and AKT phosphorylation, which was blocked by addition of linsitinib. ERK phosphorylation was also augmented after everolimus treatment and was inhibited by trametinib (fig. S6C).

To evaluate better the efficacy of our three-drug regimen, we compared it with the combination of trametinib plus paclitaxel. A phase 2 trial of the combination of MEK inhibitor selumetinib plus docetaxel showed promising efficacy in KRAS-mutant NSCLC (31), although a subsequent phase 3 study failed to confirm this (32). Our results showed that treatment with MEK, IGF1R, and mTOR inhibitors was markedly more effective than the combination of MEK inhibitor and taxane (fig. S6D). We then discontinued the treatment after four weeks and monitored tumor growth for three weeks. Disruption of the treatment resulted in tumor regrowth (fig. S6E). However, when we administered a second cycle of treatment, the triple combination again led to a marked tumor regression in most of the tumors analyzed (fig. S6F).

We evaluated the efficacy of our treatment in two further KRAS-mutant mouse lung tumor models. We used a urethane-induced lung cancer model, in which the carcinogen administration induces lung tumors carrying KRAS Q61R mutations. This model develops less aggressive tumors than the *Kras^{LSL-G12D};Tp53^{Flox/Flox}* (KP) mouse model, but with a higher number of mutations (33). The drug doses were reduced compared with the KP model experiments to avoid drug-associated toxicities linked to the different mouse genetic backgrounds (FVB/N for urethane-induced carcinogenesis, C57/Bl6J for the KP engineered model). No measurable toxicity was detected at the level of mouse body weight with the

doses used (fig. S6G). Results showed that the combination of everolimus, linsitinib, and trametinib produced a striking antitumor effect in the urethane-induced lung tumors (Fig. 5B and fig. S6H). All the tumors analyzed showed more than 50% tumor regression and, in some cases, regression was almost complete. Similarly to the KP model, tumors grew back when treatment was discontinued but again regressed when the drugs were re-administered (fig. S6I). In contrast to the KP model, regression during the second round of treatment was less pronounced than during the first cycle (Fig. 5C). This might be explained by the fact that these tumors grow more slowly after drug withdrawal, or perhaps because the higher mutation burden could increase the likelihood of selecting for outgrowth of resistant clones.

Finally, we determined the efficacy of the drug combination in the *Kras*^{LSL-G12D};*Stk11*^{Flox/Flox} mouse model. In this model, concomitant loss of *Stk11*, which codes for LKB1, results in highly aggressive tumors that show reduced response to treatment with MEK inhibitor plus docetaxel (34). Treatment of *Kras*^{LSL-G12D};*Stk11*^{Flox/Flox} mice with the three-drug combination resulted in a striking response, with robust regression of all the tumors analyzed (Fig. 5D). The three-drug treatment produced a better response than the double combinations, with no tumors growing during the treatment (fig. S6J). In summary, we have demonstrated in three different KRAS-driven lung cancer mouse models, which represent the three major genetic subgroups of KRAS-mutant lung adenocarcinoma (35), that combined MEK, IGF1R, and mTOR inhibition causes robust tumor regression.

Drug combinations with a direct KRAS inhibitor result in a profound inhibition of viability in KRAS-mutant cells

Our results suggest that a profound inhibition of the pathways downstream of KRAS is required for a durable suppression of cell proliferation and tumor regression, and that this can be achieved through combined inhibition of MEK, mTOR, and IGF1R. However, attempts at combination therapies in the clinic involving MEK inhibitors together with mTOR inhibitors have been thwarted by unacceptable adverse effects (30). We therefore decided to explore if we could achieve pathway inhibition even more selectively in cells harboring KRAS mutations, to increase the differential sensitivity between the genotypes as well as reduce potential unwanted toxicities of the three-drug combination. For this purpose, we tested the KRAS-mutant specific inhibitor ARS-1620, a selective covalent inhibitor that specifically blocks the KRAS-G12C mutant protein (20). KRAS-G12C mutations account for 40% of the KRAS mutations in NSCLC (3). H23 and H358 cells, which harbor KRAS-G12C mutations, showed a partial inhibition of cell proliferation when they were treated with ARS-1620 as single agent (Fig. 6A). The magnitude of the effect was considerably more pronounced when ARS-1620 was combined with either linsitinib or everolimus, although the inhibition of cell proliferation was still not complete. However, complete growth inhibition could be achieved when the three drugs were combined together (Fig. 6A and fig. S7A). The three-drug combination resulted in a marked induction of apoptosis and in a strengthened inhibition of viability for longer periods of time (Fig. 6B, 6C and fig. S7B). No cell growth was detected for a period of up to 17 days when cells were treated with the combination of ARS-1620, linsitinib, and everolimus. This suggests that such a combination might also delay or prevent the appearance of resistance mechanisms that emerge when inhibiting PI3K/AKT or MEK/ERK pathways with single targeted agents.

Signaling pathway analysis revealed that ARS-1620 produced not only a robust inhibition of ERK phosphorylation, but also a reduction in S6 phosphorylation. As expected, addition of IGF1R and mTOR inhibitors results in a profound and durable inhibition of AKT and S6 phosphorylation. It also reduced reactivation of RAS and ERK activation observed at 48 hours (Fig. 6D, 6E and fig. S7C), which may explain the enhanced sensitivity of KRAS-G12C mutant cell lines to the combinations with ARS-1620. As expected, ARS-1620 selectively inhibited the growth of cells harboring KRAS-G12C mutations but no inhibition in cell proliferation or signaling were observed in either cells harboring a KRAS-G12S mutation or KRAS wild-type cells (Fig. 6F, fig. S7D and S7E), whereas the KRAS-G12S mutant cells responded to KRAS knock-down (fig. S7F). In KRAS-G12C mutant cells, the combination of ARS-1620 with mTOR and IGF1R inhibitors resulted in an enhanced inhibition of cell proliferation compared with the reported combination of KRAS-G12C inhibitors plus PI3K or EGFR inhibitors (fig. S7G) (36).

H1792 cells, which also carry a KRAS-G12C mutation, responded weakly to single treatment with ARS-1620 and had a modest response when ARS-1620 was combined with either linsitinib or everolimus. Notably, a strong reduction in cell viability was obtained when the three drugs were combined together (fig. S7H and S7I). Nevertheless, the inhibition of cell growth was not as profound as that observed in H23 and H358 cells. Signaling experiments showed that ARS-1620 did not achieve the same degree of inhibition of ERK phosphorylation as trametinib (fig. S7J). These differences in pathway inhibition may be explained by the amplification of the mutant KRAS allele in H23 and H358 cells (fig. S7K). It has been described that cell adherence can attenuate the KRAS-dependency and therefore the response to ARS-1620 in KRAS-G12C mutant cells (20). Consistent with this, we observed that all KRAS-G12C cell lines that were tested, including H1792 cells, showed sensitivity to ARS-1620 when they were grown in suspension as spheroid cultures (fig. S7L). Next, we used an extended panel of cells carrying KRAS-G12C mutations to test the combination of KRAS, IGF1R, and mTOR inhibitors. In agreement with the ARS-1620 sensitivity, when cells were grown in monolayer, only H23 and H358 cells showed a profound inhibition of cell viability with the three-drug combination (Fig. 6G). In contrast, in 3D-spheroid conditions, the combination decreased cell viability uniformly by more than 95% across the entire panel of KRAS-G12C mutant cells (Fig. 6H). As expected, this strong effect was specific to cells carrying KRAS-G12C mutations.

Altogether these data suggest that in KRAS-G12C mutant tumors the combination of ARS-1620, linsitinib, and everolimus can produce a strong and durable reduction of tumor cell growth. Due to the specificity of the KRAS-G12C inhibitor, the combination enhances the differential effect between KRAS-G12C mutant and wild-type cells and can avoid the toxicity associated with inhibition of wild-type RAS pathway signaling upon trametinib treatment.

IGF1R plus mTOR inhibition increases the in vivo efficacy of KRAS-G12C inhibitors

To determine the efficacy of the triple combination of KRAS-G12C, IGF1R, and mTOR inhibitors in vivo, we were not able to use the same mouse models used to validate the combinations with MEK inhibitors, because these tumors do not carry KRAS-G12C

mutations. Therefore, we initially evaluated the effects of ARS-1620, linsitinib and everolimus in xenografts of human KRAS-G12C lung adenocarcinoma cell lines. In HCC44 xenografts, the treatment with ARS-1620 produced a partial inhibition of tumor growth, whereas the three-drug combination resulted in regression of all the tumors measured (Fig. 7A and 7B). H358 and H1373 xenografts were more sensitive to ARS-1620 treatment. However, as shown in the waterfall plot (Fig. 7B), one H358 tumor treated with ARS-1620 alone was resistant and grew to the same extent as the vehicle-treated tumors. In contrast, all H1373 tumors responded to ARS-1620 but started to progress in the third week of treatment, whereas the triple combination produced durable tumor regression (Fig. 7A and 7B). These results suggest that although treatment with ARS-1620 alone can produce tumor regression, combination with mTOR and IGF1R inhibitors can result in a more profound inhibition of tumor growth and overcome intrinsic or adaptive resistance mechanisms.

Next, we aimed to investigate the efficacy of the combinations in an immunocompetent mouse model of aggressive KRAS-mutant lung adenocarcinoma. Until very recently, most of the engineered mouse models with oncogenic KRAS alleles did not carry KRAS-G12C mutations (37), and suitable lung cancer cell lines for syngeneic transplantation driven by a KRAS-G12C mutation had not been identified. In the course of our search for appropriate cell lines, we determined that the 3LL cell line, derived from Lewis lung carcinoma and broadly used in *in vivo* experiments (38), carries both NRAS-Q61H and KRAS-G12C mutations ((39) and fig. S8A). Treatment of these cells *in vitro* with ARS-1620 resulted in a moderate reduction of ERK phosphorylation and had little effect on cell viability (Fig. 7C and 7D). Assuming that both mutant NRAS and KRAS were driving RAS pathway activation in these cells, we knocked out NRAS using CRISPR/Cas9 to promote a stronger dependency on oncogenic G12C KRAS. In contrast to the parental cells, NRAS knockout cells (3LL NRAS) exhibited a strong sensitivity to ARS-1620, which correlated with markedly improved inhibition of pathways downstream of RAS (Fig. 7C and 7D). Consistent with the results obtained in human NSCLC cell lines, treatment with ARS-1620 in 3LL NRAS cell clones showed a stronger reduction of cell proliferation when combined with linsitinib plus everolimus (Fig. 7D). As expected, the combination produced a better pathway inhibition (Fig. 7E). Moreover, treatments with ARS-1620 resulted in a stronger induction of apoptosis in 3LL NRAS cells, whereas in the parental 3LL cells only the treatments with trametinib induced apoptosis (Fig. 7F).

We decided to use the 3LL NRAS cells to validate *in vivo* the efficacy of the drug combinations with ARS-1620 in immunocompetent mice. Results showed that treatment with ARS-1620 or linsitinib plus everolimus produced a partial inhibition of tumor growth, whereas the combination of the three drugs together resulted in a striking suppression of tumor growth without measurable toxicities (Fig. 7G, fig. S8B and S8C). The triple combinations using either ARS-1620 or trametinib showed similar results, demonstrating that replacement of trametinib by ARS-1620 in the three-drug combination is a realistic option when tumors harbor KRAS-G12C mutations. Finally, we validated the *in vivo* results using a second NRAS knockout 3LL clone. Treatments with ARS-1620 or linsitinib plus everolimus enhanced mouse survival. Notably, the combination of the three drugs resulted in survival of the entire cohort until the end of the experiment (Fig. 7H). Therefore, we concluded that when tumors carry KRAS-G12C mutations, the addition of mTOR and

IGF1R inhibitors greatly improves the effectiveness of the KRAS-G12C inhibitor ARS-1620 in vivo.

Discussion

The clinical failure of agents targeting the RAF/MEK/ERK pathway downstream of RAS in KRAS-mutant cancers (32) has led to interest in the possible utility of combinations of targeted agents inhibiting the multiple pathways downstream of RAS. Here we have used a functional genomic screen to identify a combinatorial strategy to treat lung tumors harboring KRAS mutations through the use of a combination of mTOR, IGF1R, and MEK inhibitors, which results in marked tumor regression in a range of KRAS-driven mouse lung cancer models. While components of this triple drug combination involving MEK inhibitors have been found to present notable adverse effects in clinical trials, we find that substitution of MEK inhibitor with the recently developed mutant-specific KRAS-G12C inhibitor ARS-1620 allows strong inhibition of KRAS-G12C mutant tumors with reduced toxicity and shows marked potentiation of the effects of ARS-1620 alone.

Several studies have reported the need to achieve strong pathway inhibition to minimize intrinsic and adaptive resistance mechanisms and improve efficacy of targeted therapies (40–43). BRAF-mutant cancers provide a clear example of how treatment with targeted drugs has evolved to achieve this objective. Based on the observation that most tumors rapidly develop resistance to treatment with BRAF inhibitors due to reactivation of the MEK/ERK pathway, combinations of BRAF plus MEK inhibitors have been approved (44). Nevertheless, drug resistance still appears, albeit more slowly, and addition of either ERK or EGFR inhibitors to the combination is being evaluated in preclinical models and in clinical trials (41, 45). KRAS-mutant tumors also show marked short-term sensitivity to MEK and RAF inhibitors, thus, vertical inhibition of the pathway could increase the therapeutic response (46, 47). However, in contrast to BRAF-mutant tumors, where reactivation of the MAPK pathway appears to be the dominant resistance node, oncogenic KRAS can activate other pathways that can potentially compensate for loss of MEK/ERK activity. In this context, treatment with MEK inhibitors activates the PI3K/AKT pathway (14, 48), which is another major effector pathway downstream of RAS. Indeed, combinations of MEK and PI3K inhibitors have shown efficacy in KRAS-driven mouse models (6), supporting the notion that targeting both MEK/ERK and PI3K/AKT may be key to the effective treatment of KRAS-mutant tumors. However, to achieve effective pathway inhibition with acceptable toxicity, we need to better understand the pathways that are necessary for the survival of KRAS-mutant cells and the adaptive feedback mechanisms released after pathway inhibition.

We and others previously demonstrated that the combination of MEK plus IGF1R inhibition in KRAS-mutant cells causes marked inhibition of cell viability by blocking two main pathways downstream of RAS (11, 12). However, the combination results only in a partial reduction of S6 phosphorylation, used as a measure of mTORC1 signaling activity. Several studies have shown that sustained mTOR pathway activity confers resistance to targeted therapies (26, 27, 49). We hypothesize that the rather limited efficacy of the MEK and IGF1R inhibitor combination seen in long-term treatments is due predominantly to insufficient inhibition of S6 phosphorylation. Indeed, addition of mTOR inhibitors to the

drug combination results in abrogation of S6 phosphorylation and in a more profound and durable inhibition of cell viability and tumor growth. mTOR inhibitors have been approved in several cancer indications (kidney, breast, neuroendocrine), although efficacy is somewhat limited, likely in part due to the release of a p70^{S6K1}-dependent feedback loop via IRS1 which leads to the reactivation of PI3K/AKT and/or MEK/ERK signaling pathways (25, 50, 51). In KRAS-mutant lung cancer cells, the pathway reactivation is highly dependent on IGF1R signaling. Thus, combination of mTOR and IGF1R inhibitors blocks both basal AKT phosphorylation and reactivation of AKT produced by the release of the negative feedback loop, resulting in a potent inhibition of both PI3K/AKT and mTOR pathways. On the basis of these findings, we have derived a triple drug therapy that in KRAS-mutant cells results in simultaneous inhibition of the main nodes downstream of RAS. This allows the disruption of compensatory feedback, likely stalling the appearance of adaptive resistance mechanisms.

Combining MEK, IGF1R, and mTOR inhibitors causes marked tumor regression in three KRAS-driven lung cancer mouse models, two genetically engineered mouse models, KP (KRAS^{G12D} and p53 loss-of-function) and KL (KRAS^{G12D} and LKB1 loss-of-function), and one carcinogen model of urethane-induced lung tumors (KRAS^{Q61R} mutations). These three mouse models represent the three major subgroups of KRAS-mutant adenocarcinomas identified by Skoulidis et al. based on expression analysis of different patient cohorts (35). The fact that the triple combination proposed here exhibits comparable responses in the different models tested suggests that the treatment may be effective in most of the lung cancer tumors harboring KRAS mutations.

The potential success of this triple combination in a clinical setting would depend on the ability to block RAS pathway signaling output while limiting additive toxicities. Combinations of mTOR plus IGF1R inhibitors, including everolimus plus linsitinib, have been tested in phase 1 trials in unselected colorectal cancer patients without major toxicities (52), albeit without appreciable efficacy and without evaluation of target inhibition. More toxicity issues have been reported for the combination of mTOR and MEK inhibitors, because they seem to have overlapping adverse effects (30). Analysis of more than 350 clinical trials using three-drug combinations with targeted therapies suggested that, to successfully obtain safe doses, the full doses of single drugs should be reduced or alternative dosing schedules should be tested (53). With this in mind, a recent study has identified in preclinical models an intermittent schedule of RAF, MEK, and ERK inhibitors with good response and low toxicity (41). Although we have not evaluated different schedules of treatment in our mouse models, it would be informative to use these models to optimize the combination approaches to obtain maximal efficacy with minimum toxicity.

Based on available trial evidence using MEK inhibitor plus rapalog combinations, it seems likely that adding an IGF1R inhibitor to the combination may result in problematic toxicity in a clinical setting. The recently developed KRAS-G12C inhibitors offer the opportunity to target more selectively KRAS-mutant cells and have shown in vivo efficacy in a panel in KRAS-G12C mutant cell lines and in PDX tumor models (20). It is notable that all the models analyzed in that study showed suppression of tumor growth, although not all tumors presented durable regressions. This indicates that, although targeting KRAS-G12C is a promising therapeutic option, combinations with other agents may be needed to increase

efficacy. Indeed, treatment with ARS-1620 results in a strong inhibition of ERK phosphorylation but a less robust reduction in AKT and S6 phosphorylation. We hypothesized that a more sustainable inhibition of the PI3K/AKT and mTOR pathways is needed to obtain a durable response. Double combinations with either IGF1R or mTOR inhibitors greatly increase the response of KRAS-G12C mutant cancer cells to ARS-1620 in vitro, and the three-drug combination causes near-abrogation of cell viability. Previous studies using the earlier KRAS-G12C inhibitor ARS-853 demonstrated that the activity of oncogenic KRAS-G12C is responsive to growth factor stimulation and suggested that inhibition of RTKs could potentiate the effect of ARS-853 (19, 21). Our results show that inhibition of IGF1R plus mTOR reduces the reactivation of RAS signaling after treatment with KRAS-G12C inhibitors. One possible explanation is that, beyond its effects on PI3K activation via receptor tyrosine kinases, an additional aspect of the combination effects between IGF1R inhibitor and ARS-1620 is that linsitinib forces a larger proportion of the KRAS-G12C in the cell into the GDP-bound state, which is the target for ARS-1620.

Combinations of ARS-853 with different RTK inhibitors, including FGFR, EGFR, and cMET resulted in heterogeneous responses across the cell lines tested (19, 21). Combinations of ARS-1620 with RTK inhibitors or PI3K inhibitors were reported to have improved activity against G12C KRAS-mutant cell lines (36), although our previous work has failed to see selectivity of PI3K inhibitors for RAS mutant cells, either as single agents or in combination (11). In contrast, the combination of ARS-1620 with linsitinib and everolimus results in an acute loss of cell viability and durable tumor regression in all the KRAS-G12C mutant models that we tested, including those that exhibited only modest sensitivity to single agent treatment with ARS-1620, with clear selectivity over KRAS wild type cells. Our data suggest that the three-drug combination could also be effective in cells that show reduced KRAS-dependency. A recent study measured the importance of the different effector pathways in KRAS-mutant cells and classified them into three groups: cells that maintain the oncogene addiction to KRAS, cells dependent on RSK, and a smaller group that is dependent on RAL and PI3K signaling. The second group included all the LKB1-mutant cells and exhibited high mTOR activity through RSK activation (54). Based on these dependencies, we speculate that the combination of ARS-1620, linsitinib, and everolimus could suppress the activity of the essential pathways of these three subgroups and prevent any switch between dependencies, thereby reducing potential mechanisms of resistance.

There are several reasons to speculate that combinations of IGF1R and mTOR inhibitors with KRAS-G12C inhibitor can result in better responses with less toxicity compared to combinations with MEK inhibitors. Firstly, ARS-1620 alone causes a partial reduction of AKT and S6 phosphorylation, which MEK inhibitor does not, likely reflecting direct input of KRAS, but not MEK, into PI3K activation. Perhaps most critically, the KRAS-mutant allele specificity of KRAS-G12C inhibitors should result in less toxicity than MEK inhibitor because it should have no effect on wild type RAS pathway signaling in normal cells, and therefore higher doses can be administered to achieve good target inhibition. Overall, these results support the idea that co-targeting of mTOR and IGF1R can strongly increase the effectiveness of KRAS-G12C inhibitors in tumors harboring KRAS-G12C mutations, which

account for 40% of KRAS-mutant lung adenocarcinomas, and that this might be achievable in the clinic with manageable toxicities.

This work provides insight into how best to develop the use of KRAS-G12C inhibitors in the clinic, which may be a challenge relatively soon, as drugs such as AMG 510 and MRTX849 complete initial safety evaluation in phase 1 clinical trials. However, we recognize limitations of in vitro and mouse-based studies such as these when it comes to extrapolating to possible future clinical studies. As discussed above, the potential clinical toxicities of combinations of targeted agents can be unpredictable and are not necessarily uncovered by in vitro cell-based work or mouse models. In addition, although the treatment approach we propose aims to shut down growth signaling pathway reactivation by cross-talk and negative feedback, there is no reason to expect that it will not ultimately be compromised by the evolution of drug resistance due to preexisting tumor heterogeneity. In the setting of advanced cancer in patients, it is likely that other therapeutic approaches would also need to be integrated into the clinical path, such as immunotherapy, radiotherapy, and chemotherapy, and understanding how to integrate these together with G12C KRAS targeted combination treatments will be a considerable challenge.

Materials and Methods

Study design

The goal of the study was to identify combination therapies for lung tumors harboring KRAS mutations. An initial whole genome shRNA screen performed to identify complementary targets for the improvement of IGF1R and/or MEK targeting therapies identified mTOR as a potential target. Activity and selectivity of the combinations was assessed in a panel of KRAS-mutant and wild-type cell lines. Mechanistic investigation using western blot experiments was conducted to study the signaling pathways required for the survival of KRAS-mutant cells. In vitro experiments were not blinded. They were performed in at least two technical replicates and with a minimum of two biological replicates.

Therapeutic potential of the combinations was initially determined using genetically engineered mouse models. Mice were randomized by gender and tumor burden. Analysis of computerized tomography (CT) scan images to measure changes in tumor volume was done blinded. Combinations with KRAS-G12C inhibitors were tested using xenografts. Mice were randomly assigned to treatment groups to ensure similar average tumor size. Treatment cohort sizes were 4-7 mice, dependent on availability. Power calculations were not carried out. Animals were treated without knowledge of anticipated outcomes. However, due to the differences in color and density of the different drugs, treatments were not completely blinded. No data points were removed as outliers.

In vivo drug studies

All studies were performed under a UK Home office approved project license and in accordance with institutional welfare guidelines. At the end of the treatment, mice were

sacrificed with a terminal overdose of pentobarbital and local anesthetic, followed by removal of the lungs or severance of the aorta.

For autochthonous tumor formation, lung tumors were initiated using intratracheal instillation of 1×10^6 pfu (*Kras*^{LSL-G12D/+}; *Trp53*^{flox/flox} mice) or 1×10^5 pfu (*Kras*^{LSL-G12D/+}; *Stk11*^{flox/flox} mice) adenovirus expressing Cre-recombinase (Gene Transfer Vector Core) in mice between 6 and 12 weeks of age. Drug treatments in *Kras*^{LSL-G12D/+}; *Trp53*^{flox/flox} and *Kras*^{LSL-G12D/+}; *Stk11*^{flox/flox} mice started 14 weeks after adeno-Cre infection. Mice were treated with vehicle, linsitinib (25 mg/kg), everolimus (2.5 mg/kg), and/or trametinib (2 mg/kg) 5 days per week. For treatment with trametinib plus paclitaxel, trametinib was administered at 2 mg/kg 4 days per week (Mon, Tue, Thu, and Fri) and paclitaxel was administered once per week (Wed) at 10 mg/kg. For the urethane-induced lung tumor model, 8- to 16-week-old FVB/Nj mice received a single i.p. injection of urethane at 1 g/kg in PBS. Drug treatments in urethane-injected mice started 16 weeks after urethane injection. Mice were treated with linsitinib (16.6 mg/kg), everolimus (1.6 mg/kg), and trametinib (1.3 mg/kg) 5 days per week. Tumor volume was measured by micro-CT analysis using the SkyScan 1176. Micro-CT data were processed and reconstructed using N-Recon (SkyScan). Reconstructed data were subsequently imaged using DataViewer, and tumor volumes were calculated using the CTan program (SkyScan).

For toxicity assessment, body weight was measured during the treatments and pathology screening necropsies were performed. For the necropsy, *Kras*^{LSL-G12D/+}; *Trp53*^{flox/flox} treated-mice were fixed in 4% neutral buffered formalin, processed, and 20 different organs were embedded in paraffin wax. Hematoxylin-eosin stains were examined by a pathologist (EH).

For tumor studies with human cell lines, 6- to 8-week-old female athymic nude mice NU(NCr)-*Foxn1*^{nu} (Charles River) were used. Five million human NSCLC cells (H358, HCC44, and H1373) were resuspended in PBS, mixed 1:1 with growth-factor-reduced matrigel, and injected subcutaneously into one flank of 6- to 8-week-old athymic nude mice. Similarly, 400,000 3LL- NRAS cells were injected into one flank of 6- to 8-week-old male C57BL/6 mice. When tumors reached an average size of 200 mm³, mice were randomized and treated 5 days per week with ARS-1620 (100 or 200 mg/kg for i.p or oral gavage administration, respectively) and/or linsitinib (16.6 mg/kg) plus everolimus (1.6 mg/kg). Treatments were administered six hours apart, starting with the KRAS inhibitor. Tumors were measured using calipers, and volume was estimated using the formula width² x length x 0.5.

IGF1R inhibitor (linsitinib), mTOR inhibitor (everolimus), and MEK inhibitor (trametinib) were prepared in 0.5% (hydroxypropyl) methyl-cellulose / 0.2% Tween-80 and administered by oral gavage (5 µl/g) 5 days per week (unless otherwise stated). Paclitaxel was prepared in PBS and administered i.p. (4 µl/g) one day per week. ARS-1620 was solubilized in 100% Labrasol (Gattefosse) and administered by oral gavage (5 µl/g) 5 days per week. Alternatively, it was solubilized in 25% DMSO, 25% PEG400, and 50% water and administered i.p. (4 µl/g).

Statistical analysis

Data are presented as mean $\pm$ SD unless otherwise stated. Statistical tests were done using an unpaired, two-tailed Student's *t* test, unless otherwise stated. If variance of groups was significantly different, the Mann-Whitney test was used. To compare two growth curves, two-way ANOVA was performed. All statistical tests were done using Prism 7 software. Significance is presented as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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One sentence summary

Combined targeting of KRAS-G12C, mTOR, and IGF1R enhances and extends the response to recently developed KRAS-G12C inhibitors in lung cancer models.

Fig. 1. A whole-genome shRNA screen identifies linsitinib and trametinib sensitizers.

(A) Schematic of the pooled whole-genome shRNA screen to identify sensitizers to trametinib and/or linsitinib in KRAS-mutant NSCLC cells. (B) Genes of the mTOR pathway identified in the shRNA screen. In green, genes that increased sensitivity to linsitinib when knocked down, and in orange, genes that produced resistance. (C) Fold change ($\log_2$) of the number of reads in drug-treated cells vs vehicle-treated cells. Each bar represents one shRNA targeting the same gene. For *MTOR* and *TSC2*, the best five scoring shRNAs have been plotted. For *RRAGC* all the shRNAs in the screen have been plotted. (D-E) H23 cells were infected with the indicated shRNAs. After puromycin selection, cells were treated for six days with either serial dilutions of linsitinib (*MTOR* and *RRAGC*, D) or serial dilutions of linsitinib plus 1.5 nM trametinib (*TSC2*, E), and cell viability was measured. The arrow in panel E indicates the viability when cells were treated only with 1.5 nM trametinib. Mean $\pm$

SD of biological replicates and representative of three independent experiments. (F) H23 cells were infected with the indicated shRNAs. After puromycin selection, cells were treated for 24 hours with DMSO or 1 μ M linsitinib, and cell lysates were probed with the indicated antibodies. Right panels show the western blot quantification. The numbers in each shRNA indicate the last two numbers in the shRNA name (file S1).

Fig. 2. Combination of IGF1R, mTOR and MEK inhibitors decreases the viability of KRAS-mutant NSCLC cells.

(A-B) KRAS-mutant cells were treated with serial dilutions of everolimus (A) or AZD8055 (B) together with 1 μ M linsitinib, 1 nM or 5 nM trametinib (1 nM for H23 cells and 5 nM for H358 and H1792), or the combination of both, and cell viability was measured after 6 days. Mean $\pm$ SD of 2-3 independent experiments. (C) Apoptosis induction (caspase-3 cleavage) in H1792 cells treated for 48 hours with serial dilutions of everolimus or AZD8055 in presence or absence of 1 μ M linsitinib, 5 nM trametinib, or the combination of both. Mean $\pm$ SD of biological replicates and representative of two independent experiments. (D-E) KRAS-mutant (D) or non-transformed AT2 lung (E) cells were treated with several drug combinations (40 nM everolimus, 1 μ M linsitinib, 1 nM or 5 nM trametinib) and

stained with crystal violet at various time points. (F-G) Viability data of 10 KRAS-mutant (F) and 7 KRAS wild-type (G) cells treated for 6 days with 40 nM everolimus or 20 nM AZD8055 in presence or absence of 1 μ M linsitinib +/- 5 nM trametinib. Mean $\pm$ SD, unpaired Student's t-test.

Fig. 3. Combination of IGF1R with mTOR inhibitors results in a strong inhibition of PI3K/AKT and mTOR pathways.

(A) KRAS-mutant cells were treated for 24 h with 40 nM everolimus, 20 nM AZD8055, 1 μ M linsitinib, or the combinations. Cell lysates were probed with the indicated antibodies. (B) Quantification of the western blots shown in panel A. (C) NSCLC cell lines were treated for 24 h with 40 nM everolimus, 20 nM AZD8055, 1 μ M linsitinib, or the combinations. Cell lysates were probed with the indicated antibodies. For all western blots, see fig. S4C. (D) Quantification of the western blots shown in panel C. Data have been normalized to the DMSO-treated cells (Ctrl). (E) Relative phosphorylation of AKT in cells treated with mTOR

plus IGF1R inhibitors compared with cells treated with mTOR inhibitor alone. Data are represented as mean $\pm$ SD, unpaired Student's t-test. (F-G) KRAS-mutant (F) and KRAS wild-type (G) cells were treated for 24 h with different drug combinations (1 μ M linsitinib, 40 nM everolimus, and/or 5 nM trametinib). Cell lysates were probed with the indicated antibodies.

Fig. 4. mTOR inhibition causes activation of the IGF1R pathway in KRAS-mutant cells. (A) H1792 cells were treated with DMSO (vehicle), 100 nM everolimus, or 80 nM AZD8055 for 24 h. Cell lysates were assayed using a phospho-RTK array kit. Phosphorylated RTKs that change their amounts with the drug treatment are highlighted with boxes. (B) KRAS-mutant cells were treated for 24 h with various concentrations (nM) of everolimus or AZD8055, and cell lysates were probed with the indicated antibodies. (C) KRAS wild-type cells were treated with DMSO, 100 nM everolimus, or 80 nM AZD8055 for 24 h. Cell lysates were assayed using a phospho-RTK array kit. Phosphorylated RTKs that change their amounts with the drug treatment are highlighted with boxes. Shorter exposure of phospho-arrays for H2170 cells is shown in fig. S5B. (D) Cell lysates from four KRAS-mutant and four KRAS wild-type cells growing at steady-state conditions were probed with the indicated antibodies. (E) IGF1 and IGF2 concentrations in supernatant of NSCLC cell lines growing in steady-state conditions.

Fig. 5. Combination of mTOR, IGF1R, and MEK inhibitors promotes tumor regression in *KRAS*-driven lung tumors.

(A) *Kras^{LSL-G12D};Trp53^{Flox/Flox}* mice were treated with linsitinib (Lins; 25 mg/kg), everolimus (Ev; 2.5 mg/kg), and/or trametinib (Tr; 2 mg/kg) (4-5 mice per group). A waterfall representation of the response of each tumor after 4 weeks of treatment is shown. The inset box shows an enlargement of the combination treatments. (B-C) Mice with urethane-induced lung tumors were treated with vehicle or linsitinib (17 mg/kg), everolimus (1.7 mg/kg), and trametinib (1.3 mg/kg) (6-7 mice per group). Waterfall representation of the response of each tumor after 4 weeks of treatment (B) or after a second cycle of 4 weeks

of treatment (C). (D) *Kras*^{LSL-G12D};*Stk11*^{Flox/Flox} mice were treated with vehicle, linsitinib (25 mg/kg), everolimus (2.5 mg/kg), and/or trametinib (2 mg/kg) (4 mice per group). A waterfall representation of the response of each tumor after 3 weeks of treatment is shown. The inset box shows an enlargement of the combination treatments. Statistics were done using Mann-Whitney test.

Fig. 6. Drug combinations with a direct KRAS inhibitor inhibit the viability in KRAS-mutant cells.

(A) KRAS-G12C mutant cells were treated with the KRAS-G12C inhibitor ARS-1620 (1 μ M) as single treatment or in combination with the indicated drugs. Cell confluence was followed over time using Incucyte. (B) KRAS-mutant cells were treated with different drug combinations and apoptosis (caspase-3 cleavage) was monitored over time using Incucyte. (C) KRAS-mutant cells were treated with several drug combinations and stained with crystal violet at different time points. (D) KRAS-mutant cells were treated for 24 h with several drug combinations, and cell lysates were probed with the indicated antibodies. (E) KRAS-mutant cells were treated with either ARS-1620 or the combination of ARS-1620 + linsitinib + everolimus. Cell lysates were probed with the indicated antibodies or were used to measure RAS-GTP by RAF-RBD pull-down. (F) KRAS wild-type cells were treated with

several drug combinations and stained with crystal violet at different time points. (G-H) NSCLC cells were grown in 2D-adherent monolayers (G) or 3D-spheroid suspension (H) and treated with 1 μ M ARS-1620 (Ars), 500 nM linsitinib plus 40 nM everolimus (Lins +Ev), or the combination of the three-drugs (A+L+Ev). Cell viability was measured after 5 days. Mean $\pm$ SD of 2-3 independent experiments, unpaired Student's t-test. When not otherwise indicated, treatments were done using 1 μ M linsitinib, 40 nM everolimus, 1 μ M ARS-1620, and/or 5 nM trametinib.

Fig. 7. Combination of KRAS-G12C inhibitor with IGF1R plus mTOR inhibitors increases in vivo efficacy.

(A) Tumor volume of HCC44, H358, and H1373 xenografts treated with ARS-1620, linsitinib plus everolimus, or the three-drug combination (5-7 mice per group, mean±SEM). Growth curves were compared using two-way ANOVA. (B) Waterfall representation of the response of each individual tumor after 21 days of treatment. In H1373, # indicates values after 17 days of treatment. Mice were euthanized at day 17 because they had reached the maximum tumor volume. (C) 3LL cells (parental and 2 NRAS knockout clones) were treated with DMSO or 1 μM ARS-1620 for 24 hours. Cell lysates were probed with the

indicated antibodies. (D) 3LL cells were treated with serial dilutions of ARS-1620 in presence or absence of 1 μ M linsitinib plus 40 nM everolimus. Cell viability was measured after 5 days. Mean $\pm$ SEM of three independent experiments. (E) 3LL cells were treated for 24 hours with different combinations containing 1 μ M ARS-1620, 1 μ M linsitinib, 40 nM everolimus, and/or 5 nM trametinib. Cell lysates were probed with the indicated antibodies. (F) Relative apoptosis induction (caspase-3 cleavage) in 3LL cells after 72 h of treatment with the same treatments as in panel E. Mean $\pm$ SD of two independent experiments. (G) Tumor volume of 3LL NRAS-63 cells grown subcutaneously in C57BL/6 mice. Mice were treated with different drug combinations containing ARS-1620, linsitinib, everolimus, and/or trametinib (5-7 mice per group, mean $\pm$ SEM). Growth curves were compared using two-way ANOVA. (H) Survival analysis of C57BL/6 mice bearing subcutaneous 3LL NRAS-86 tumors treated with ARS-1620, linsitinib plus everolimus, or the three-drug combination (4-5 mice per group). All mice were treated with ARS-1620 (100 mg/kg intraperitoneally or 200 mg/kg oral gavage), linsitinib (17 mg/kg), everolimus (1.7 mg/kg), and/or trametinib (1.3 mg/kg).